Phytochemical investigation of *Cinnamomum iners* against *Naja kaouthia* and *Calloselasma rhodostoma* snake venoms on enzyme-induced hemorrhagic necrosis

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Pharmacy in Pharmaceutical Sciences

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ชื่อวิทยานิพนธ์	การศึกษาทางพฤกษเคมีของกิ่งอบเชยไทยต่อการต้านฤทธิ์ของเอนไซม์ที่ทำให้เกิดเนื้อตายจากพิษงูเห่าไทย และงูกะปะ
ผู้เขียน	นางสาวกาญจนา จีนชูแก้ว
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ปีการศึกษา	2552

บทคัดย่อ

การศึกษาพฤกษเคมีของกิ่งอบเชยไทยที่มีฤทธิ์ในการต้านพิษงู และต้านฤทธิ์ที่ทำให้เกิดเนื้อตายจากพิษงูของงูเห่าไทย และงูกะปะ ซึ่งสารสกัดชั้นเอทิลอะซิเทต และเมทานอล ให้ผลดีในการทดสอบฤทธิ์ในการต้านพิษงู จากนั้นได้นำเอาส่วนสกัดเอทิลอะซิเทต และเมทานอลมาแยกได้สารบริสุทธิ์ 3 ชนิด คือ *β*-sitosterol, *β*-sitosterol-3-*O*-*β*-D-glucopyranoside และcompound 3 ซึ่งพิสูจน์โครงสร้างสารด้วยข้อมูลทางสเปกโทรสโกปี และเปรียบเทียบกับสารที่มีอยู่แล้ว เมื่อศึกษาฤทธิ์ต้านพิษงู และต้านฤทธิ์ที่ทำให้เกิดเนื้อตายจากพิษงูของงูเห่าไทย และงูกะปะ พบว่าสารบริสุทธิ์ *β*-sitosterol, *β*-sitosterol-3-*O*-*β*-D-glucopyranoside และcompound 3 มีฤทธิ์ต้านแอคติวิตี้ของเอนไซม์โปรติโอไลติคของพิษงูกะปะที่ความเข้มข้น 10 มิลลิกรัมต่อมิลลิลิตร คือ 43.7%, 27.8% และ58.8% ตามลำดับ มีฤทธิ์ต้านแอคติวิตี้ของเอ็นไซม์ฟอสโฟไลเปส เอทู ของพิษงูเห่าไทยที่ความเข้มข้น 10 มิลลิกรัมต่อมิลลิลิตร คือ 69.6 %, 53.8% และ61.7% ตามลำดับ และมีฤทธิ์ต้านแอคติวิตี้ของเอนไซม์ฟอสโฟไลเปสเอทู ของพิษงูกะปะที่ความเข้มข้น 10 มิลลิกรัมต่อมิลลิลิตร คือ 46.8%, 7.9% และ92.8% ตามลำดับ

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ABSTRACT

Phytochemical investigation of *Cinnamomum iners* against *Naja kaouthia* and *Calloselasma rhodostoma* snake venoms on enzyme-induced hemorrhagic necrosis. The EtOAc and MeOH fractions were revealed high activity against snake venom. Then, isolation of the EtOAc and MeOH fraction yielded *ß*-sitosterol and *ß*-sitosterol-3-*O*-*ß*-D-glucopyranoside and compound 3**.** The structure of all isolated compounds was elucidated by spectroscopic techniques and comparison of compounds with an authentic samples of compound 1, 2 and 3 confirmed their identify. Three compounds, *ß*-sitosterol, *ß*-sitosterol-3-*O*-*ß*-D-glucopyranoside and compound 3 were showed the inhibitory capabilities to the proteolytic enzyme of CR venom (10 mg/ml) at the percentage of 43.7, 27.8 and 58.8 respectively, phospholipase A_2 (PLA_2) activity against NK venom 69.6 %, 53.8% and 61.7% at the concentration of 10 mg/ml. respectively and also against CR venoms 46.8%, 7.9% and 92.8% at the concentration of 10 mg/ml, respectively.

Keywords: *Cinnamomum iners*/ *Naja kaouthia* (NK)/ *Calloselasma rhodostoma* (CR)/ *ß*-sitosterol/ *ß*-sitosterol-3-O-*ß*-D-glucopyranoside/ compound 3/ SDS-PAGE/ proteolytic activity/ phospholipase A_2 (PLA_2) activity

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Kanchana Jeenchoogaew

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LIST OF ABBREVIATION AND SYMBOLS

δ	chemical shift in ppm
°C	degree Celsius
CD_3OD	deuterated methanol
$CDCl_3$	deuterated Chloroform
$CHCl_3$	chloroform
COSY	correlation spectroscpy
CR	Malayan Pit Viper (*Callosellasma rhodostoma*)
DW	distilled water
DMSO-d_6	deuterated dimethylsulphoxide
d	doublet (for NMR signal)
EtOAc	ethyl acetate
EtOH	ethanol
g	gram
Hexane	*n*-hexane
HMBC	heteronuclear multiple bond correlation
HMQC	heteronuclear multiple-quantum coherence
HPLC	high performance liquid chromatography
hr	hour
i.p.	intrapertoneal
IR	infrared
J	coupling constant
Kg	kilogram
Kda	kilodalton
L	liter
LD_{50}	fifty percent lethal dose
m	multiplet (for NMR signals)
MeOH	methanol
µg	microgram
mg	milligram
min	minute
NMR	nuclear magnetic resonance

LIST OF ABBREVIATION AND SYMBOLS (continued)

/	per
%	percent
ml	milliliter
MS	mass spectroscopy
NK	Thai cobra (*Naja kaouthia*)
PLA_2	phospholipase A_2
s	singlet (for NMR signals)
s.c.	subcutaneous
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
t	triplet (for NMR signals)
TLC	thin layer chromatography
UV	ultraviolet
UV/Vis	ultraviolet/visible
w/v	weigh/volume

CHAPTER 1
INTRODUCTION

1.1 General introduction

Snakebite is one of the most important public health problems of tropical countries. In Thailand, although the annual incidences of snakebites are inaccurate. The morbidity and mortality incidences are estimated at 13 and 0.01 per 100,000 populations, respectively (http//:epid.moph.go .th) of venomous snakes. The most common species in Thailand are *Calloselasma rhodostama* (Malayan pit viper, 43.9%), *Trimeresurus* spp. (green pit viper, 39.0%), *Naja kaouthia* (Thai cobra, 10.7%), *Doboia russelli* (Russell's viper, 2.2%) (Pochanugool et al., 1998; Chanhome et al., 2000). The bite by Malayan pit viper is high morbidity whereas by Thai cobra is high mortality but both snakes cause the most severe local tissue necrosis in 100% and 50% of the bites, respectively. (Yingprasertchai et al., 2003; Jintakul and Chanhome, 1995). Venoms of Thai cobra and Malayan pit viper seriously effect to the bodily system of the envenomed victims (Assakura et al., 1992). Symptoms of local pain, local bleeding, inflammation and complications including local wound necrosis are usually the main problems at the bite site (Ode and Asuzu, 2006; Alam and Gomes, 2003; Bjarnason and Fox, 1994; Markland, 1998). The venom components responsible for these local effects are phospholipase A_2 (PLA_2) and protease enzymes. (Chippaux et al., 1991). The general treatment for snakebite is the administration of antivenom, which is the only available specific treatment. However, antivenom does not provide enough protection against venom which induced haemorrhage and local tissue damage (Sutherland, 1977). An alternative treatment of snakebite is herbal medicine, which have been used among traditional healers especially in tropical areas where there are plentiful sources of medicinal plants, as therapy for snakebite for a long time. Thai herbs can be use as snakebite remedies. The herbs normally indigenized at the area surrounding the residence of the snakebite victims (Pratepvi monmoly, 1979).

Cinnamomum iners Reinw. ex Blume, known as "Ob Chuei" (in Thai), belongs to the family Lauraceae, is a plant widespread throughout Southeast Asia (Wannissorn et al., 2005; Mastura et al., 1999).

This plant has used as traditional herbal medicine and have many pharmacological activities such as anti-bacterial, anti-inflammatory, analgesic and antipyretic. (Alzoreky and Nakahara, 2003.; Pormjit, 1989). Puripatthavong et al., (2004) reported that *Cinnamomum iners* Reinw. ex Blume was active against snake venoms. However, a few of this plant was scientifically studied, especially in term of anti-snake venoms. Therefore, this study was to clarify the antagonistic activity and antihaemorrhagic necrosis of *Cinnamomum iners* Reinw. ex Blume against the snake venoms from *Naja kaouthia* and *Calloselasma rhodostoma*.

1.1 Objective

This study was focused on the phytochemical study and the screening in term of their SDS-PAGE electrophoresis, proteolytic and phospholipase A_2 enzyme activities. The inhibition effects from twigs of *C. iners* against some effects of *Naja kaouthia* and *Calloselasma rhodostoma* venoms were examined using *in vitro* assays.

The specific aims of this study were:

- To purify the constituents of the twigs of *C. iners* and determine the chemical structures of the isolated compounds by spectroscopic method.
- To identify the most promising pure compounds expressing a functional character in assay for SDS-PAGE electrophoresis, proteolytic enzyme activity and phospholipase A_2 enzyme activity.

CHAPTER 2
LITERATURE REVIEW

2.1 Snakes

Venomous snake bite is important medical problem in Thailand since economic activities of country are mainly agricultural areas where abundant of snakes (Songsumard, 1995). The snakes responsible for such high mortality rate are *Calloselasma rhodostoma* (Malayan pit viper, 40%), *Trimeresurus albolabris* (Green pit viper, 30%), *Naja kaouthia* (Thai cobra, 12%), *Daboia russelii siamensis* (Russell's viper, 10%) and others. Snakes in Thailand are classified into two families of venomous snakes, families Elapidae and Viperidae (Pithayanukul et al., 2005).

2.1.1 *Naja kaouthia*

Scientific name: *Naja kaouthia*
Common name: Thai cobra or Monocled Cobra
Family: Elapidae

2.1.1.1 General information

Naja kaouthia is a ground dwelling snake in the flat country, which can however climb and swim very well. The *Naja kaouthia* is equally at home in a wide variety of places: forest and shrub areas, as well as plantations, rice fields, pastures, villages and cities. The *Naja kaouthia* is active at night and day, but more often at night. These snakes eat rodents, frogs, toads, ducks and chicks. When threatened they straighten up, spread the neck flat, and hiss. If you react calmly, they usually take flight. The *Naja kaouthia* mates in Thailand in the months of December and January. After about two months of mating, the females lay between 10 and 30 eggs. The eggs are laid in moderately damp soil, under heaps of leaves or stones, in the spaces of hollow trees, in rat holes, and in spaces under houses. Depending on the temperature, the eggs incubate from between 50 and 60

days. The young animals are long, between 25-30 cm and identical in colouring and appearance to the adults. *Naja kaouthia* is a species of snake. Body scales are smooth and arranged in 19-21 (usually 21) longitudinal rows at mid-body. Throat is pale or scarcely any dark mottling which often followed by a single dark band. Ventrolateral throat spots are distinct, remainder of venter is either pale or increasingly cloudy with darker pigmentation towards the rear. In adults, hood markings are usually in distinct, usually a pale, oval or circular marking with 1 or 2 dark spots. In the center surrounded by a narrow dark outer border (Figure 2-1.). Fangs are not modified for spitting. Venom discharge orifice is large. The distribution of *Naja kaouthia* ranges in Bangladesh, Myanmar, Cambodia, India, Laos, Malaysia, Thailand and Vietnam (Chanhome et al., 1998.; Jintakune and Chanhome., 1995.; Mahanta et al., 2001).

Figure 2-1. Thai cobra (*Naja kaouthia*) (http//:www.redcross.or.th)

2.1.2 *Callosellasma rhodostoma*

Scientific name: *Callosellasma rhodostoma*

Common name: Malayan Pit Viper

Family: Viperidae

2.1.2.1 General information

Malayan pit viper is widely distributed in Thailand, Malaysia, Laos, Java, Vietnam and Cambodia (Daltry et al., 1996). It is the most common cause of snakebite in Malaysia and Thailand (Tsai et al., 2000). The envenoming by Malayan pit viper causes high morbidity and some mortality especially among rubber and coffee plantation workers in soutern Thailand (figure 2-2.).

Figure 2-2. Malayan Pit Viper (*Callosellasma rhodostoma*) (http//:www.naturephoto.cz.htm)

Malayan Pit Viper is a relatively short, a flatten body and a large triangular–shaped head with a pointed and slightly upturned snout. There is a well defined dark post–ocular patch, sometimes with a white edge above and below. Its low limit, on the supralabials, is scalloped. The top of the head is covered with nine large smooth symmetrical scales and is normally dark. The ground color of body varies from vary dark brownish–black to various shades of reddish, yellowish and

grayish (figure 2-2.). The dorsal pattern is of alternating dark, triangular markings with their apices towards the vertebral line and below these, a series of dark spots on the flanks (Kritnanon et al., 1991).

2.2 Chemical composition of snake venoms

Approximately 89-95% of viperid and 25-70% of elapid venoms, including digestive hydrolases (protease, exo and endopeptidase, phosphomonoesterase, phosphodiesterase and phospholipases), hyaluronidases and activator or inactivators prey's physiological mechanism (Stocker et al., 1991). Most of venoms contain phosphomonoesterase, phosphodiesterase, ribonucleases, deoxyribonucleases, nucleotidases, amino acid oxidases, lactate dehydrogenases and phospholipase A_2 (Das, 1996; Cox, 1991). Elapid venoms contain acetylcholine esterase, phospholipase B and glycerolphosphatase while viperid venoms have endopeptidase, arginine esterhydrolase, thrombin like enzyme and protrombin activating enzyme. The various enzymes identified in snake venoms are listed in Table 2-1. (Braud et al., 2000; Kemparaju et al, 1999).

2.2.1 The function of some of the key enzymes (Meier and White, 1995)

Aceylcholinesterase : Attacks the nervous system, relaxing muscles to the point where the victim has very little control.

Amino acid oxidase : Plays a part in digestion and the triggering of other enzymes, (is responsible for venom's characteristic light yellowish colouring).

Polypeptide toxins : Directly disrupt nerve-impulse transmission, usually causing heart or respiratory failure. Taxon with the compound in its venom: cobra (*Naja*) (cardiotoxin)

Hyluronidases : Catalyse reactions that break mucopolysaccharide links in connective tissues, thereby enhancing diffusion of venom. Several types of snake contain this venom.

Proteases : Catalyse reactions that disrupt protein peptide bonds in tissues, causing blood-vessel wall damage and haemorrhaging and muscle-fibre deterioration. Vipers, pit vipers contain this venom.

Phospholipases : Catalyses reactions that harm musculature and nerves. Almost all venomous species.

Thrombin like enzymes : Inhibit blood clotting. Vipers, pit vipers, a few elapids (but rare) contain this venom.

Phosphodiesterase : Accounts for the negative cardiac reactions in victims, most notably a rapid drop in blood pressure.

Other enzymes : ribonucleases, deoxyribonucleases, nucleotidases, lactate dehydrogenases, acidic and basic phosphatases.

Table 2-1. Enzymes found in snake venoms (Iwanaga and Suzuki, 1979; White, 2005; You et al., 2006)

All snake venoms	**Viperid venoms**	**Elapid venoms**	**Other venoms**
Phospholipase A_2	Endopeptidase	Acetylcholinesterase	Catalase
Phosphodiesterase	Arginine esterhydrolase	Phospholipase B	Amylase
Proteolytic enzymes	Thrombin like enzyme	Glycerophosphatase	β-Glucosaminidase
Ribonuclease	Prothrombin activator		Lactate dehydrogenase
Phosphomonoesterase			Heparinase like enzyme
Deoxyribonuclease			
L-amino acid oxidase			
Hyaluronidase			
5'-Nucleotidase			
NAD-nucleotidase			

2.3 Effects of snake venom

Effects of snake venom in the tissues envenomated by the bite are called local effects. Other actions arise from toxins transported through the blood vessels or through lymph vessels, and are called systemic effects (Peterson, 2006).

2.3.1 Systemic effects

2.3.1.1 Neurotoxicity

Snake venom neurotoxins often act upon the acetylcholine receptor system, with different components causing post-synaptic antagonism and acetylcholinesterase activity. Other components may cause direct pre-synaptic nerve cell destruction. There are two groups of neurotoxins, namely the pre-synaptic and the post-synaptic neurotoxins (Chang et al., 1997).

- **Pre-synaptic neurotoxin :** The pre-synaptic neurotoxin act on the proximal terminal of the nerve synapse by blocking the release of acetylcholine. The physiological transmitter and eventually destroying the nerve terminal, preventing transmission of nervous impulses across the synaptic gap (Karlsson et al., 1971).
- **Post-synaptic neurotoxin :** The post-synaptic neurotoxin act on the distal terminal of the nerve synapse by competitively inhibiting the binding of acetylcholine, again preventing the physiological transmission of nervous impulses across the synaptic gap (Lee, 1970).

2.3.1.2 Hemotoxicity

Hemotoxic effects are mediated by proteolytic enzyme and peptides that can cause local tissue destruction directly and by intimal injury to blood vessels followed by thrombosis and necrosis. Activation of the coagulation cascade can occur at multiple points resulting in net anticoagulation. Direct lysis of red blood cells can cause acute hemolytic anemia and produce subcutaneous tissue destruction and loss of digits (Stocker et al., 1991).

2.3.1.3 Myotoxicity

Myotoxins can cause compromise of muscle compartments from direct myonecrosis as well as from local pressure effects. Secondary edema can develop rapidly in tissues from both cytokine release and from hemorrhage into local tissues. Systemic effects can include pulmonary edema. Intravascular injection can cause a more severe systemic reaction with diffuse bleeding from thrombocytopenia and hypofibrinogenemia. In severe cases of envenomation, myonecrosis can cause tissue damage, producing loss of fingers, toes, legs and arms (Valentin and Lambeau., 2000; Lambeau et al., 1990).

2.3.2 Local effects

Venoms are polymolecule basic proteins which diffuse only slowly within the tissues and so produce at first severe symtoms of poisoning in the region of the bite. They have toxic effect on many different types of cell causing the destruction of cell membranes (Jabeen et al., 2005).. Necrosis may occur anywhere on the body but it is commonest around the site of the bite such as finger, toe, hand and foot. It may lead to gangrene and limb loss. Skin graffs and long periods of donvalescence may be required to deal with theinjure. (Melo and Ownby, 1999).

2.4 Perspective (venom components with local action)

The venom consists of proteins, polypeptides, and enzymes that cause necrosis and hemolysis. Proteolytic and phospholipase enzymes commonly found in snake venom. Most damaging effects are the result of proteolytic and phospholipase enzymes activity (Gay et al., 2005; Borges et al., 2001). Proteolytic enzymes catalyze the breakdown of proteins integral to membrane and tissue integrity. They can be more than one proteolytic enzyme in snake's venom include peptide hydrolases, proteases and proteinases. Phospholipase hydrolyzes phospholipids, the primary components of biological membranes (Kemparaju et al, 1999). Among the phospholipase, PLA_2 enzymes are the most widespresd an extensively studied of all venom enzyme. Under experimental

conditions, they damage mitocondria, red blood cells, leukocytes, platelets, peripheral nerv ending, skeletal muscle, vascular endothelium and other membranes (Jagadeesha et al., 2002).

2.4.1 Proteolytic enzyme

The proteolytic enzyme are known as peptide hydrolase, proteases, endopeptidases, peptidases and proteinases. They may be several proteolytic enzyme in a single venom. They are responsible for local changes in vascular permeability leading to oedema, blistering and bruising and to necrosis (Mukherjee and Maity, 2002). Its activity is similar to trypsin that digests the protein and peptide causing the tissue damage and necrosis (Mochian et al., 2006). Catalyse reactions disrupt protein peptide bonds in tissues, causing blood-vessel wall damage and haemorrhaging and muscle-fiber deterioration (Lallol, 2005).

2.4.2 Phospholipase A_2 (PLA_2) enzymes

The enzyme noted phospholipase A_1 and phospholipase A_2 are classified as acyl hydrolases. phospholipase C and phospholipase D are classified as phosphodiesterases (Figure 2-3.). PLA_2 enzyme are separated into at least two structural classes. The first isolated from venom of primitive reptiles such as cobra (Elapids) and sea snake (Hydrophids), posess a unique disulfide bond between half-Cys residues 11 and 69. PLA_2 from viper pit viper venom constitutent the class II enzyme having a C-terminal extension of about 6 residues and terminating by a half-Cys disulfide bonded to half-Cys 50 which bordors the active site. PLA_2 from lizard and bee venoms are class III. This PLA_2 enzyme in being a glycoprotein and althouth it retains a conserved Ca^{2+} binding site, it lacks the proton-relys system proposed for the active site of pancreatic (Kirakasus, 1998).

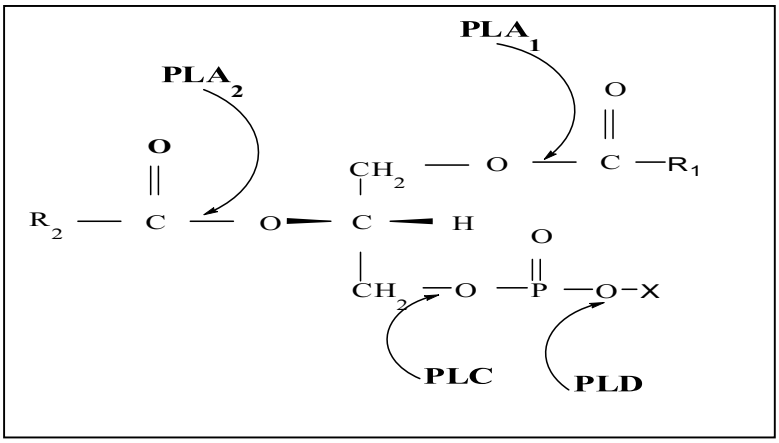


Figure 2-3. Sites of action of phospholipase A_1, phospholipase A_2, phospholipase C and phospholipase D

PLA_2 enzymes are major components in snake venom (Dennis, 1994; Jemeel et al, 2006). They display a wide range of biological effect. PLA_2s are esterolytic enzyme which breakdown membrane phospholipids such as lecithin into fatty acid and lysolecithin. This causes cellular membrane damage. In human being, all these enzyme cause oedema, blister formation and local tissue necrosis. PLA_2 the middle (*sn*-2) position of glycerol (Figure 2-4.), producing primarily arachidonic acid (precursor to the prostaglandins and leukotrienes) and lyposhospholipids (precursor to platelet-activating factor) (Kini, 2005; Camgo et al., 2005).

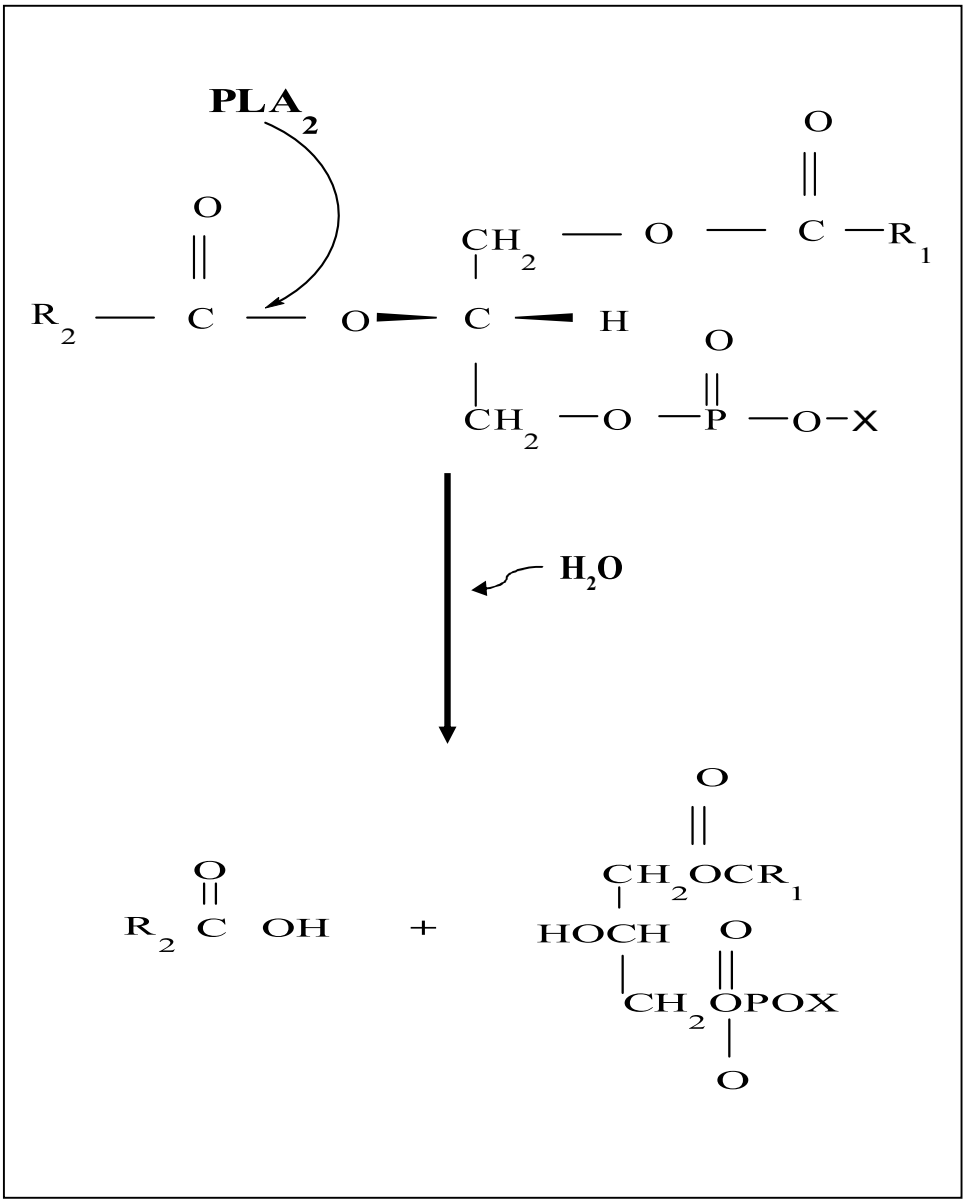


Figure 2-4. The specific reaction catalyzed by PLA_2 at *sn*-2 position of the glycerol backbone

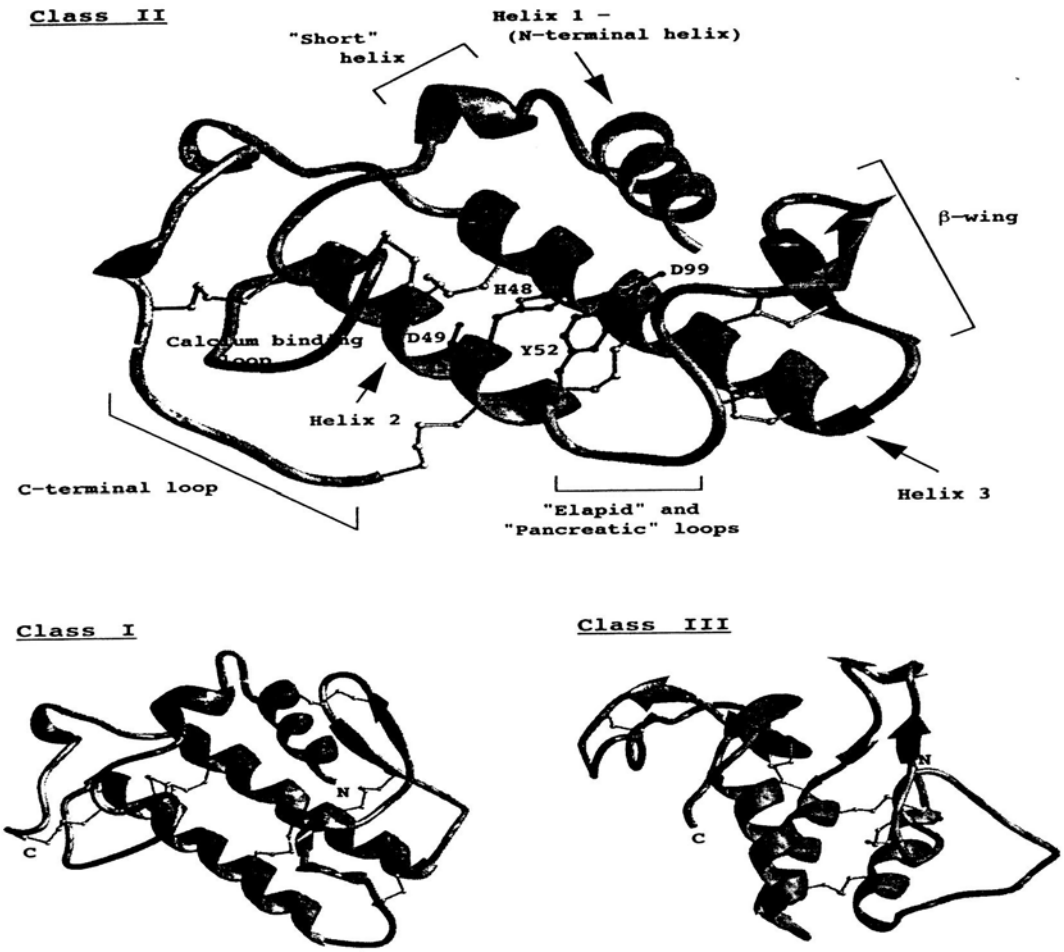


Figure 2- 5. Structure of phospholipase A_2

Group I : phospholipase A_2 from elapid snake venom.

Group II : phospholipase A_2 from viperid snake venom.

Group III : phospholipase A_2 from lizard and bee venom.

2.5 Treatment of snake envenomation

Antivenom (or antivenin or antivenene) is a biological product used in the treatment of venomous bites or stings. Antivenom is created by injecting a small amount of the targeted venom into an animal such as a horse, sheep, goat, or rabbit. The subject animal will undergo an immune response to the venom, producing antibodies against the venom's active molecule which can then be harvested from the animal's blood and used to treat envenomation in others (Theakston et al., 2003). Antivenoms bind to and neutralize the venom, halting further damage, but do not reverse damage already done. Thus, they should be administered as soon as possible after the venom has been injected, but are of some benefit as long as venom is present in the body. Since the advent of antivenoms, some bites which were previously inevitably fatal have become only rarely fatal provided that the antivenom is administered soon enough (Isbister, 2002). First Aid for Snake Bite, a lot of promptness and instant action is required in this case. Call the emergency medical service before you start the first aid process. Keep in mind, you have to save the victim. Catching the snake is not important, as you pose a risk of getting a snake bite again. Following are the first aid tips:

- Keep the victim calm and assure him that he will be alright. Help him come out of the shock as an increase in the heart rate or tremors may lead to more complications.
- Remove the clothes and jewelery around the affected body part.
- Immobilize the affected area without pressurizing it.
- Wash the wound immediately using warm water and soap.
- Do not elevate the casualty and keep the bite below the heart level.
- Observe the patient keenly.
- Never try to suck the venom with your mouth as you usually do when someone is hurt and the wound bleeds.
- You should not try to remove the venom by cutting into the wound or by applying suction
- Do not apply any ice on the wound.
- Do not give alcohol or hot beverages to the patient.
- Take the patient to the hospital as soon as possible, for further treatment.
- Inform the doctor about any signs that you noticed after the snake bite. Conditions like drowsiness, bleeding from gums or dropping-eyelids should be reported to the doctor.

2.6 Problem of antivenom treatment

Unfortunately the conventional antivenoms currently available are not only expensive, but also do not effectively neutralize venom induced hemorrhage and local necrosis. Some of the antivenoms cause allergic reaction in patients (Grant et al., 2000; Gutierrez et al., 2005; Ferreira et al., 1992). The allergic reactions of antivenom usually occur more than 20% by developing either early (within a few hours) or late reaction (5 days or more) after being given antivenom (Warrell, 1995).

Antivenom development in animal, such as horse or sheep, is time consuming, expensive and requires special storage condition (Chippaux and Goyffon, 1998). Antivenom sometimes does not provide enough protection against venom induced hemorrhage and nephrotoxicity, which is the important cause of death (Sutherland, 1977; Corregan et al., 1987; Gilon et al., 1989; Warrell, 1999) and local tissue damage (Figure 2-6.) (Leon et al., 2000). Antivenom administration via intravenous route needs experienced technician (Chippaux and Goyffon, 1998).

2.7 Studies of Thai plants against snakebite

Many Thai medicinal plants were recommended against snakebite such as *Sasevieria metallica* (Agavaceae), *Eucharis grandiflora* (Amaryllidaceae), *Crinum rubra* (Amaryllidaceae), *Curcuma aeruginosa* (Zinguberaceae), *Curcuma longa* (Zinguberaceae), *Cleome* (Capparidaceae), *Trigono stemonreidiodes* (Euphorbiaceae), *Typhopium trilobatum* (Araceae), *Eclipta prostrata* (Astraceae) and *Musa paradisiaca* (Musaceae) (Table 2-2.) (Bunjob et al., 2000; Krachanglikit et al., 1982; Pratepvimolmolee, 1989; Pitthayanukul et al., 2004). However, a few of plants were studied scientifically (Ferreira et al., 1992; Ramasamy et al., 2004).

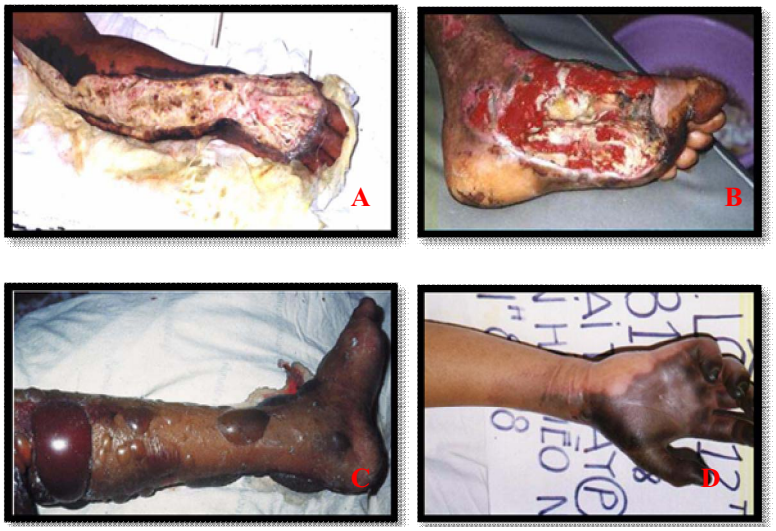


Figure 2-6. Envenoming by Thai cobra and Malayan pit viper (http//: www. venom supplies.com)

A: Thai cobra bite, extensive necrosis of skin subcutaneous tissues after the bite.

B: Thai cobra bite, extensive necrosis of the foot subcutaneous tissues 14 day after the bite.

C: Malayan pit viper bite, local edema, blistering, bruising, bleeding and early necrosis 4 day after the bite.

D: Malayan pit viper bite, darkening of an area of necrotic skin at the site of the bite.

Table 2-2. Plants used for snakebite treatment (Puripattanavong et al., 2005)

Species	**Family**
Adhatoda vasica	Annonacea
Aglaia andamanica	Meliaceae
Aglaia dookkoo	Meliaceae
Albizia procera	Mimosoideae
Allium ascalonicum	Alliaceae
Allium cepa	Alliaceae
Allium sativum	Alliaceae
Androgdraphis paniculata	Acanthaceae
Angelica sylvestris	Umbelliferae
Artemisia vulgaris	Compositae
Artocarpus heterophyllus	Moraceae
Artocarpus altilis	Moraceae
Barleria lupulina	Acanthaceae
Boesenbergia pandurata	Zingiberaceae
Capsicum frutescens	Solanaceae
Cardiospermum halicacabum	Sapindaceae
Chromolaena odoratum	Compositae
Cinnamomum iners	Lauraceae
Citrus aurantifolia	Rutaceae
Citrus grandis	Rutaceae
Clerodendrum inerme	Labiatae
Clinacanthus nutans	Acanthaceae
Croton tiglium	Euphorbiaceae
Curcuma amarissima	Zingiberaceae
Curcuma longa	Zingiberaceae
Curcuma zedoaria	Zingiberaceae
Datura metel	Solanaceae

Table 2-2. (cont.) Plants used for snakebite treatment (Puripattanavong et al., 2005)

Species	**Family**
Desmos Chinensis	Annonaceae
Elephantopus scaber	Compositae
Euphorbia tirucalli	Euphorbia
Eurycoma longifolia	Simaroubaceae
Fibraurea tinctoria	Menispermaceae
Gossypium arboreum	Malvaceae
Heterosmilax pertenuis	Smilacaceae
Houttuynia cordata	Saururaceae
Impomoea aquatica	Convolvulaceae
Justicia ventricosa	Acanthaceae
Languas galanga	Zingiberaceae
Lansium domesticum	Meliaceae
Levisticum officinale	Umbelliferae
Melastoma malabathricum,	Melastomata
Melastoma malabathricum	Melastomataceae
Mentha cordifolia	Labiatae
Mimosa sp.	Mimosoideae
Morus alba	Moraceae
Morinda citrifolia,	Rubiaceae
Nardostachys jatamansi,	Valerianaceae
Neriem indicum,	Apocynaceae
Ocimum americanum	Labiatae
Ocimum basilicum	Labiatae
Ocimum tenuiflorum	Labiatae
Picrorrhiza kurroa	Scrophulariaceae
Piper chaba	Piperraceae
Piper nigrum	Piperaceae

Table 2-2. (cont.) Plants used for snakebite treatment (Puripattanavong et al., 2005)

Species	Family
Piper nigrum	Piperaceae
Physalis angulata	Solanaceae
Saussurea lappa	Compositae
Stemona tuberosa	Stemonaceae
Strychnos nux-vomica	Strychnaceae
Tacca leontopetaloides	Taccaceae
Terminalia chebula	Combretaceae
Thunbergia laurifolia	Acanthaceae
Vitex trifolia	Verbenaceae
Zingiber cassumunar	Zingiberaceae

2.8 Plants proven to be active in anti-snake venom and their active compounds

Plants list in table 2.2 are herbs believed to be antidotes to snakebite. Puripattanavong et al. (2005) was studied 111 sample extracts of Thai plants which were active against snake venoms. Antagonistic activities shown in this study were obtained from *Desmos chinensis* (Annonaceae), *Melastoma malabathricum* (Melastomataceae), *halicacabum* (Sapindaceae), *Cinnamomum iners* (Lauraceae) and *Cardiospermum Gossypium arboretum* (Malvaceae).

Preliminary study used the extract from *Diodia scandns* Gronov ex L. (Rubiaceae) on toxic effects of saw scaled viper (*Echis carinatus*) which is the most common snake in the savanna of Nigeria by Onuauchi (1989). The water-soluble fraction of the aerial parts of plants was used to pretreat albino mice i.p., 30 min before administration of venom at the dose of 2 mg/ml i.p. This fraction reduced the mortality from 50% to 10% at concentration of 1.5 mg/ml i.p.

Andrographis paniculata (Acanthaceae) is the common plants found throughout in India. Nazimudeen et al. (1978) reported its use as a snakebite antidote. After extraction of the air-dried whole plant with 90% ethanol, the ethanol-soluble fraction was used in their study. Mice were

treated with this fraction 4 g/kg or 2 g/kg, i.p., 30 min prior to the administration of an LD_{50} (320 μg/kg) of cobra venom (*Naja naja*).

Pereira et al. (1991) reported on oral pretreatment of mice against twice the lethal dose (5 mg/ml s.c.) of *Bothrops jararaca* venom. Of the 18 species of 13 plant families was tested, extracts of *Phylanthus klotzschianus* (Euphobiaceae), *Casearian sylvestris* (Flacourtaceae) and *Apoleia leiocarpa* (Leguminosae) conferred 100% protection up to 48 hr after administration.

Abubakar et al. (2000) reported the extract of the *Guiera senegalensis* was found to detoxify the venoms from two common northern Nigerian snake species, *Echis carinatus* and *Naja nigricollis*, in separated experiments. The extract of *G. senegalensis* contains high amount of tannin. This might be the plausible mechanism of the detoxifying action of the plant extract and its success against snake envenomation.

Manhanta and Mukherjee (2001) studied neutralization of lethality, myotoxicity and toxic enzymes of *Naja kaouthia* venom by *Mimosa pudica* root extracts. This study indicated that the aqueous of root *M. pudica* was effective in neutralizing the lethality, myotoxicity and tested toxic enzymes (protease, PLA_2 and acetylcholinesterase) of *Naja kaouthia* venom. Significant neutralization of toxic enzyme of *Naja kaouthia* venom by water extracts of *M. pudica* roots might lead to inhibition of lethality of venom.

Recently, Alam and Gomes (2003) reported the root extracts of *Vitex negundo* and *Emblica officinalis* possess the potent neutralizing capacity to snake venom. The methanolic extracts of these plants antagonized lethal activity of the *Vipera russelli* and *Naja kaouthia* venoms induced both in *in vitro* and *in vivo* studies.

2.9 Compound responsible for antivenom activity

2.9.1 Steroids

Sitosterol (*ß*-sitosterol) is the most abundant of the phyto-steroids. The compound occurs either as such or in the form of its, frequently accompanied by its mono-unsaturated analogue, stigmasterol also either free or as its glucoside. Administeration to animals and humans, as well as in *in vitro* tests, sitosterol has been found to display a large array of pharmacological properties, among

them anti-inflammatory just a few of the scores of plants reputed for anti-snake venom activity(Melo et al., 1994). ß-Sitosterol and stigmatosterol from *Eclipta prostrata* show (Asteraceae) inhibited myotoxicity of crolid venom (More, 1991).

2.9.2 Pentacyclic triterpenes (free or as glycosides)

Pentacyclic triterpenes with anti-snake venom activity are abounded. In the following important examples will be list by Mors et al. (2000) as well as the anti-snake venoms plants in with they occure. Examples of these are lupeol, α-amyrin, oleanolenic acid, betinlin and bredemeyeroside (Melzig and Bormann, 1998). Among these bredemeyeroside B as new triterpenoid saponin has been isolated from the roots of *Bredemeyera flovibunda* (Polygavaceae) which used as remedy for the treatment of snakebite in Brazillian folk medicine (Daros et al., 1996).

2.9.3 Phenolic compounds

Phenolic compounds are important constituents of anti-snake venom plants which presently subdivided into the following categories : hydroxybenzoic acids, cinnamic acid derivatives, coumarins, curcuminoids, flavonoids and polyphenols (vegetable-tannins) (Deepa and Veerabasa, 2000).

2.9.4 Hydroxybenzoic acids and their methyl ethers

2-Hydroxy-4-methoxy benzoic acid was identified as a snake venom neutralizing factor in the Indian anti-snake venom plant, *Hemidesmus indicus* (Alam et al., 1994). 2,6-Dihydroxybenzoic acid is a component of the bulbs of *Gloriosa superba* used against snakebite in India (Alam et al., 1994).

2.9.5 Cinnamic acid derivatives

Caffeic acid has been found in anti-snake venom plants such as several species of *Prestonia coalita* and *Pinus sylvestris* was applied externally on the site of snake bites as an antidote to the venom. Isoferulic acid is presented in another anti-snake venom plant, *Cimicifuga racemosa,* known as "snake weed", "snake root" or "black snake root" in north America (Agoro, 1978).

2.9.6 Curcuminoids

Three diarylheptanoids-curcumin (diferuyl-methane), demethoxycurcumin and bis-demethoxy-curcumin make up the yellow dye of the rhizomes of turmeric, *Curcuma longa*, and other *Curcuma* species. Turmeric is also widely used as medicinal plant in oriental tradition, the treatment of snake bite poisoning being one of its uses. The extract of the plant was shown to inactivate almost completely the neurotoxin of the cobra, *Naja siamensis* (Ferreira et al., 1992). Another unsaturated ketone of turmeric, ar-turmerone, was shown to inhibit the lethal action of rattlesnake venom, in addition to exhibit other important biological activities (Cherdchu and Karlsson, 1983).

2.9.7 Coumarins

Coumarin occure often in considerable amounts in anti-snake venom plants. Exsamples are *Dipteryx odorata, Dipteryx punctata* and *Liatris squarrosa*. Coumarin showed increase survival mice from *Brothrops jararaca* snake venom when administered 1 hr orally before envenomation Pereira et al. (1994). The other compounds in this group as herniarin and ayapin are isolated from the Amezonian anti-snake venom plant *Eupatorium triplinerve* which shows to exhibit considerable hemostatic activity (Bose and Sen, 1941).

2.9.8 Flavonoids

Flavonoids have been held responsible for anti-inflammatory, anti-hepatotoxic, anti-hypertensive, antiarrhytmic, hypocholesterolemic, anti-allergic and anti-tumour (Alcaraz and Holt, 1985). Qurcetin and several of it glycoside are the flavonoids most often encountered in anti-snake venom plants (Iglesias et al., 2000). Rutin was against vascular effects cause by *Bothrops atrox* venom (More et al., 2000). Thus, morin presented in the anti-scorpion sting plant *Artocarpus integrifolia* and in the anti-snake venom plant. (Bjarnason and Fox, 1994; Pessini et al., 2001).

2.9.9 Coumestans

Eclipta prostrata was studied for snake poison antidotes over ten years ago. The plant is well known as an anti-snake poison both in China and Brazil. Wedelolactone was found to be the main compounds of coumestan to neutralize the lethal effect of South American rattlesnake (*Crotalus durissus terrificus*) venom in mice (Mors et al., 1989). Thus, wedelolactone was shown to exert several well defined pharmacological actions such as antimyotoxic, antihemorrhagic, antiproteolytic and antiphospholipasic (Melo et al., 1994).

2.9.10 Tannins

The first scientific approach to a natural anti-snake venom remedy presently concerns not only a micromolecule but also a much larger polyphenol. The juice of the stem and rootstock of the banana tree (*Musa paradisiaca*) has been used against snake bite in the Caribbean area (Abubakar et al., 2000).

2.9.11 Polysaccharides

Polysaccharides have been shown to exhibit mainly anti-inflammatory and immunomodulating activities. These properties were extended to anti-snake venom actions. The bark of *Casearia sylvestris* is known as such as a remedy throughout Brazil. Its aqueous extract yielded

besides sitosterol and stimasterol and a mixture of polysaccharides, which could be resolved into five distinct units three being neutral and two of acidic nature (Mors et al., 2000). *Calendura officinalis* anti-snake venom plant was already mentioned to contain steroids and triterpenes has its wound healing action attributed to the presence of an immunostimulating heteroglycan (Varlan et al., 1989).

2.10 Ob-Chuei-Thai (*Cinnamomum iners* Reinw. ex Blume)

2.10.1 Botany

Scientific name: *Cinnamomum iners* Reinw. ex Blume
Common name: Chouang, Sikhai, Medang Teja, Medang kemangi, Teja Badak, Teja Lawang, Kayun Manis Hutun
English name : Wild Cinnamom
Family: Lauraceae

The genus *Cinnamomum* (family : Lauraceae) consists of 100 species of trees and shrubs distributed in Asia such as *C. camphora*, *C. cassia*, *C. zylanicum*, *C. aromaticum* and many of them are aromatic and flavouring (Pomjit, 1989). *C. iners* is one such species widespread throughout Thailand, Peninsular Malaysia, Sumatra, Java, Borneo (Sarawak, Brunei, Sabah, West-, Central- and East-Kalimantan), Philippines. It is cultivated up to an altitude of 500 metres above mean sea level where the mean temperature is 27 °C and annual rainfall is 2000-2400 mm. (Wannissorn et al., 2005).

2.10.2 Characteristics

C. iners is a small or medium sized tree (Figure 2-7.), sometimes planted as an ornamental tree because of its pretty foliage. The bark is grey and smooth, with horizontal, wavy bands and is 6 to12 mm. thick. The wood is light, yellowish brown, moderately hard, shining, smooth, and scented. The young leaves are reddish-pink and tender, while the mature leaves are dark green. The leaves are opposite as a rule, smooth, leathery, lanceolate, oblong linear-oblong, rarely

ovate 8 to16 cm. long and up to13 cm. wide rounded at the base and pointed at the tip. The blades are 3-nerved. The flowers are borne in panicles and appear creamy when young. The fruits are blue-black and dispersed by birds. The branchlets are nearly smooth. The flowers are yellowish and 4 mm. in length. The fruit is about 10 mm. long with the base sunk in the perianth. (Vutthumdate, 1987).

Figure 2- 7. Character of stem (A), leaves (B), flowers (C) and fruits of *Cinnamomum iners* Reinw. ex Blume

2.10.3 Phytochemistry

The chemical constituents in *C. iners* were previously reported (Samitinun et al., 1982). The chemical constituents included monoterpene, sesquiterpene alkaloids, condensed tannins, flavonoids, triterpenes, and antraquinones (Table 2-3)

Table 2-3. The chemical components in *C. iners*

Part of plant	Chemical component
Leaves	Phenolic acids, cinnamaldehyde, eugenol, hydroxyl- chalcone cinnamophilin and coumarin (Dung, 1996)
Bark of stem	alkaloids, condensed tannins, flavonoids, antraquinones and triterpenes (Gardner et al., 2000)
Stem	saponin and terpene (Braud et al., 2000)
Root	saponin, terpene, cinnamaldehyde and safrol (Gardner et al., 2000)

The volatile oil are main components in all parts of *C. iners* (Table 2-3) such as apilo, camphore, β-caryophyllene, eugenol, α-pinene, safrol, geraniol, cinnamaldehyde, linalool, eugenol, limonene, β-pinene and piperitone (Figure 2-8.) (Boonyaprapatsorn and Choogchaicharoan-porn, 2000).

Table 2-4. Volatile components in all parts of (flowers, leaves, bark, stem, bark of stem and root) of *C. iners* (Phutdhawong et al., 2007)

cis-lina lool oxide, *trans*-linaool oxide, linalool, borneol, terpinen-4-ol, α-terpineol, 2,6-octadien-1-ol geraniol, propanoic acid, (-)-bornyl acetate, *cis*-α-bergamotene, cyclohexene, 3-allyl-6-methoxyphenol, α-copaene, β-elemene, dodecanal, caryophyllene, α-cubebene, (+)-aromaden- drene, 2-propen-1-ol, α-humulene, α-amorphene, germacrene-D, caryophyllene oxide, viridiflorol, aromadendrene, alloaromadendrene, β-selineneal, nerolidol, palustrol, α-muurolene, β-bisabolene, ∞-

cadinene, epiglobulol, α-cadinol, unidentified, spathulenol, tetradecanal, calarene, hexahydro, isospathulenol, α-copaene, cadinol, α-longipinene and benzyl benzoate

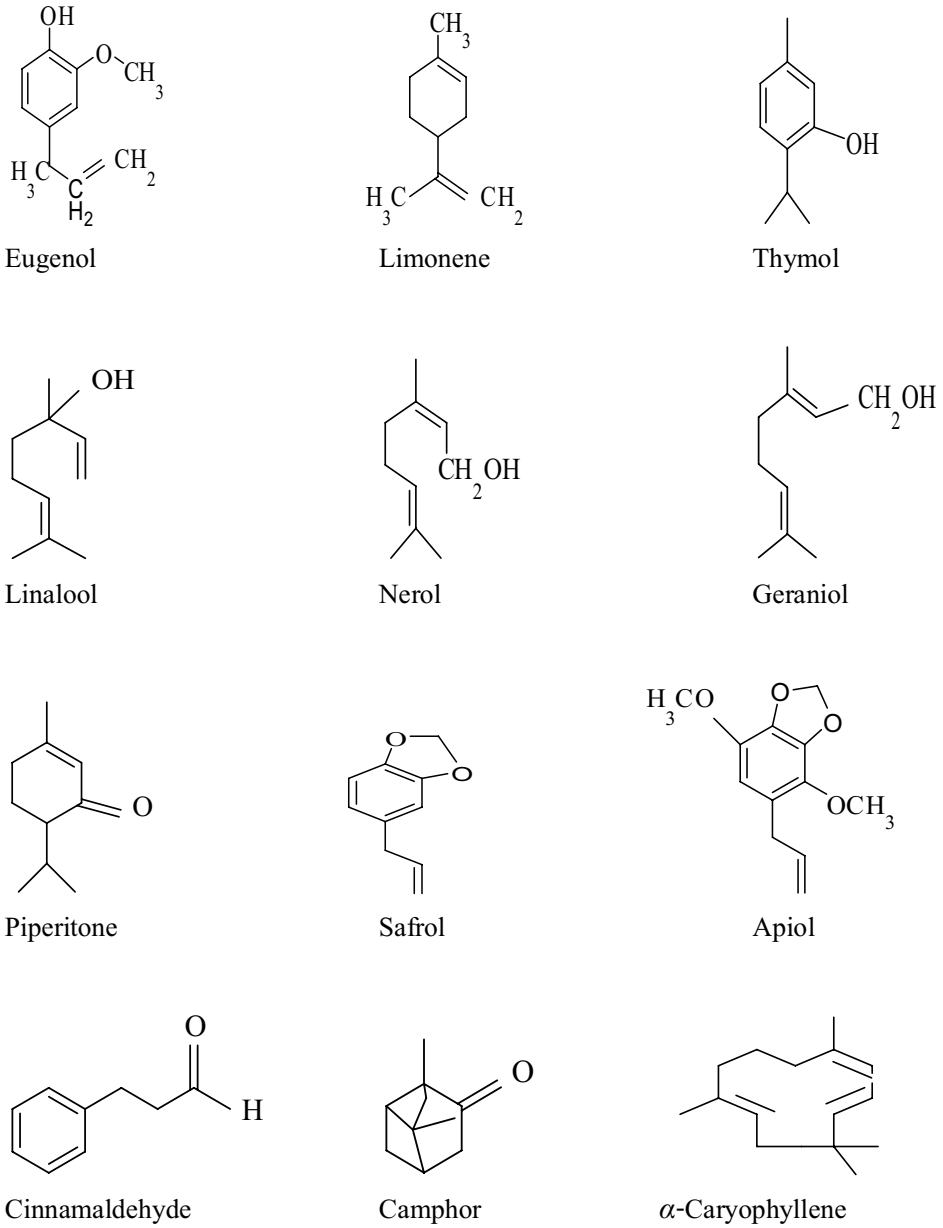


Figure 2-8. Structure of some chemical constituents of *C. iners*

2.11 Ethnopharmacology

Cinnamomum genus has demonstrated the antibacterial (Alzoreky and Nakahara, 2003), the antifungal (Baruah et al, 1996), the anti-clotting actions (Kosuge et al., 1984), the anti-inflammatory action (Kubo et al, 1996), blood sugar control, the anti-oxidant activity (Lin et al., 1995), the anti-tumor property (Shin et al., 1992) and cardiac diseases (Itokawa et al., 1984).

C. iners possesses various biological activities such as antioxidant, antimicrobial, antidiabetic and antiallergic (Iida et al., 1997, Mastura et al., 1999, Anon, 1976; Lee and Ahn., 1998). The volatile oil use as flavoring agent in food, beverages, preparations, cosmetics, toiletries and perfumes. Also used in aromatherapy (Rob and Jayewardene., 1975).

C. iners is good detoxifying herp and acts as a pain reliever. Various terpenoides found in volatile oil are believed to account for cinnamon's medicinal effects. Important among these compound are eugenol and cinnamaldehyde. The volatile oil also shows antimicrobial activity against *Psudomonas*, *Aspergillus parasiticus, Stephylococcus aureus, Candida, Saccharomyces cerivisiae* and gram positive (Toda et al., 1990; Chiou et al., 1992).

C. iners oil helps break down fats in the digestive system, possiply by boosting the activity of digestive enzymes. Volatile oil also has a potential role in the treatment of diabetes. *C. iners* contains a chemical called methoxy hydroxyl chalcone which can reduce the blood glucose level (Akhtar, 1979). Methoxy hydroxyl chalcone possess insulin-like activity (Anderson et al., 2004) and have demonstrated a dose-dependent increase in glucose utilization in animal muscle tissue (Qin et al., 2003; 2004). In a clinical trial with type 2 diabetes patients, power extract (1 to 6 g/day for 40 days) decreased blood glucose levels (Khan et al., 2003, Onderoglu et al., 1999).

C. iners extracts exhibited antioxidant action with an ethanol extract being more effective than an aqueous extract (Lin et al., 2003). Studies have evaluated the relative antioxidant action of cinnamon against other herbs and spices, and against alpha-tocopherol. In a study of wound-healing action of an ethanol extract of cinnamon, the significant increase in wound healing was attributed to antioxidant activity (Lee and Shibamoto, 2002; Murcia et al., 2004; Jayaprakasha et al., 2003).

Laboratory experiments suggest anti-inflammatory action of certain chemical components in *C. iners*. Cinnamaldehyde inhibited nitric oxide production, which has been implicated in the inflammatory disease process (Lee et al., 2002; 2005) and has demonstrated

inhibition of cyclo-oxygenase (COX-2) catalyzed prostaglandin E_2 biosyn- thesis (Huss et al., 2002; Friedman et al., 2000). Curinary *C. iners* is on the food and drug administration's list of

herps generally regarded as safe. The amounts of *C. iners* normally used in food are non toxic, although some people develop allergic reactions after eating this spice. Chronic use may cause inframmation in the mouth. Ingestion of volatile oil cause redness and burning of the skin. *C. iners* handlers have a high incidence of asthma, skin irritation and hair loss. Toothpastes and oilment containing *C. iners* may cause stomatitis and dermatitis in some cases. Do not use in case of fever and pregnary (Bisset, 1994).

.

CHAPTER 3
MATERAILS AND METHODS

3.1 Plant materials

The fresh twigs of *Cinnamomum iners* Reinw. ex Blume (twigs size 3-4 cm diameter) were collected from Thai herbal garden, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai campus, Songkhla, in March, 2005. A voucher specimen (accession number : SKP 096-03-09-01) was deposited at the Herbarium of Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai campus, Songkhla, 90112, Thailand.

Figure 3-1. *Cinnamomum iners* Reinw. ex Blume (Lauraceae)

3.2 Venoms and antivenoms

The lyophilized venoms and antivenoms of *Naja kaouthia* (NK, Family : Elapidae) and *Calloselasma rhodostoma* (CR, Family : Viperidae) were provided by Queen Saovabha Memorial Institute, Thai Red Cross Society, Bangkok, 10330, Thailand. The venom was dissolved in distilled water and kept at 4 °C for further study. The venom concentration was expressed in terms of dry weight (mg/ml stock venom solution)

Figure 3-2. Snake venom and antivenom

(A) *Naja kaouthia* and *Calloselasma rhodostoma* venoms.

(B) *Naja kaouthia* and *Calloselasma rhodostoma* antivenoms.

3.3 Chemical and reagents

3.2.1 Chemical and reagents for extraction

95% Ethanol, commercial grade (L.B Science®, Thailand)

n-Hexane, commercial grade (L.B Science®, Thailand)

Chloroform, commercial grade (L.B Science®, Thailand)

Ethyl acetate, commercial grade (L.B Science®, Thailand)

Methanol, commercial grade (L.B Science®, Thailand)

Acetone, commercial grade (L.B Science®, Thailand)

Methanol, AR grade (Merck®, Germany)

Acetonitrile, HPLC grade (J.T. BAker®, USA)

Distilled water for HPLC

Note : All of commercial solvents were distillated before used.

2.3.2 Chemical and reagents for SDS-PAGE

Acrylamide (Amersham Bioscience AB, Sweden)

N, N'-methylenebisacrylamide (Pharmacia Biotech, Sweden)

Tris : Tris (hydroxymethyl) aminomethane (USB™, Ohio)

SDS: Sodium dodecyl sulfate (Pharmacia Biotech, Sweden)

Glycine (USB™, Ohio)

Brilliant blue R-250 (USB™, Ohio)

Bromophenol blue dye (USB™, Ohio)

APS: Ammonium persulfate (Sigma®, USA)

TEMED : N', N', N', N'-tetramethylethylenediamine (Invitrogen™, USA)

EDTA : Ethylenediaminetetraacetic acid (USB™, Ohio)

HCl : Hydrochloric acid (Merck®, Germany)

NaOH : Sodium hydroxide (Merck®, Germany)

Glycerol (Merck®, Germany)

Sucrose (Merck®, Germany)

Acetic acid (L.B Science®, Thailand)

Kaleidoscope prestained standards catalog number 161-0324 (Bio-Red, USA)

2.3.3 Chemical and reagents for phospholipase A_2 enzyme activity assay and proteolytic enzyme activity assay

$CaCl_2$: Calcium chloride (Merck®, Germany)

NaCl : Sodium Chloride (Merck®, Germany)

KCl : Potassium chloride (Merck®, Germany)

Na_2HPO_4 : Sodium hydrogen phosphate anhydrous (Merck®, Germany)

KH_2PO_4 : Potassium dihydrogen phosphate (Merck®, Germany)

TCA : Trichloroacetic acid (Merck®, Germany)

Casein (USB™, Ohio)

RBC (Human red blood cell)

Hen's egg yolk

3.4 Instrumentation

3.4.1 Apparatus used for extraction and elucidation structure

Silica gel (Merck®, Germany) was used for normal column chromatography and silica gel 60 GF_{254} no. 5544 (Merck®, Germany) was used for thin-layer chromatography (TLC). Reverse phase HPLC was performed using Apollo® C-18 (250 X 4.6 mm I.D., particle size 5 μm) guard column. Melting point was determined on Buchi® 520 apparatus. UV spectra were taken on Shimadzu® UV 2401 PC spectrophotometer. IR spectra were measured as KBr disc on Jasco® IR 810 spectrophotometer. All NMR spectra were observed on a Varian Inova® 500 specphotometer (Germany) using TMS as internal standard with ^{1}H and ^{13}C nuclei and were recorded chemical shift parameter (δ) value in ppm down field from TMS (0.00).

3.4.2 Apparatus used for SDS-PAGE

SDS-PAGE using Refraction™ Twin Vertical Series (Galileo Bioscience, USA). All samples were applied on 12.5% polyacrylamide slab gels and then were stained with Coomassie brilliant blue R-250 to determine the bands of protein.

3.4.3 Apparatus used for proteolytic enzyme activity and phospholipase A_2 enzyme activity

The absorbance at 280 nm for proteolytic enzyme activity and at 580 nm for PLA_2 enzyme activity were measured using a Spectronic® Genesys 5 (Miltonroy, USA). Incubation samples (37 °C), using waterbath (Memmert, Geramany) were carried out.

3.5 Methods

3.5.1 Isolation of chemical constituents from the twigs of *C. iners*

3.5.1.1 Extraction

The twigs (4.3 kg) of *C. iners* were washed with distillated water. The cleaned plant materials were cut into small pieces and dried at 50 °C in oven and then grounded. The dried twigs powder (2.7 kg) was macerated three times with 95% ethanol (4×2L) at room temperature for three days and filtered. The pooled filtrate was evaporated *in vacuo* and then brown residue (193.3 g) was obtained. This residue (148.8 g) was pre-adsorbed on Keisel- ghur® in a glass percolator and further eluted with *n*-hexane, until the filtrate was clear. The pooled of *n*-hexane filtrate was evaporated by Rotavap® to give 3.1 g Hexane fraction (2.1% yielded). The remaining air dried residue was then exhaustively eluted with chloroform, ethyl acetate and methanol, respectively. After evaporating by Rotavap®, 2.5 g (1.7% yielded) of $CHCl_3$ fraction, 20.7 g (13.9% yielded) of EtOAc fraction and finally 119.9 g (80.6% yielded) of MeOH fraction were obtained (Figure 3-3).

The Hexane, $CHCl_3$, EtOAc and MeOH fractions were collected in refrigerator (ca. 7 °C) before further purification.

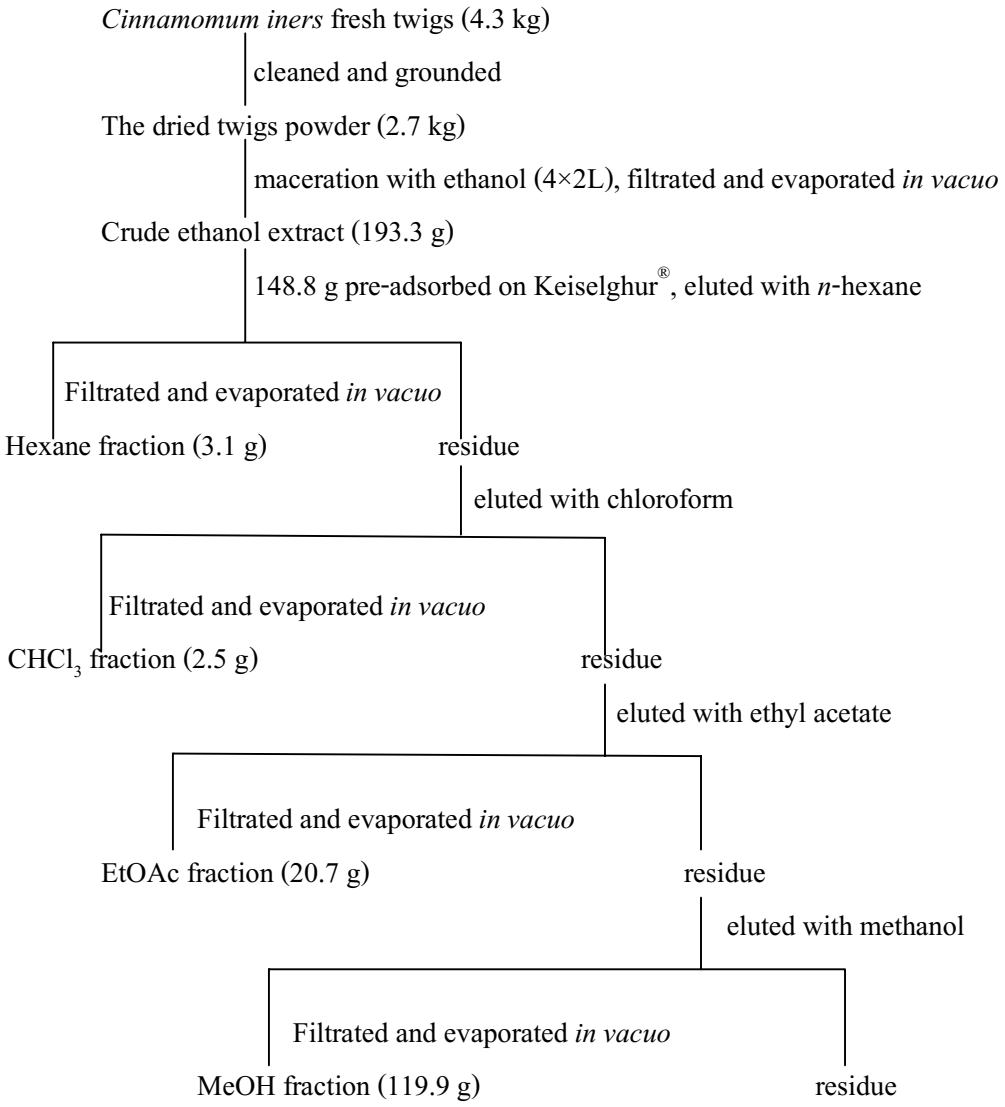


Figure 3-3. Extraction and isolation scheme of *C. iners* Reinw. ex Blume

3.5.1.2 Isolation and purification of ethyl acetate fraction from *C. iners*

The EtOAc fraction (EA-I) (1.3 g) was subjected to column chroma- tography (CC) (silica gel, 200 g) by eluting with Hexane : EtOAc (4:1 to 1:4) to furnish 6 fractions [(EA-I-1) – (EA-I-6)]. Fraction EA-I-3 (800 mg) was then re-chromatographic to a new CC (silica gel, 200 g) eluting with Hexane : EtOAc (9:1 to 1:4) to obtain 6 fractions [(EA-I-3-1) - (EA-I-3-6)]. Fraction EA-I-3-4 (50 mg) was purified by preparative TLC and gave a pure compound 1 (15 mg). Fraction EA-I-5 was further isolated with CC (silica gel, 200 g) and eluting with $CHCl_3$: MeOH (4:1 to 10:0) to obtain 4 fractions [(EA-I-5-1) - (EA-I-5-4)].

The EtOAc fraction (EA-II) (11.9 g) was chromatographic by CC (silica gel, 500g) eluting with $CHCl_3$: MeOH (9:1 to 1:9) to totally 7 fractions [(EA-II-1) - (EA-II-7)] were collected and combined on the basis of the TLC profiles after detection with UV lamp and using 50% sulfuric acid in water as a spraying reagent. After purification, compound 2 (20 mg) was separated from fraction (EA-II-6) (80 mg). Fraction (EA-II-3) (3.3 g) was purified by CC on silica gel (200 mg) with $CHCl_3$: MeOH (4:1 to 1:4) to afford 5 fractions [(EA-II-3-1)-(EA-II-3-5)]. Fraction EA-II-3-1 (200 mg) was re-chromatographic over silica gel (200 g) using $CHCl_3$: MeOH (9:1 to 10:0) to give 3 fractions [(EA-II-3-1-1) - (EA-II-3-1-3)] but all isolated fractions were not shown pure compound (Figure 3-4).

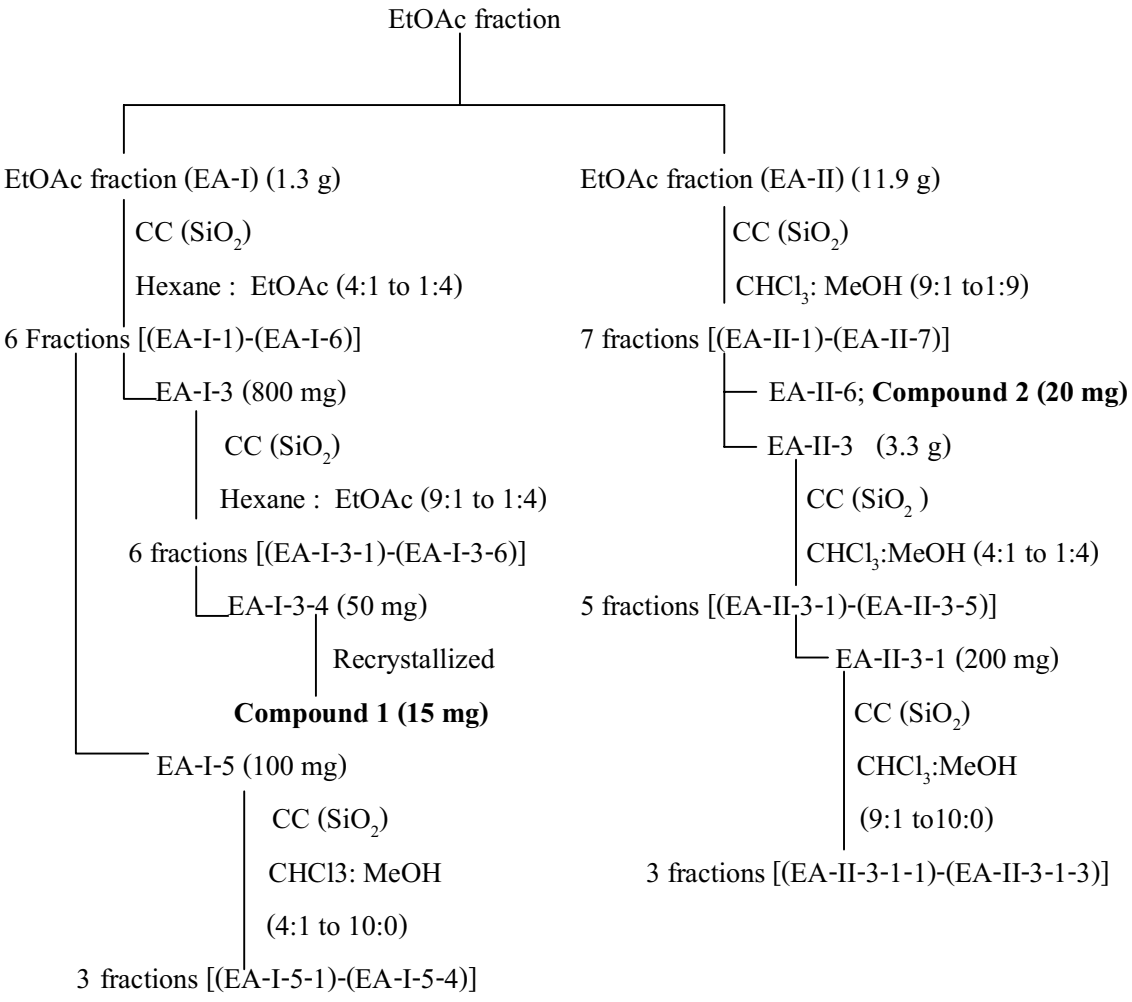


Figure 3-4. Isolation and purification of compound 1 and 2 from EtOAc fraction

3.5.1.3 Isolation and purification of methanol fraction from *C. iners*

The MeOH fraction (ME-I) (30.0 g) was subjected to CC on silica gel column (800 g) and eluted with $CHCl_3$: MeOH (4:1 to 1:4) to afford 20 fractions [(ME-I-1)-(ME-I-20)]. Fraction ME-I-10 (1.2 g) was applied to sephadex-LH 20 CC with MeOH as eluent to give 6 fractions [(ME-I-10-1) - (ME-I-10-6)]. The result, all fractions were not isolated the pure compound.

A part of fraction ME-I-4 (4.0 g) from ME-I was further subjected to CC using $CHCl_3$: MeOH (4:1 to 1:9) to afford 3 fractions [(ME-I-4-1) - (ME-I-4-3)]. The ME-I-4-2 fraction (700 mg) was repeatedly subjected to CC using silica gel column (4:1 to 1:9) to give 3 fractions [(ME-I-4-2-1) - (ME-I-4-2-3)]. Fraction ME-I-4-2-2 (300 mg) was resubjected to RP-18 HPLC with isocratic of CH_3CN : H_2O from 10:90 to 100:0 or MeOH : H_2O from 10 : 90 to 100:0 with flow rate 1 ml/min. The compound was not isolated from this method. The residue methanol (1.2 g) (ME-II) was fractionated by CC on silica gel eluted with $CHCl_3$: MeOH (4:1 to 10:0) to give 6 fractions [(ME-II-1) - (ME-II-6)]. Fraction ME-II-4 was re-loaded to CC on silica gel using $CHCl_3$: MeOH (4:1 to1:9) to give 5 fractions [(ME-II-4-1)-(ME-II-4-5)]. Fraction ME-II-4-1 was further purified using silica gel column with $CHCl_3$: MeOH (4:1 to 1:9) to afford 6 fractions [(ME-II-4-1-1)-(ME-II-4-1-6)]. Fraction ME-II-4-1-2 was purified by CC on silica gel with $CHCl_3$: MeOH (4:1 to 1:9) as eluent to afford 4 fractions [(ME-II-4-1-2-1)-(ME-II-4-1-4)]. Compound 3 (30 mg) was purified and recrystallized with MeOH from fraction ME-II-4-1-2-2 (Figure 3-5).

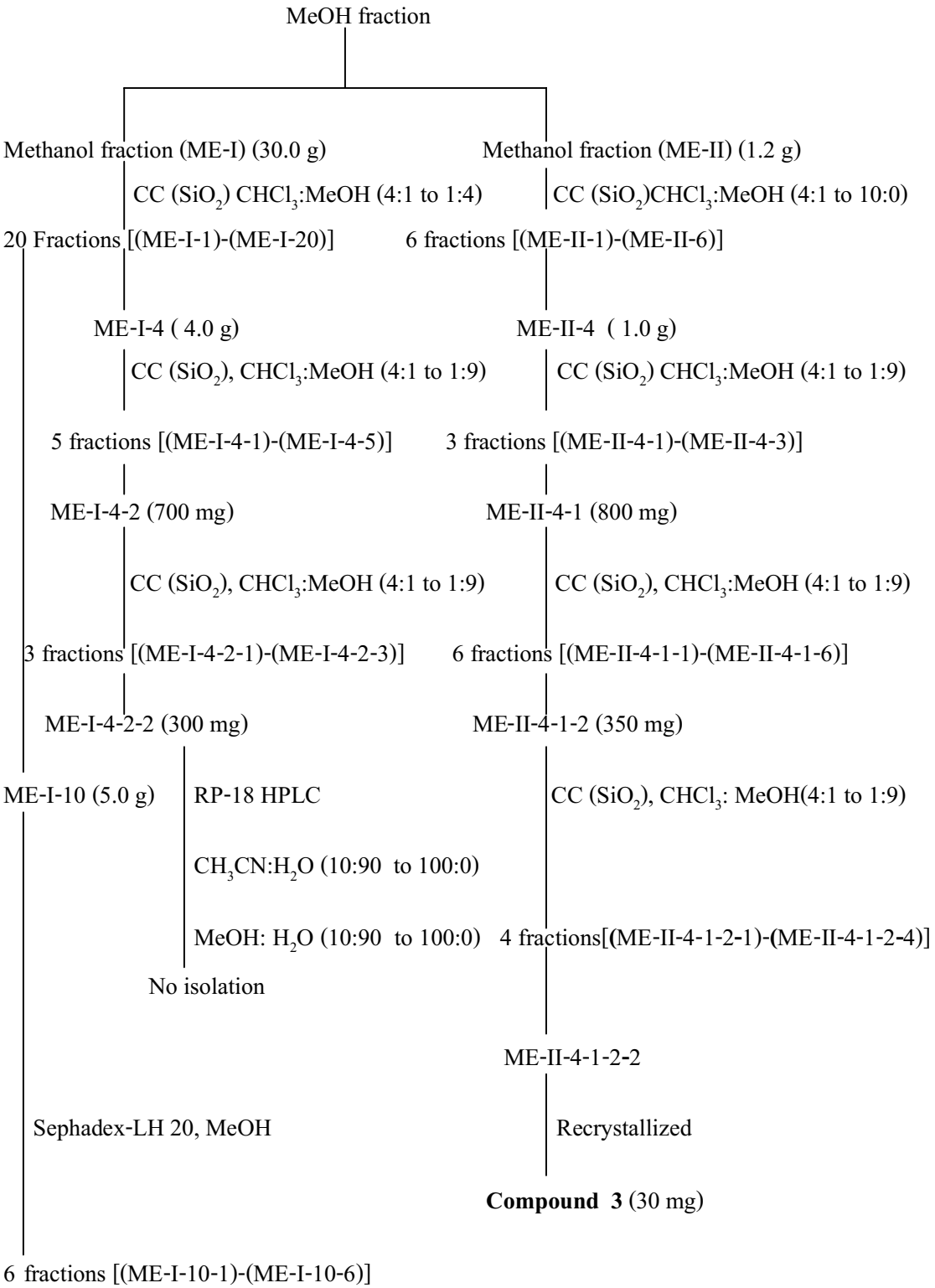


Figure 3-5. Isolation and purification of compound 3 from MeOH fraction

3.5.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis was performed on 12.5% polyacrylamide gel according to the method of Bollage and Stuart (1993). NK or CR venoms were dissolved to 10 mg/ml solution in distilled water. Various concentrations of the extracts (50, 10 and 2 mg/ml) or compounds (10, 5 and 2 mg/ml) were dissolved in 95% ethanol and mixed with NK or CR venom. After incubation for 1 hour at room temperature, 0.1% bromophenol blue and sucrose were added into the mixtures. They were then centrifuged and supernatant was loaded on bottom of well. Allow the gels to run at 50 mA for 1 hour. Then, gels were stained with 0.2% (w/v) Coomassie brilliant blue R-250 and destained with 10% acetic acid in water. The blue color of each band will be appeared and finally, gels were dry in oven.

3.5.2.1 Procedures of SDS-PAGE

- Clean glass plate and spacers and set up gel plates (Figure 3-6)
- Preparation of polyacrylamide gels (separating and stacking gel) (Figure 3-7 and Table 3-1).
- Load samples into each well, start running the gel at 50 mA and continue until the dye front (bottom line) reaches the end of the gel (Figure 3-8).
- After SDS-PAGE, the gel was transferred into a plastic container
- Coomassie staining solution was poured onto the gel, and it was incubated at room temperature for 2 hours with shaking (Figure 3-9)
- The gel was then destained with destaining buffer and after destaining, the gel was dried in oven.

Table 3-1. Combination of all reagents except the TEMED for the 12.50% separating gel and the

Reagents	Separating Gel	Stacking gel
TEMED	10.00 µl	5.00 µl
30% acrylamide / Bis	2.90 ml	0.90 ml
1.5 M Tris, pH 8.8	1.75 ml	-
0.5 M Tris, pH 6.8	-	1.00 ml
DW	1.00 ml	1.90 ml
1% SDS	0.70 ml	0.40 ml
10% APS	0.35 ml	0.20 ml

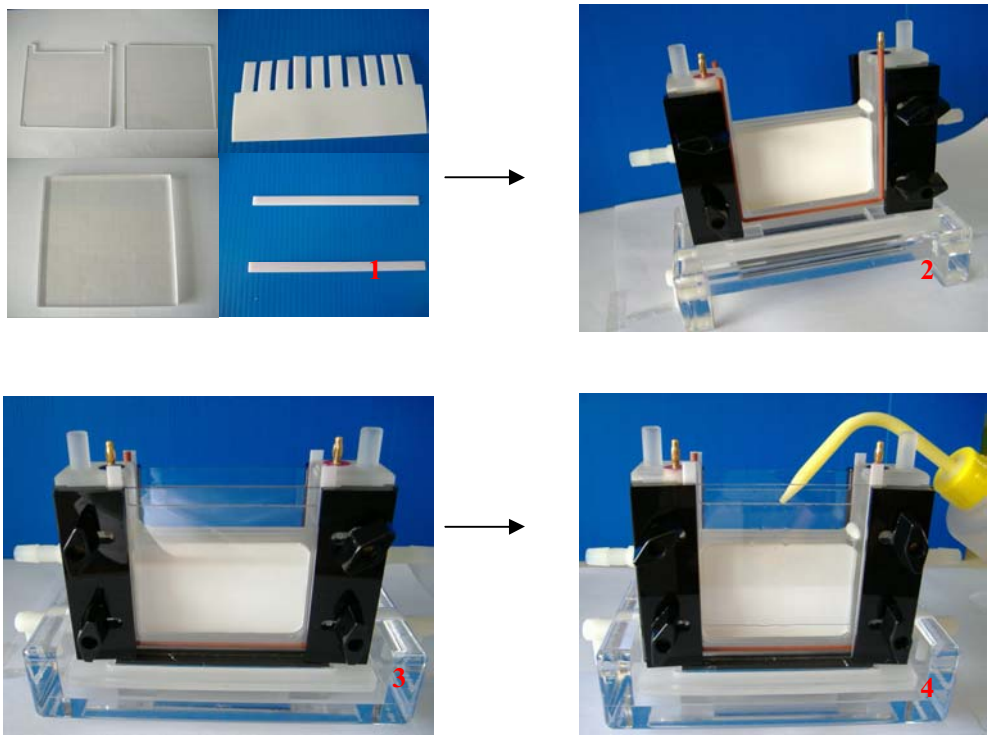


Figure 3-6. Clean and set up gel plate

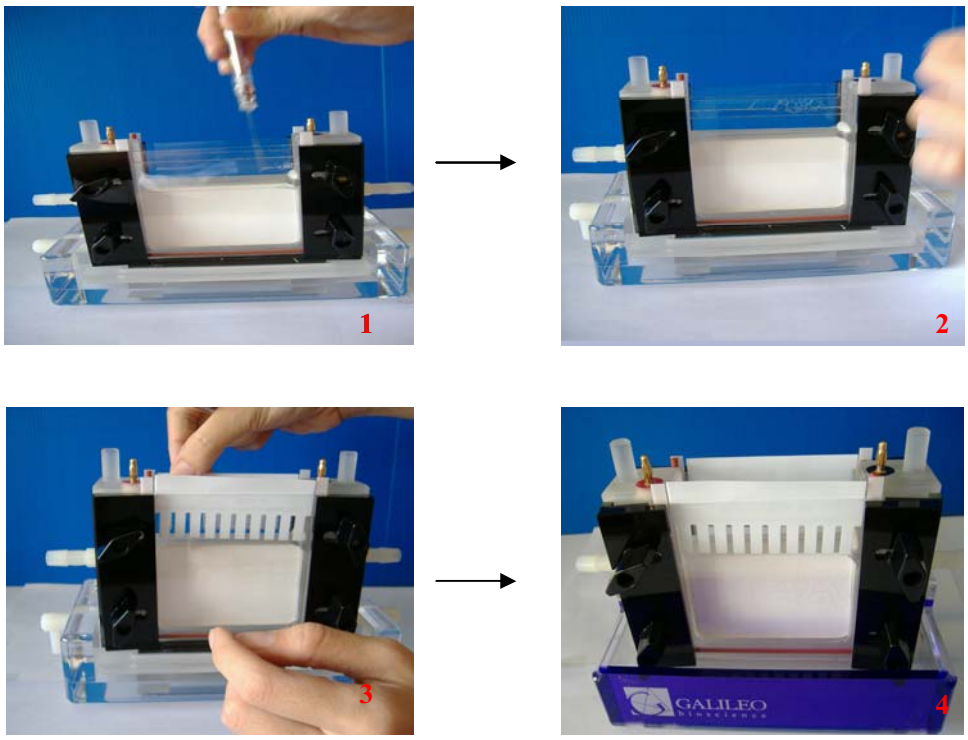


Figure 3-7. Preparation of separating and stacking gel

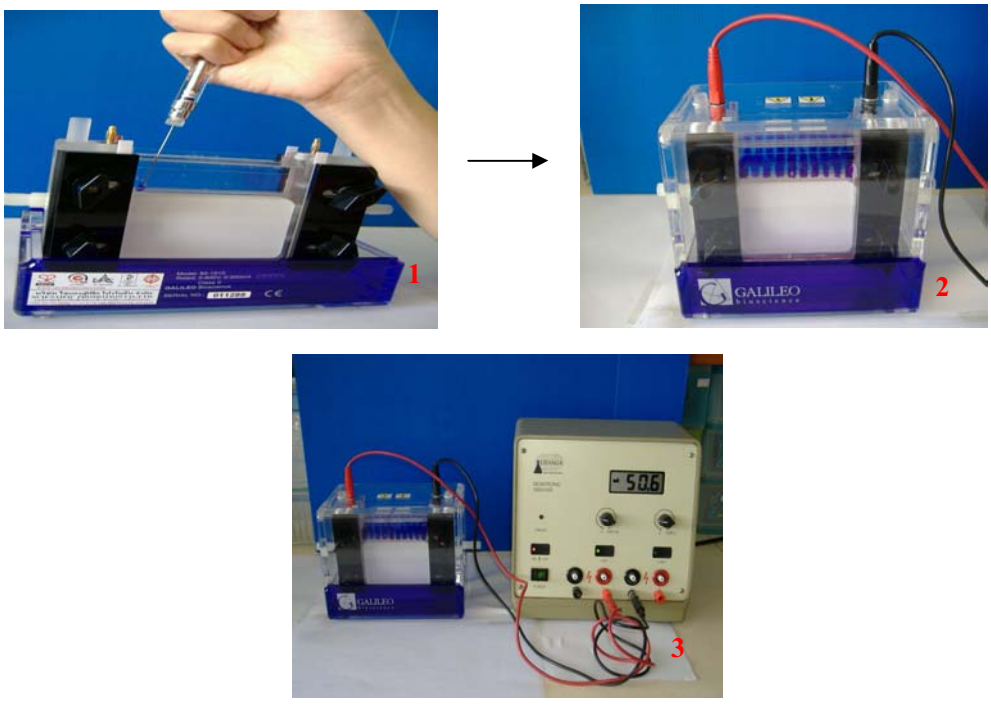


Figure 3-8. Load samples and running the gels

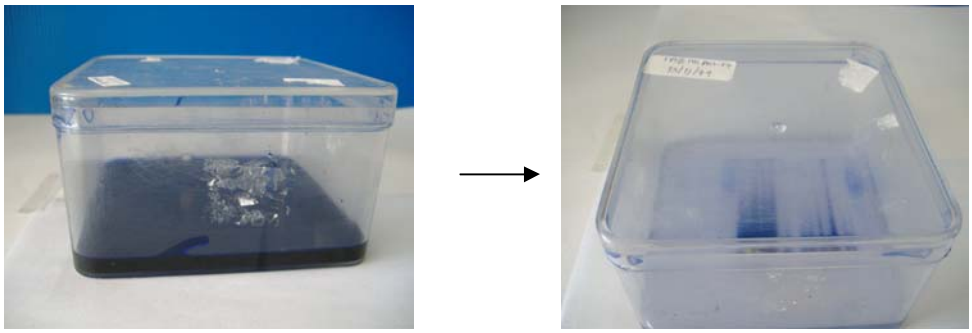


Figure 3-9. Staining and drying gels

3.5.3 Proteolytic enzyme activity

Proteolytic enzyme activity was determined using casein as substrate, described by Mandlbaum et al., 1982. CR venoms were dissolved to make a 2 mg/ml solution in distilled water. The samples were dissolved in 95% ethanol in concentration of 50, 10 and 2 mg/ml or compounds (10, 5 and 2 mg/ml). Various concentrations of extracts were mixed with CR venom and then added 2.0 ml of 1% casein in 0.2 M PBS (pH 8.8) and 50.0 µl of 8 mM $CaCl_2$ solution. After incubation at 37 °C for 90 min, the reaction was stopped by addition 1.0 ml of 5% TCA. After centrifuged (5,000 rpm) for 10 min at 25 °C, the supernatant was aliquot and then centrifuged again with 10,000 rpm for 5 min at 25 °C. Finally, the supernatant was measured the absorbance at 280 nm. Activity was expressed as percentage of caseinolytic activity (Figure 3-10).

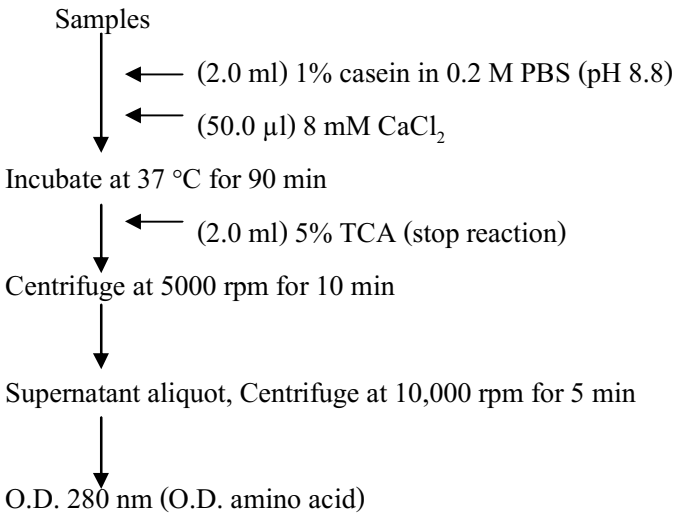


Figure 3-10. Protocol of proteolytic enzyme activity

3.5.4 Phospholipase A_2 (PLA_2) enzyme activity

PLA_2 activity was determined by an indirect hemolytic test (De Hass et al., 1968) using human erythrocytes and hen's egg-yolk emulsion as substrate. The extracts were dissolved in 95% ethanol in concentration of 50, 10 and 2 mg/ml. or compounds (10, 5 and 2 mg/ml). CR venoms were dissolved to 80 mg/ml solution in distilled water. NK venoms were dissolved to 160 mg/ml solution in distilled water. CR venom or NK venom was mixed with various concentrations of extracts and incubated for 1 hour at room temperature. Then, 50.0 µl egg-yolk emulsions were added and incubatied at 37 °C for 10 min. After incubation, 50.0 µl of 60 mM EDTA, 3.2 ml of PBS (pH 7.5) and 1.2 ml of 2% (v/v) red blood cell in PBS (pH 7.5) were added, respectively, and stand in hot air oven at 37 °C for 60 min. Finally, the mixtures were centrifuged at 5,000 rpm for 10 min at 25 °C and the supernatant was measured the absorbance at 580 nm (Figure 3-11).

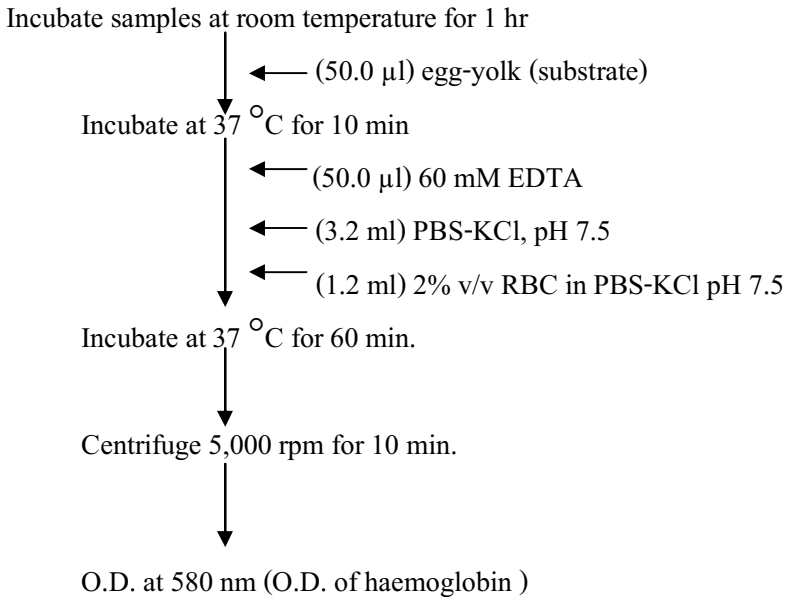


Figure 3-11. Protocol of phospholipase A_2 enzyme activity

3.5.5 Statistical analysis

Statistical analysis was performed using Student's test (Microsoft Excel Software). A value of $p < 0.05$ was considered significant.

CHAPTER 4
RESULT AND DISCUSSION

4.1 Phytochemical investigation on *Cinnamomum iners*

The fresh twigs of *Cinnamomum iners* were investigated and isolated. The crude extract was further fractionated using many solvents with increasing polarity, as described in Chapter 3. Compound 1 was isolated and identified as *ß*-sitosterol from EtOAc fraction (EA-I) (1.3 g). Furthermore, *ß*-Sitosterol-*3-O-ß*-D-glucopyranoside (compound 2) was separated, identified and characterized form EtOAc fraction (EA-II) (11.9 g). The MeOH fraction (ME-II) (1.2 g) afford compound 3 (compound 3). The structures of all compounds (1-3) were determined using IR, ^{1}H-NMR, ^{13}C-NMR, COSY, HMBC, HMQC and mass spectrums and described as follow.

4.1.1 Identification of compound 1

Compound 1 was isolated and purified from the EtOAc fraction of ethanol extract, as presented in figure 3-4 (Chapter 3). This compound was white crystalline, m.p. 136 °C (Literature* 136-138 °C), R_f 0.32 (EtOAc : Hexane, 1:9). The EIMS of compound 1 showed the molecular ion peak at *m/z* 414, in agreement with the formula $C_{29}H_{50}O$ (Figure 4-1) (calcd. *m/z* 414.3661).

The ^{1}H NMR ($CDCl_3$, 500 MHz) of compound 1 showed a characteristic steroidal pattern (Table 4-1). A downfield proton at δ 3.52 was consistent with C-3 proton germinal to hydroxyl group. Therefore, the C-3 hydroxyl group was assigned to be *ß* and germinal hydrogen was α-oriented. Two 3H singlets at δ 0.68 and 1.01 were assigned to the C-18 and C-19 methyl protons, respectively. A triplet at δ 5.35 was assigned to the olefinic C-6 proton. Other signals appeared as doublets at δ 0.92, 0.83 and 0.81 were assigned to the C-21, C-26 and C-27 methyl protons, respectively. Another 3H triplet at δ 0.84 was assigned to the C-29 methyl protons.

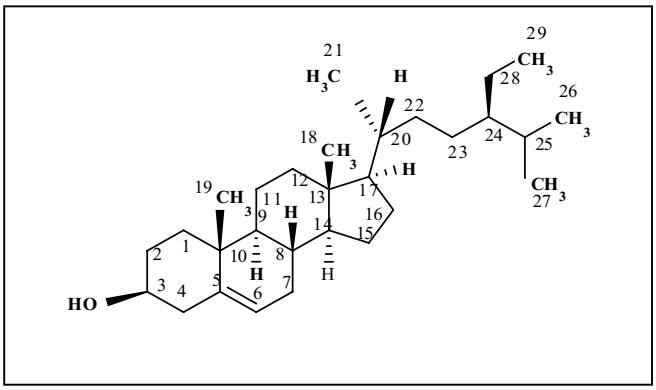


Figure 4-1. Structure of compound 1 (*β*-Sitosterol)

In comparison with authentic *β*-Sitosterol (provide by Assist. Prof. Dr. Jindaporn Puripattanavong) in term of m.p., ^{1}H and ^{13}C NMR spectrum data (Table 4-2), the compound 1 was identified and established as *β*-sitosterol (Chang et al., 1981).

Table 4-1. ^{1}H NMR data of compound 1 (500 MHz, $CDCl_3$)

Hydrogen No.	δ (ppm)	M	J (Hz)	Literature* δ (ppm)	$\Delta\delta$ (ppm)
3	3.52	m	-	3.52	0.00
6	5.35	dd	2.0, 5.1	5.35	0.00
18	0.68	s	-	0.68	0.00
19	1.01	s	-	1.00	0.01
21	0.92	dd	4	0.92	0.00
26	0.83	d	8.3	0.81	0.02
27	0.79	d	8.5	0.80	0.01
29	0.82	t	6.6	0.85	0.03

(Literature* Chang et al., 1981)

Table 4-2. ^{13}C NMR data and ^{1}H-^{13}C correlation of compound 1 (500 MHz, $CDCl_3$)

Carbon No.	δ (ppm)	HMBC	HMQC	Literature* δ (ppm)	$\Delta\delta$ δ (ppm)
1	37.3	1	19	37.4	-0.1
2	31.9	2	-	31.9	0.0
3	71.8	3	4	71.8	0.0
4	42.3	4	6	42.3	0.0
5	140.7	-	4,19	140.7	0.0
6	121.7	6	4	121.7	0.0
7	31.6	7	-	31.6	0.0
8	31.9	8	6	31.9	0.0
9	50.2	9	19	50.1	0.1
10	36.5	-	6,19	36.5	0.0
11	21.1	11	-	21.0	0.1
12	39.8	12	18	39.7	0.1
13	42.3	-	18	42.3	0.0
14	56.8	14	17,18	56.8	0.0
15	24.3	15	-	24.3	0.0
16	28.3	16	15, 18, 21, 22	28.2	0.1
17	56.1	17	-	56.0	0.1
18	11.9	18	-	11.8	0.1
19	19.4	19	21,22	19.4	0.0
20	36.2	20	17	36.1	0.1
21	18.7	21	21	18.7	0.0
22	33.7	22	-	33.9	-0.2
23	26.1	23	-	26.0	0.1
24	45.9	24	26, 27, 30	45.8	0.1
25	30.5	25	26, 27	29.2	1.3
26	19.8	26	27	19.8	0.0
27	19.3	27	26	19.1	0.2
28	24.2	28	23, 29	23.1	1.1
29	11.8	29	-	11.9	-0.1

(Literature* Chang et al., 1981)

4.1.2 Identification of compound 2

Compound 2 was isolated and purified from the EtOAc fraction of ethanolic extract, as presented in figure 3-4 (Chapter 3). It was white powder, m.p. 280 °C, (Literature* 280-286 °C), R_f 0.65 (1:4, MeOH:$CHCl_3$). The ESI-MS showed the molecular ion peak at *m/z* 576 corresponded to the molecular formula $C_{35}H_{60}O_6$ (calc. *m/z* 575.0198) (Figure 4-2.).

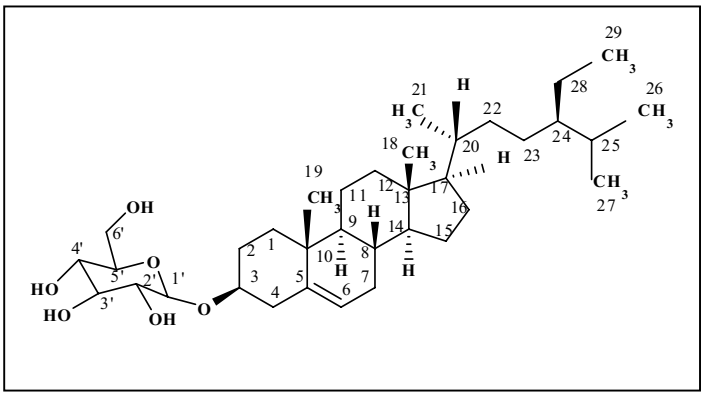


Figure 4-2. Structure of compound 2 (*ß*-Sitosterol-*3-O-ß*-D-glucopyranoside)

The ^{1}H NMR spectrum ($CDCl_3$: CD_3OD, 500 MHz) pattern was similar to that *ß*-sitosterol with some additional peak relating to a carbohydrate moiety (Table 4-3). A downfield broad triplet at δ 5.40 was assigned to the C-6 olefinic proton. The multiplet at δ 3.43 assigned for the proton of C-3. The 3H singlet appeared at δ 0.95, 1.05, 0.85, 0.82, 0.84 and 0.69 corresponding to the C-21, C-19, C-29, C-26, C-27 and C-18 methyl protons, respectively. The C-1' anomeric proton appeared at δ 4.40 as a doublet indicating the presence of a *ß*-sugar. ^{13}C-NMR pattern was also similar to *ß*-sitosterol with additional six peaks confirming the glucose ring (Table 4-4). Furthermore, the structure was supported by ^{1}H-^{1}H COSY, HMBC and HMQC. Compound 1 was also identified co-TLC comparison with an authentic sample of *ß*-sitosterol-*3-O-ß*-D-glucopyranoside (Kojima et al., 1990).

Table 4-3. ^{1}H NMR data of compound 2 (500 MHz, $CDCl_3$: CD_3OD)

Hydrogen No.	δ (ppm)	M	J (Hz)	Literature* δ (ppm)	$\Delta\delta$ (ppm)
3	3.43	m	-	3.45	0.02
6	5.4	dd	-	5.36	0.04
18	0.69	s	-	0.68	0.01
19	1.05	s	-	1.01	0.04
21	0.95	dd	6.60	0.94	0.01
26	0.82	d	7.00	0.81	0.01
27	0.84	d	7.50	0.84	0.00
29	0.85	t	7.5	0.86	-0.01

(Literature* Kojima et al., 1990)

Table 4-4. ^{13}C NMR data and ^{1}H-^{13}C correlation of compound 2 (500 MHz, $CDCl_3$)

Carbon No.	δ (ppm)	HMBC	HMQC	Literature* δ (ppm)	$\Delta\delta$ (ppm)
1	39.5	1	19	37.6	1.9
2	30.6	2	-	30.3	0.3
3	80.9	3	1, 2, 4, 1'	78.3	2.6
4	40.1	4	-	39.4	0.7
5	142.1	-	4,19	141.0	1.1
6	121.7	6	-	122.0	-0.3
7	32.4	7	6	32.2	0.2
8	32.3	8	6	32.1	0.2
9	50.6	9	19	50.4	0.2
10	37.1	-	6,19	37.0	0.1
11	21.6	11	-	21.4	0.2
12	40.3	12	18	40.0	0.3
13	42.8	-	18	42.6	0.2
14	57.1	14	18	57.0	0.1
15	24.7	15	-	24.6	0.1
16	27.2	16	-	28.7	-1.5
17	56.7	17	18, 21, 22	56.3	0.4

Table 4-4. (cont.) ^{13}C NMR data and ^{1}H-^{13}C correlation of compound 2 (500 MHz, $CDCl_3$)

Carbon No.	δ (ppm)	HMBC	HMQC	Literature* δ (ppm)	Δδ (ppm)
18	12.1	18	-	12.0	0.1
19	19.4	19	-	19.3	0.1
20	36.5	20	21	36.5	0.0
21	19.2	21	-	19.1	0.1
22	34.6	22	21	34.3	0.3
23	30.5	23	-	26.4	4.1
24	49.5	24	26, 27, 29	46.1	3.4
25	29.9	25	26, 27	29.5	0.4
26	20.0	26	27	20.1	0.1
27	19.6	27	26	19.5	0.1
28	23.7	28	29	23.4	0.3
29	12.3	29	-	12.2	0.1
Glucose					
1'	102.8	1'	2'	102.6	0.2
2'	75.4	2'	4'	75.4	0.0
3'	78.3	3'	1', 5'	78.7	-0.4
4'	72.2	4'	6'	71.7	0.5
5'	78.6	5'	1', 3'	78.5	0.1
6'	62.3	6'	-	62.9	-0.6

(Literature* Kojima et al., 1990)

4.1.3 Identification of compound 3

Compound 3 (Figure 4-3) was isolated and purified from the methanol fraction of ethanolic extract (Chapter 3). The structure of this compound right now was not completed because the signals presented in ^{13}C NMR (δ 82.3, 70.8, 65.5, 65.0, 64.5 and 22.2) cannot suitable for the structure elucidation. However, the diequatorial coupling anomeric protone at δ 4.41 and 5.09 (Table 4-5) were attributed to glucose (Glc) and rhamnose (Rha), respectively (Joconnet et al., 1999). The precence of rhamnosyl moiety was also inferred methyl signal at δ 17.56 and attachment of thise glucose to C-1'' position of *1-O*-rhamnose. The ^{13}C NMR spectrum, together with signals characteristic of glucose and rhamnose, which exhibited two anomeric carbons at δ

103.1 and 99.7 for rhamnosyl and glucosyl, repectively (Table 4-6). The ^{13}C NMR signal at δ 99.7, 71.5, 76.1, 70.7, 77.2 and 63.9 corresponed to the carbons of β-D-glucose and signal at δ 103.1, 70.0, 69.1, 71.2, 65.9 and 21.9 corresponed to the carbons of a α-L-rhamnose (Neszmely et al., 1978). In conclusion, compound 3 was consisted of β-D-glucose, α-L-rhamnose and unknown fractments (Figure 4-3)

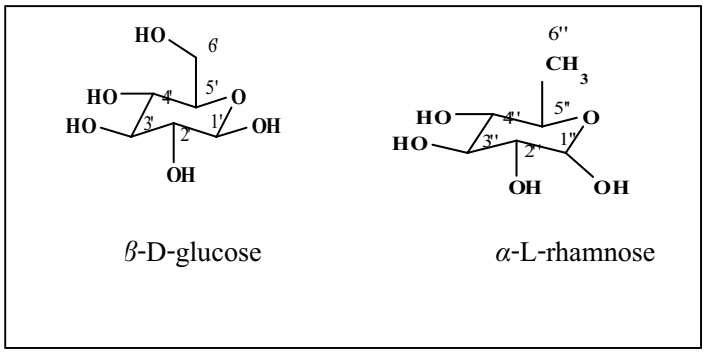


Figure 4-3. Fractments of compound 3

Table 4-5. ^{1}H NMR and ^{13}C NMR data of glucose (500 MHz, DMSO-d_6+D_2O)

Position	Hydrogen			Literature*	Carbon	Literature*
	δ (ppm)	**M**	***J* (Hz)**	**δ (ppm)**	**δ (ppm)**	**δ (ppm)**
Glucose			-	-		-
1'	4.40-5.10	-	-	4.42	99.7	99.2
2'	3.71	d	9.5	3.64	71.5	71.9
3'	3.79	d	9.5	3.76	76.1	76.2
4'	3.80	d	9.5	3.82	70.7	70.4
5'	3.61	m	-	3.57	77.2	76.5
6'	4.04	-	6.96	4.04	63.9	61.5

Literature*: DMSO- d_6 only, (Literature* Neszmely et al., 1978)

Table 4-6. ^{1}H NMR and ^{13}C NMR data of rhamnose (500 MHz, DMSO-d_6+D_2O)

Position	Hydrogen			Literature*	Carbon	Literature*
	δ (ppm)	M	J (Hz)	δ (ppm)	δ (ppm)	δ (ppm)
Rhamnose			-	-		-
1"	4.40-5.10	-	-	5.09	103.1	101.1
2"	3.90	m	-	3.92	70.0	70.3
3"	3.63	dd	9.0, 9.5	3.63	69.1	69.3
4"	3.46	dd	9.0, 9.5	3.46	71.2	71.6
5"	3.85	s	-	3.84	65.9	68.1
6"	1.24	d	6.86	1.27	21.9	17.5

Literature*: DMSO- d_6 only, (Literature* Neszmely et al., 1978)

4.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The present investigation against NK and CR venoms was tested by crude extracts, extract fractions and pure compounds from twigs of *C. iners*. The crude EtOH extract and each fractions of *C. iners* were investigated for efficiency to bind or neutralize protein bands of NK and CR venoms by SDS-PAGE.

The SDS – PAGE analysis of the extracts can diminish the band of NK or CR venoms by comparison bands of venom with standard protein markers (MW 7.1 – 210 kDa), in comparison bands of protein venom with standard mass markers, the lower band of CR venom is band of soybean trypsin inhibitor (31.8 kDa) and the upper band of NK venom is myosin (210.0 kDa) (Figure 4-4). SDS-PAGE analysis, if the extract has the enzyme inhibition activity the band of venom disappear (positive, (+)), but the extract of plant can not neutralize venom because the band of venom still on the page (negative, (-)) (Table 4-7, Table 4-8).

Table 4-7. SDS-PAGE of NK and CR venoms test by EtOH fraction and each fraction

Samples	NK venom			CR venom		
	Conc. of extract			Conc. of extract		
	50 mg/ml	10 mg/ml	2 mg/ml	50 mg/ml	10 mg/ml	2 mg/ml
EtOH	+	+	+	+	+	-
Hexane	-	-	-	-	-	-
$CHCl_3$	-	-	-	-	-	-
EtOAc	+	+	-	+	+	-
MeOH	+	+	+	+	+	+
EA-I-1	+	+	-	+	+	-
EA-I-2	+	-	-	+	-	-
EA-I-3	+	+	-	+	+	-
EA-I-4	-	-	-	-	-	-
EA-I-5	+	+	-	+	+	-
EA-II-1	+	+	-	+	-	-
EA-II-2	+	-	-	+	-	-
EA-II-3	+	+	-	+	+	-
EA-II-4	+	-	-	-	-	-
EA-II-5	+	+	-	+	+	-
EA-II-6	+	-	-	+	-	-
EA-II-7	+	-	-	+	-	-

Table 4-8. SDS-PAGE of NK and CR venoms test by MeOH fraction and each fraction

Samples	NK venom			CR venom		
	Conc. of extract			Conc. of extract		
	50 mg/ml	10 mg/ml	2 mg/ml	50 mg/ml	10 mg/ml	2 mg/ml
ME-I-1	+	-	-	-	-	-
ME-I-2	-	-	-	-	-	-
ME-I-3	+	+	-	+	+	-
ME-I-4	+	+	-	+	+	-
ME-I-5	+	-	-	+	-	-
ME-I-6	-	-	-	-	-	-
ME-I-7	+	-	-	-	-	-
ME-I-8	+	-	-	+	-	-
ME-I-9	+	-	-	+	-	-
ME-I-10	+	+	-	+	+	-
ME-I-11	+	-	-	+	-	-
ME-I-12	+	-	-	+	-	-
ME-II-1	-	-	-	-	-	-
ME-II-2	+	-	-	+	-	-
ME-II-3	-	-	-	-	-	-
ME-II-4	+	+	-	+	+	-
ME-II-5	+	-	-	+	-	-
ME-II-6	+	+	-	+	+	-

Table 4-9. SDS-PAGE of NK and CR venoms test by compound 1(*β*-Sitosterol), compound 2 (*β*-Sitosterol-*3*-*O*-D-glucopyranoside) and compound 3

Compound No.	NK venom			CR venom		
	Conc. of extract			Conc. of extract		
	50 mg/ml	10 mg/ml	2 mg/ml	50 mg/ml	10 mg/ml	2 mg/ml
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	-	-	-	-	-	-

From SDS-PAGE pattern (Figure 4-5, 4-6 and 4-7), the crude EtOH extract, EtOAC and MeOH fractions of *C. iners*, showed good neutralize the protein bands of NK and CR venoms. Unfortunately, the Hexane and $CHCl_3$ fractions showed negative test for neutralizing the protein bands of NK and CR venoms (Figure 4-8, 4-9). The EtOAc and MeOH fractions significantly neutralized NK and CR venoms. However, three compounds (*β*-sitosterol, *β*-sitosterol-*3*-*O*-*β*-D-glucopyranoside and compound 3) were isolated and identified from EtOAc and MeOH fractions, they showed negative test for protein neutralization of NK and CR venoms (Figure 4-10, 4-11 and 4-12). The *in vitro* experiment by means of SDS-PAGE was carried out to support the result from proteolytic and PLA_2 enzyme activity.

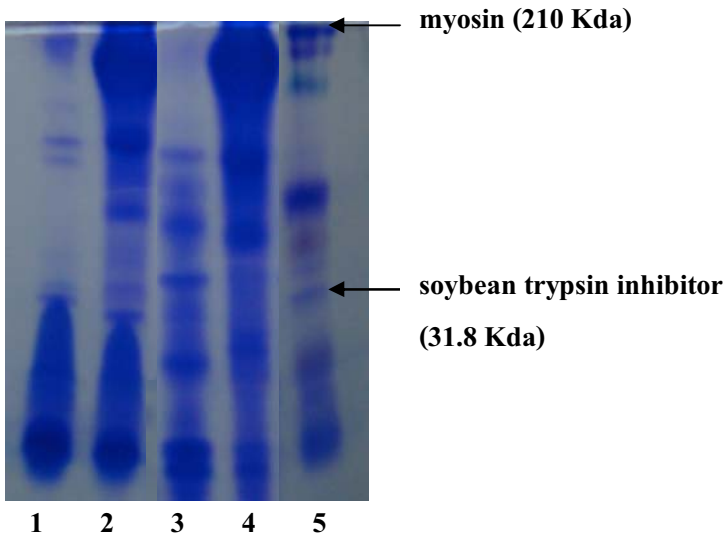


Figure 4-4. SDS-PAGE pattern of venom, antivenom and standard protein markers. Lane 1, NK venom; Lane 2, NK venom+antivenom; Lane 3, CR venom; Lane 4, CR venom+antivenom**;** Lane 5, standard protein markers: myosin (210 kDa) and soybean trypsin inhibitor (31.8 kDa)

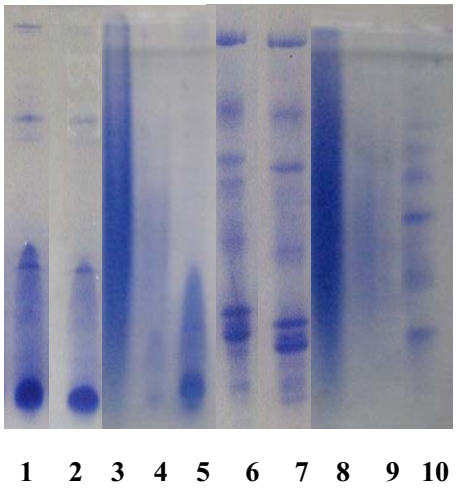
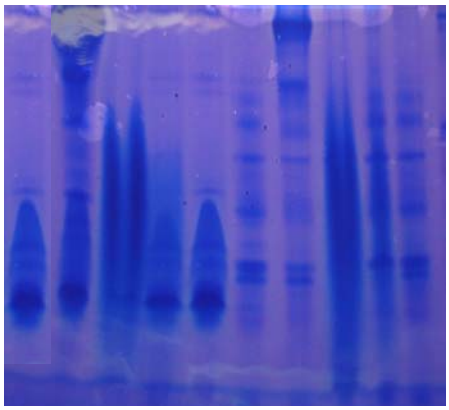
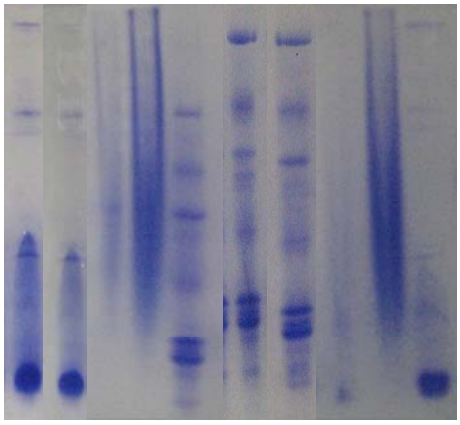


Figure 4-5. SDS-PAGE patterns of EtOH extract; Lane 1, NK venom; Lane 2, NK venom+antivenom; Lane 3, CR venom; Lane 4, CR venom+antivenom**;** Lane 1-3, NK venom+EtOH extract fraction (conc. 50, 10 and 2 mg/ml), respectively; Lane 4-6, CR venom+EtOH extract fraction (conc. 50, 10 and 2 mg/ml), respect



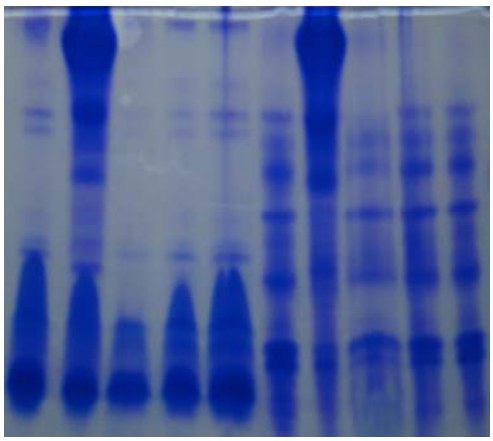
1 2 3 4 5 6 7 8 9 10

Figure 4-6. SDS-PAGE patterns of EtOH extract; Lane 1, NK venom; Lane 2, NK venom+antivenom;Lane 3, CR venom; Lane 4, CR venom+antivenom**;** Lane 1-3, NK venom+EtOH extract fraction (conc. 50, 10 and 2 mg/ml), respectively; Lane 4-6, CR venom+EtOH extract fraction (conc. 50, 10 and 2 mg/ml), respectively.



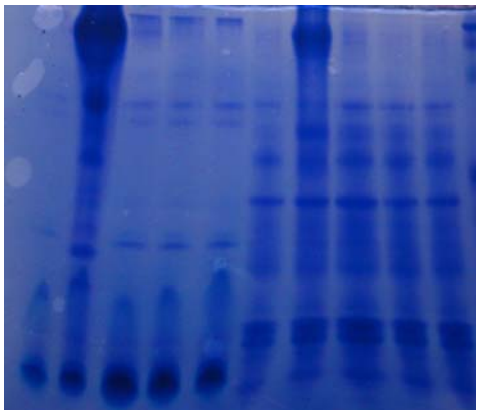
1 2 3 4 5 6 7 8 9 10

Figure 4-7. SDS-PAGE patterns of MeOH extract; Lane 1, NK venom; Lane 2, NK venom+antivenom; Lane 3, CR venom; Lane 4, CR venom+antivenom**;** Lane 1-3, NK venom+MeOH extract fraction (conc. 50, 10 and 2 mg/ml), respectively; Lane 4-6, CR venom+MeOH extract fraction (conc. 50, 10 and 2 mg/ml), respectively.



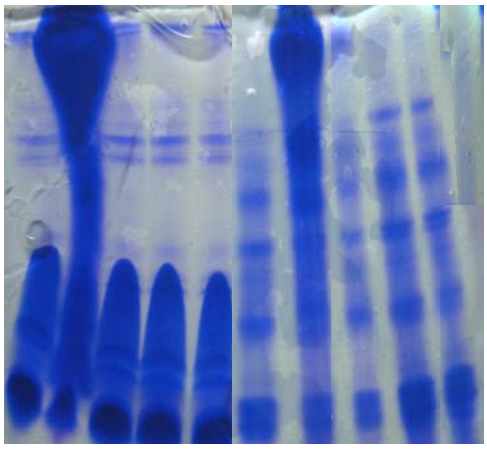
1 2 3 4 5 6 7 8 9

Figure 4-8. SDS-PAGE patterns of Hexane extract; Lane 1, NK venom; Lane 2, NK venom+antivenom; Lane 3, CR venom; Lane 4, CR venom+antivenom**;**Lane 1-3, NK venom+Hexane extract fraction (conc. 50, 10 and 2 mg/ml), respectively; Lane 4-6, CR venom+Hexane extract fraction (conc. 50, 10 and 2 mg/ml), respectively.



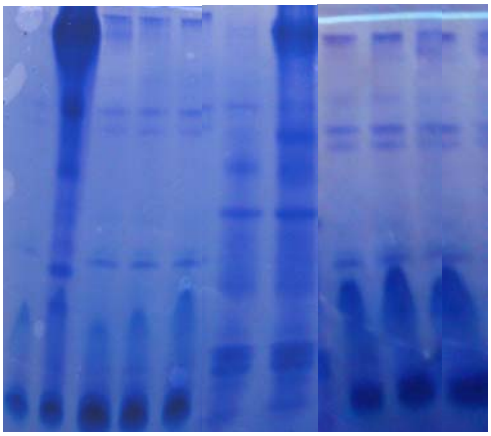
1 2 3 4 5 6 7 8 9 10

Figure 4-9. SDS-PAGE patterns of $CHCl_3$ extract; Lane 1, NK venom; Lane 2, NK venom+antivenom; Lane 3, CR venom; Lane 4, CR venom+antivenom**;** Lane 1-3, NK venom+$CHCl_3$ extract fraction (conc. 50, 10 and 2 mg/ml), respectively; Lane 4-6, CR venom+$CHCl_3$ extract fraction (conc. 50, 10 and 2 mg/ml), respectively.



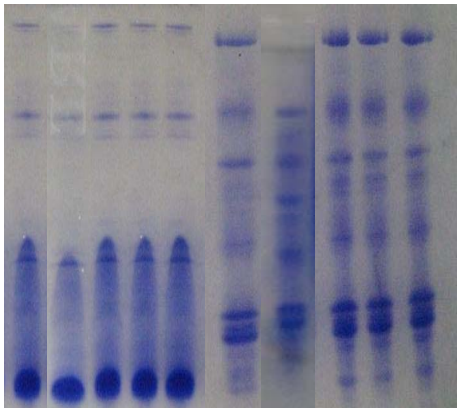
1 2 3 4 5 6 7 8 9 10

Figure 4-10. SDS-PAGE pattern of compound 1; Lane 1-3, NK venom+compound 1 (conc. 10, 5 and 2 mg/ml), respectively. Lane 3-6, CR venom+compound 1 (conc. 10, 5 and 2 mg/ml), respectively.



1 2 3 4 5 6 7 8 9 10

Figure 4-11. SDS-PAGE pattern of compound 2. Lane 1, NK venom; Lane 2, NK venom+antivenom; Lane 3, CR venom; Lane 4, CR venom+antivenom**;** Lane 1-3, NK venom+compound 2 (conc. 10, 5 and 2 mg/ml), respectively. Lane 4-6, CR venom+compound 2 (conc. 10, 5 and 2 mg/ml), respectively.



1 2 3 4 5 6 7 8 9 10

Figure 4-12. SDS-PAGE pattern of compound 3. Lane 1, NK venom; Lane 2, NK venom+antivenom; Lane 3, CR venom; Lane 4, CR venom+antivenom**;** Lane 1-3, NK venom+compound 3 (conc. 10, 5 and 2 mg/ml), respectively. Lane 4-6, CR venom+ compound 3 (conc. 10, 5 and 2 mg/ml), respectively**.**

4.3 Proteolytic activity

The proteolytic enzyme or protease is the one type of enzyme that can be found in snake venom. The activity of this enzyme is similar to trypsin which digests the proteins and peptides, therefore the tissues are destroyed and leading to the necrosis. In this experiment, a protein, casein, and calcium chloride were used as substrate and co-factor, respectively. After incubation at 37 °C for 90 minutes, the reaction was stopped with trichloroacetic acid. Finally, the absorbance of amino acids was measured. The OD of snake venom was equal 100 degree of proteolytic activity and antivenom was used as a positive control. Samples with venom were defined to proteolytic activity of snake venom. In comparison with antivenom, if the activity of sample with snake venom was lower than 50 degree or similar to antivenom, it means that the extract can inhibit the proteolytic enzyme. Because of the low amount of proteolytic enzyme in NK venom, the proteolytic enzyme activity of NK venom was not investigated.

The proteolytic activity of EtOH and their derived fractions of *C. iners* were investigated and analyzed (Figure 4-13). As the result, the good fractions for activity proteolytic enzyme of CR venom were crude EtOH extract and MeOH fractions at conc. 50, 10 and 2 mg/ml, respectively. The EtOAc fraction exhibited activity against proteolytic at conc. 50 and 10 mg/ml but the Hexane and $CHCl_3$ fractions only positive results at conc. 50 mg/ml. All fraction [(EA-I-1)-(EA-I-6)] and EA-II-1, EA-II-2, EA-II-3, EA-II-4, EA-II-6 were found to be active against proteolytic activity produced from CR venom at conc. 10 mg/ml. (Figure 4-14). ME-I-2, ME-I-4, ME-I-5, ME-I-6, ME-I-7, ME-I-8, ME-I-9, ME-I-10, ME-I-11, ME-II-3, ME-II-4, ME-II-5 and ME-II-6 were found to be active against proteolytic activity produced from CR venom at conc. 10 mg/ml (Figure 4-15).

In *in vitro* studies showed that compound 2 significantly inhibited proteolytic activity at conc. 10 and 5 mg/ml but compound 1 and 3 inhibited proteolytic activities at only conc. 10 mg/ml. However compound 1, 2 and 3 showed weak inhibitory proteolytic activities against CR venom at conc. 2 mg/ml (Figure 4-16).

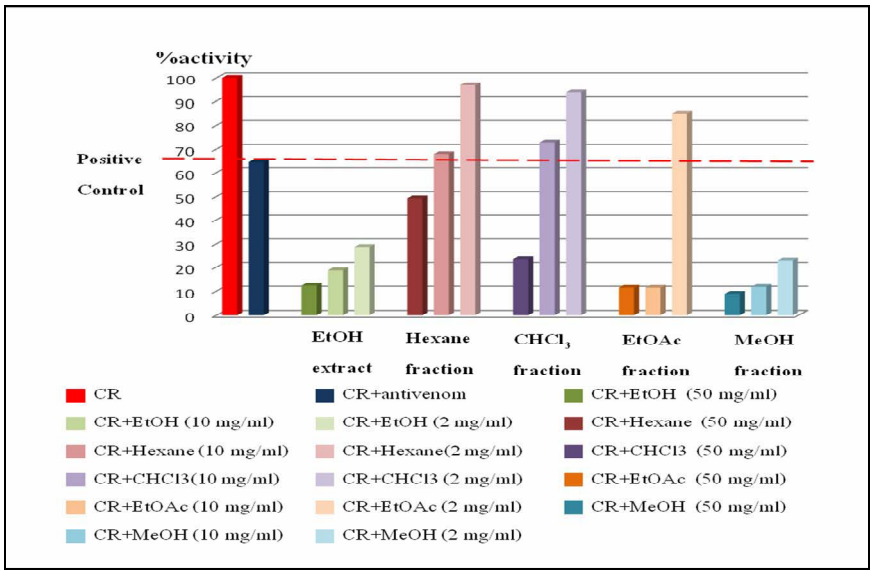


Figure 4-13. Result chart of the proteolytic enzyme activity of Malayan pit viper venom tested by 5 fractions from *C iners*.

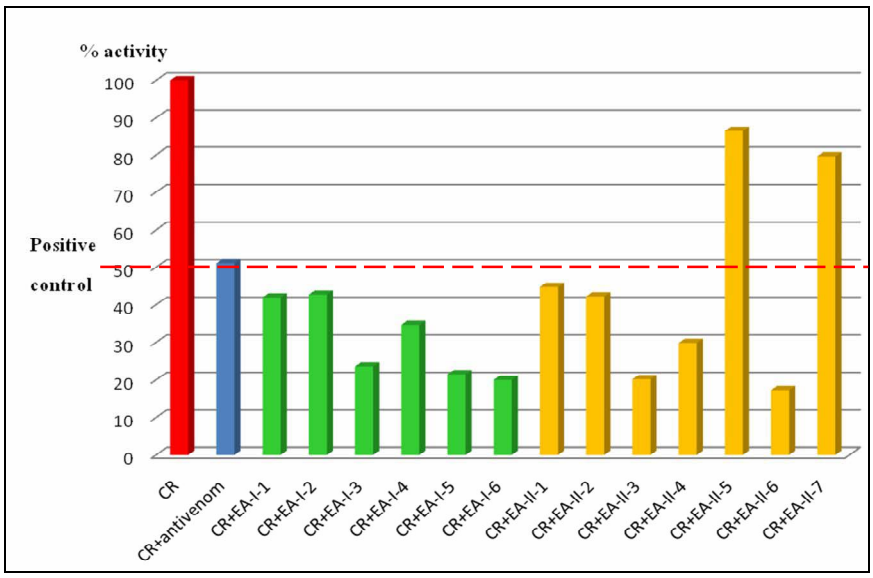


Figure 4-14. Result chart of the proteolytic enzyme activity of Malayan pit viper venom tested by [(EA-I-1)-(EA-I-6)] and [(EA-II-1)-(EA-II-7)] fractions at conc. 10 mg/ml

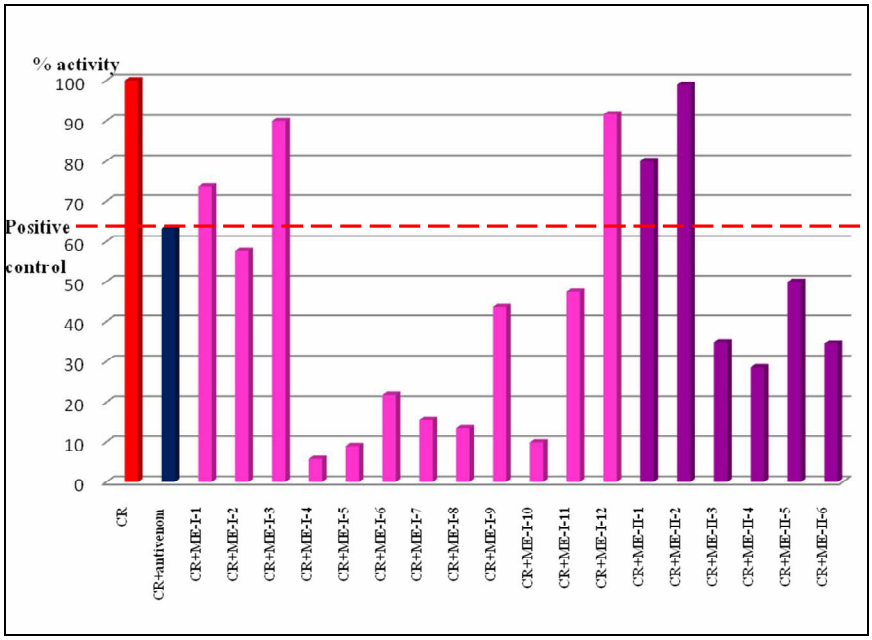


Figure 4-15. Result chart of the proteolytic enzyme activity of Malayan pit viper venom tested by [(ME-I-1)-(ME-I-12)] and [(ME-II-1)-(ME-II-6)] fractions at conc. 10 mg/ml

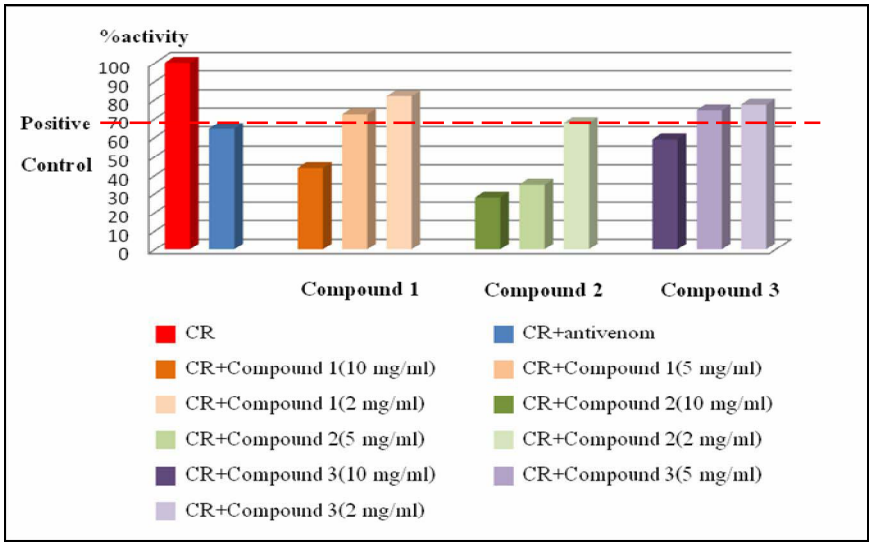


Figure 4-16. Result chart of the proteolytic enzyme activity of Malayan pit viper venom tested by compound 1, 2 and 3 from *C. iners*.

4.4 Phospholipase A_2 (PLA_2) activity

The PLA_2 is an enzyme that can be found in the various snake venoms. When snake bite, the lysophospholipids were produced and disrupt cellular membrane and caused lyses of red blood cells. In this experiment, egg yolk and calcium chloride were used as substrate and co-factor, respectively. The PLA_2 enzyme in snake venom digested lecithin (in egg yolk) and changed to lysolecithin form in 60 minutes at 37 $^{\circ}$C. The hemoglobin in red blood cells was lyses by lysolecithin and then measured the absorbance of the rest hemoglobin. The absorbance of interaction of snake venom and antivenom will be set to equal 0 and 100 degree of enzyme activity, respectively. All of extract fractions and isolated compounds showed the activity against snake venom in comparison with antivenom.

The PLA_2 activity of crude EtOH extract and their derived fractions of *C. iners* twig were obtained. As the result, the good fractions for activity test against NK venom were EtOAc and MeOH fraction at conc. 50, 10 and 2 mg/ml, respectively. In comparison with NK-antivenom (Figure 4-17.), the good fractions against PLA_2 activity of CR venom were crude EtOH extract, $CHCl_3$, EtOAc and MeOH fractions at conc. 50, 10 and 2 mg/ml, respectively. (Figure 4-21.).

Nine semi pure fractions, EA-I-3, EA-I-5, EA-I-6, EA-II-3, EA-II-5, ME-I-3, ME-I-4, ME-I-10 and ME-II-4 were found to be active against PLA_2 activity produced from NK venom at conc. 10 mg/ml (Figure 4-13, Figure 4-14). EA-I-3, EA-I-5, EA-II-3, EA-II-6, ME-I-5, ME-I-10 and ME-II-4 fractions were found to be active against PLA_2 activity produced from CR venoms at conc. 10 mg/ml (Figure 4-22, Figure 4-23).

PLA_2 activity showed that compound 1, 2 and 3 at all concentrations did not significantly inhibit this activity of both NK (Figure 4-20) and CR (Figure 4-24) venoms.

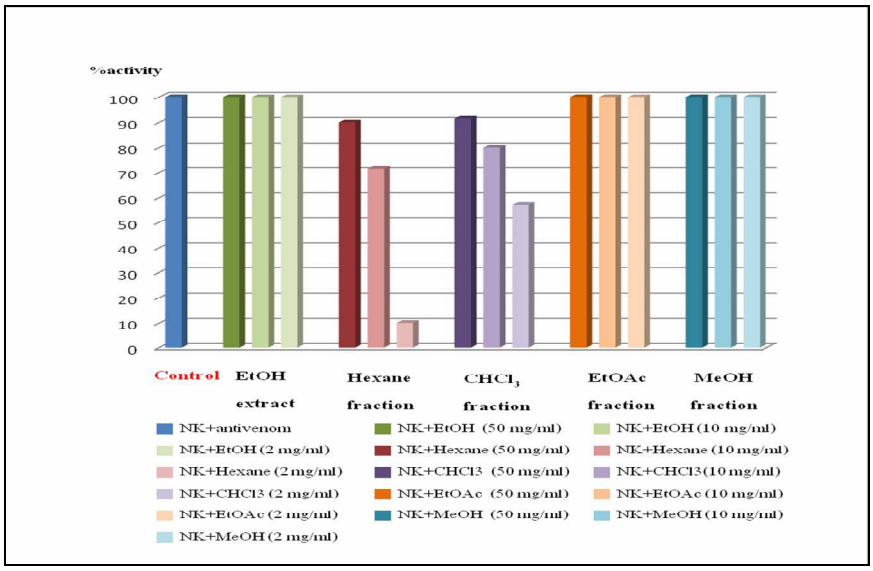


Figure 4-17. Result chart of the Phospholipase A_2 (PLA_2) enzyme activity of Thai cobra (NK) venom tested by 5 fractions from *C. iners*

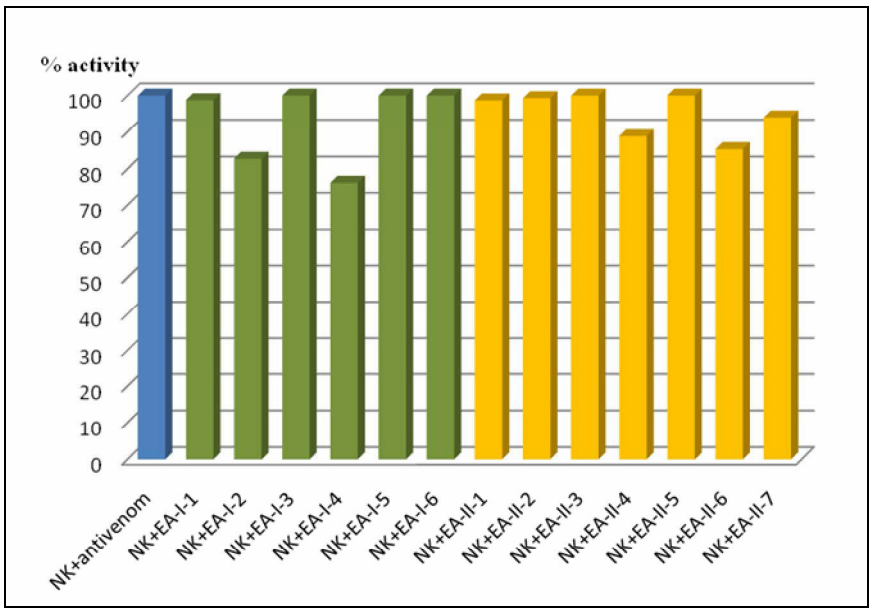


Figure 4-18. Result chart of the Phospholipase A_2 (PLA_2) enzyme activity of Thai cobra (NK) venom tested by [(EA-I-1)-(EA-I-6)] and [(EA-II-1)-(EA-II-7)] fractions at conc. 10 mg/ml

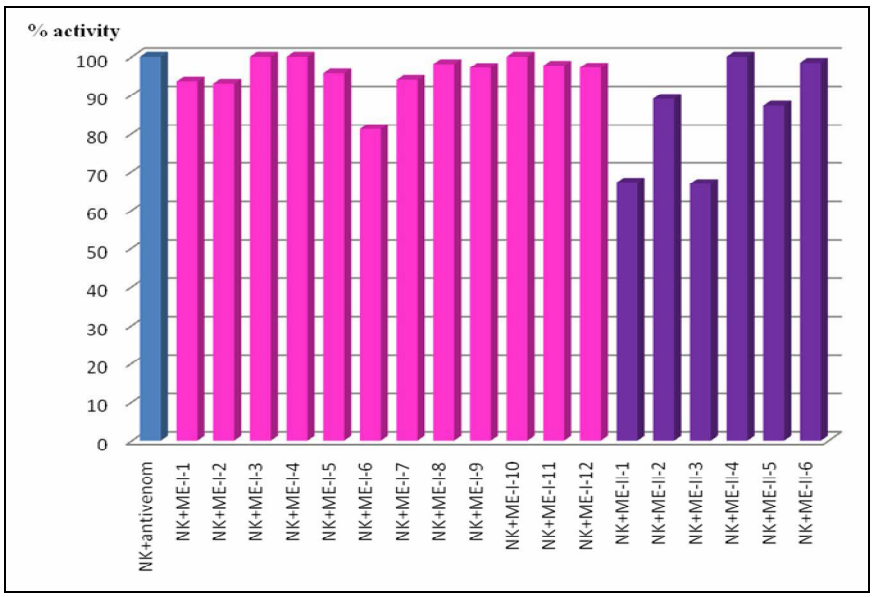


Figure 4-19. Result chart of the Phospholipase A_2 (PLA_2) enzyme activity of Thai cobra (NK) venom tested by [(ME-I-1)-(ME-I-12)] and [(ME-II-1)-(ME-II-6)] fractions at conc. 10 mg/ml

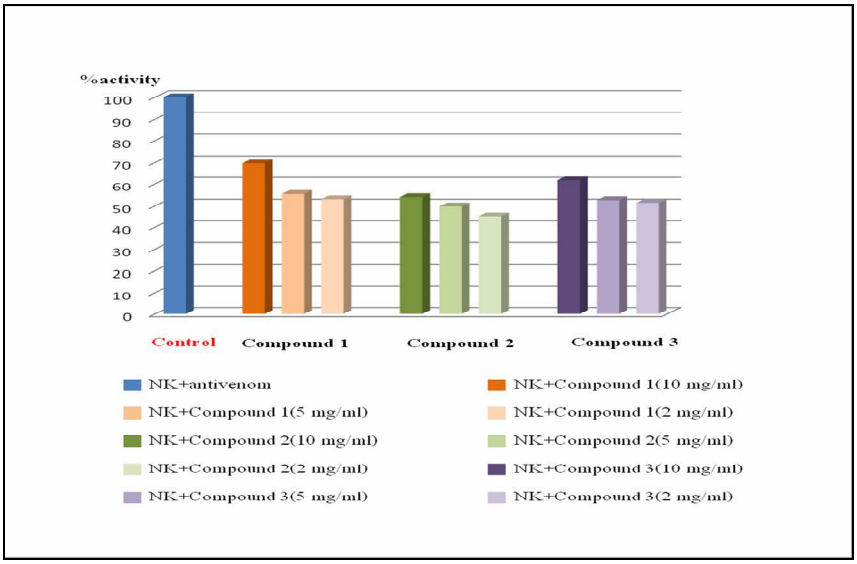


Figure 4-20. Result chart of the Phospholipase A_2 (PLA_2) enzyme activity of Thai cobra (NK) venom tested by compound 1, 2 and 3 from *C. iners*

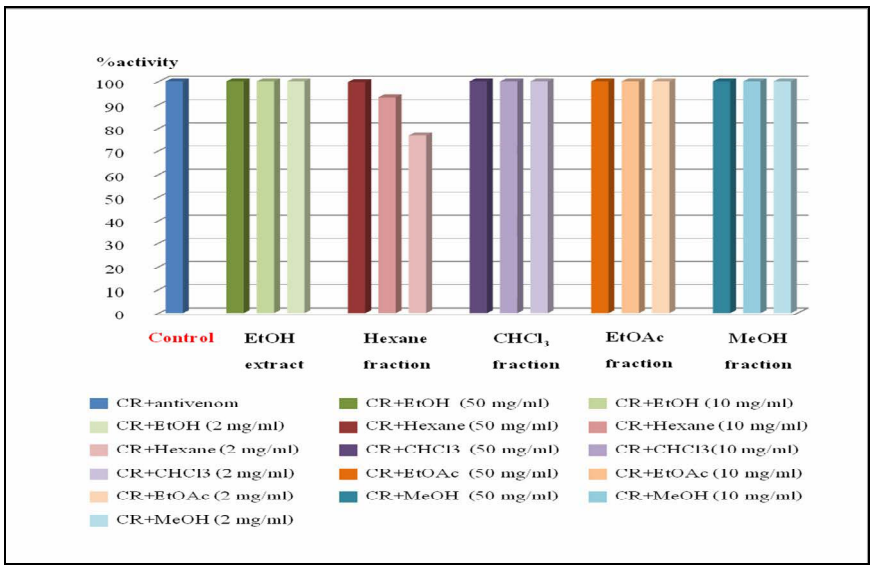


Figure 4-21. Result chart of the Phospholipase A_2 (PLA_2) enzyme activity of Malayan pit viper (CR) venom tested by 5 fractions from *C. iners*

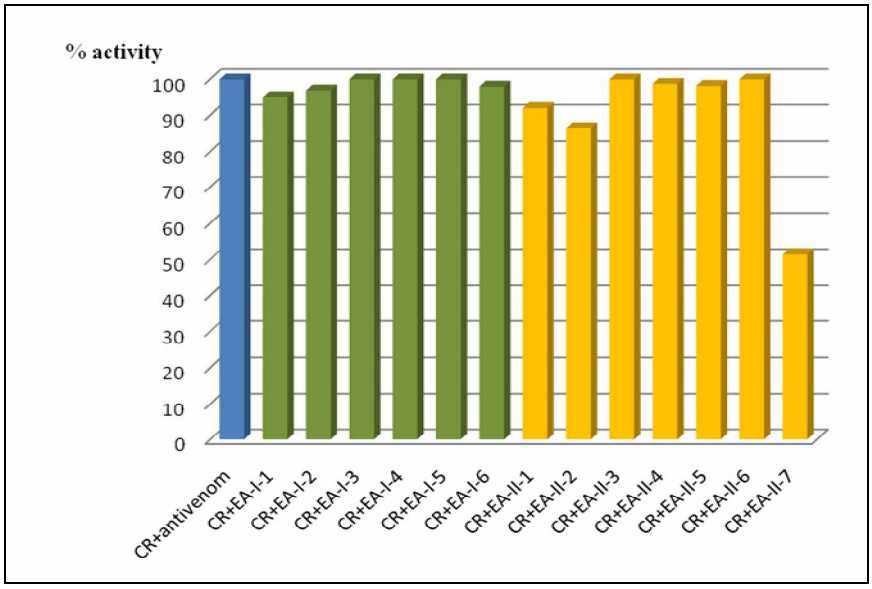


Figure 4-22. Result chart of the Phospholipase A_2 (PLA_2) enzyme activity of Malayan pit viper (CR) venom tested by [(EA-I-1)-(EA-I-6)] and [(EA-II-1)-(EA-II-7)] fractions at conc. 10 mg/ml

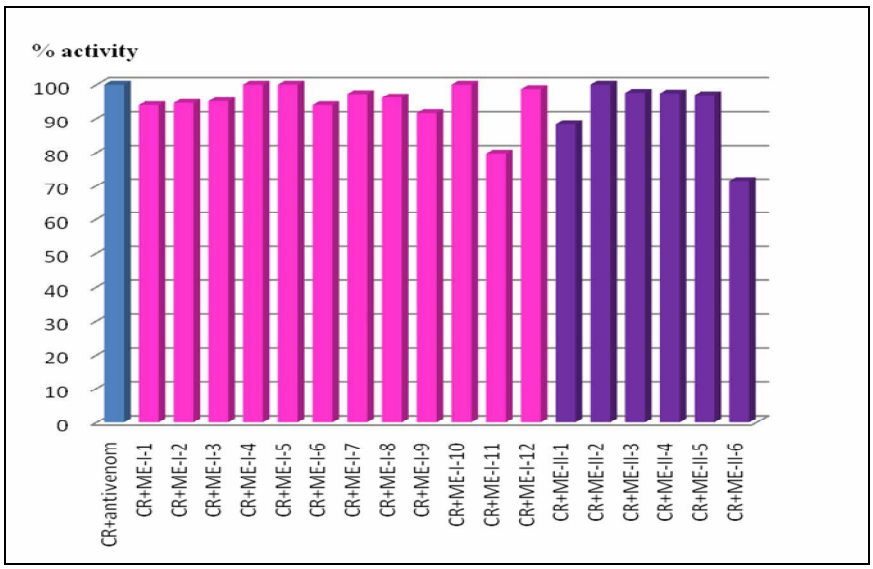


Figure 4-23. Result chart of the Phospholipase A_2 (PLA_2) enzyme activity of Malayan pit viper (CR) venom tested by [(ME-I-1)-(ME-I-12)] and [(ME-II-1)-(ME- II-6)] fractions at conc. 10 mg/ml

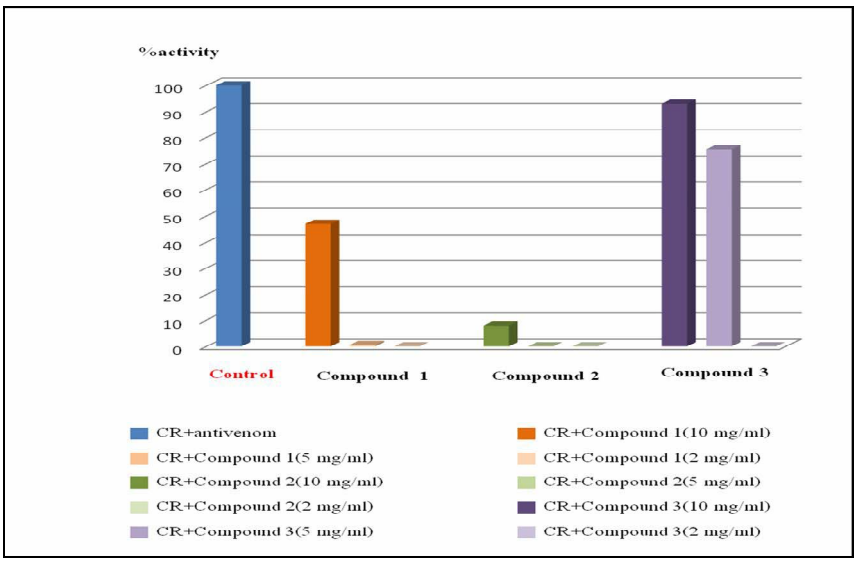


Figure 4-24. Result chart of the Phospholipase A_2 (PLA_2) enzyme activity of Malayan pit viper (CR) venom tested by compound 1, 2 and 3 from *C. iners*.

CHAPTER 5
CONCLUSIONS

The fresh twigs of *Cinnamomum iners* were collected from Thai herbal garden, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai Campus, Songkhla, Thailand. The crude ethanol extract of *C. iners* was prepared and then successively partitioned with *n*-Hexane, $CHCl_3$, EtOAc and MeOH, respectively. The EtOAc and MeOH fractions were repeatedly subjected to column chromatography to afford two known sterols and a disaccharide. Compound 1 (*ß*-sitosterol) and 2 (*ß*-sitosterol-3-*O*-*ß*-D-glucopyranoside) were isolated and identified by comparison of ^{1}H and ^{13}C NMR, MS, UV and IR data with the literature value. Compound 3 was consisted of *ß*-D-glucose, *α*-L-rhamnose and unknown fractments.

In vitro study, anti-NK and CR venom activities screenings of ethanol (EtOH), *n*-Hexane (Hexane), chloroform ($CHCl_3$), ethyl acetate (EtOAc) and methanol (MeOH) extracts of the twigs of *C. iners* were investigated and tested by mean of SDS-PAGE, inhibition of proteolytic and PLA_2 enzyme activities. The EtOH, EtOAC and MeOH extracts showed high efficiency to neutralize protein bands of NK and CR venoms in SDS–PAGE and inhibition of proteolytic and PLA_2 enzyme activities. Furthermore, after fractionation, EA-I-3, EA-I-5, EA-II-3, EA-II-6, ME-I-4, ME-I-10 and ME-II-4 fractions showed good results against NK and CR venoms.

After purification and determination, 3 compounds were separated. The active *ß*-sitosterol and *ß*-sitosterol-3-*O*-*ß*-D-glucopyranoside were isolated and purified from the EA-I-3 and EA-II-6 fractions, respectively. Furthermore, compound 3 was separated and identified from the ME-II-4 fraction. However, all isolated compounds can not diminish the protein bands of NK and CR venoms, the inhibition of proteolytic and PLA_2 enzyme activities gave the good result. The *ß*-sitosterol and compound 3 showed proteolytic activities against CR venom depend on its concentration 43.7%, and 58.8% at the concentration of 10 mg/ml; 72.2% and 74.3% at concentration of 5 mg/ml and 81.9% and 77.3% at concentration of 2 mg/ml, respectively). The *ß*-sitosterol-3-*O*-*ß*-D-glucopyranoside showed more proteolytic activity against CR venom 27.8%, 35.0% and 67.0% at the concentration of 10, 5 and 2 mg/ml, respectively. Furthermore, *ß*-sitosterol, *ß*-sitosterol-3-*O*-*ß*-D-glucopyranoside and compound 3 showed moderately PLA_2 activity against NK venom 69.6 %, 53.8% and 61.7% at the concentration of 10 mg/ml; 55.3%, 49.7% and 52.5% at concentration of 5 mg/ml and 52.9%, 45.1% and 51.3% at concentration of

2 mg/ml, respectively and also against CR venoms 46.8%,7.9% and 92.8% at the concentration of 10 mg/ml; 0.6%, 0% and 75.2% at concentration of 5 mg/ml, respectively.

The further investigation and purification of EA-II-5 and ME-I-10 fractions works are necessary for the better understanding of the inhibitory effects for *Naja kaouthia* and *Calloselasma rhodostoma* venoms.

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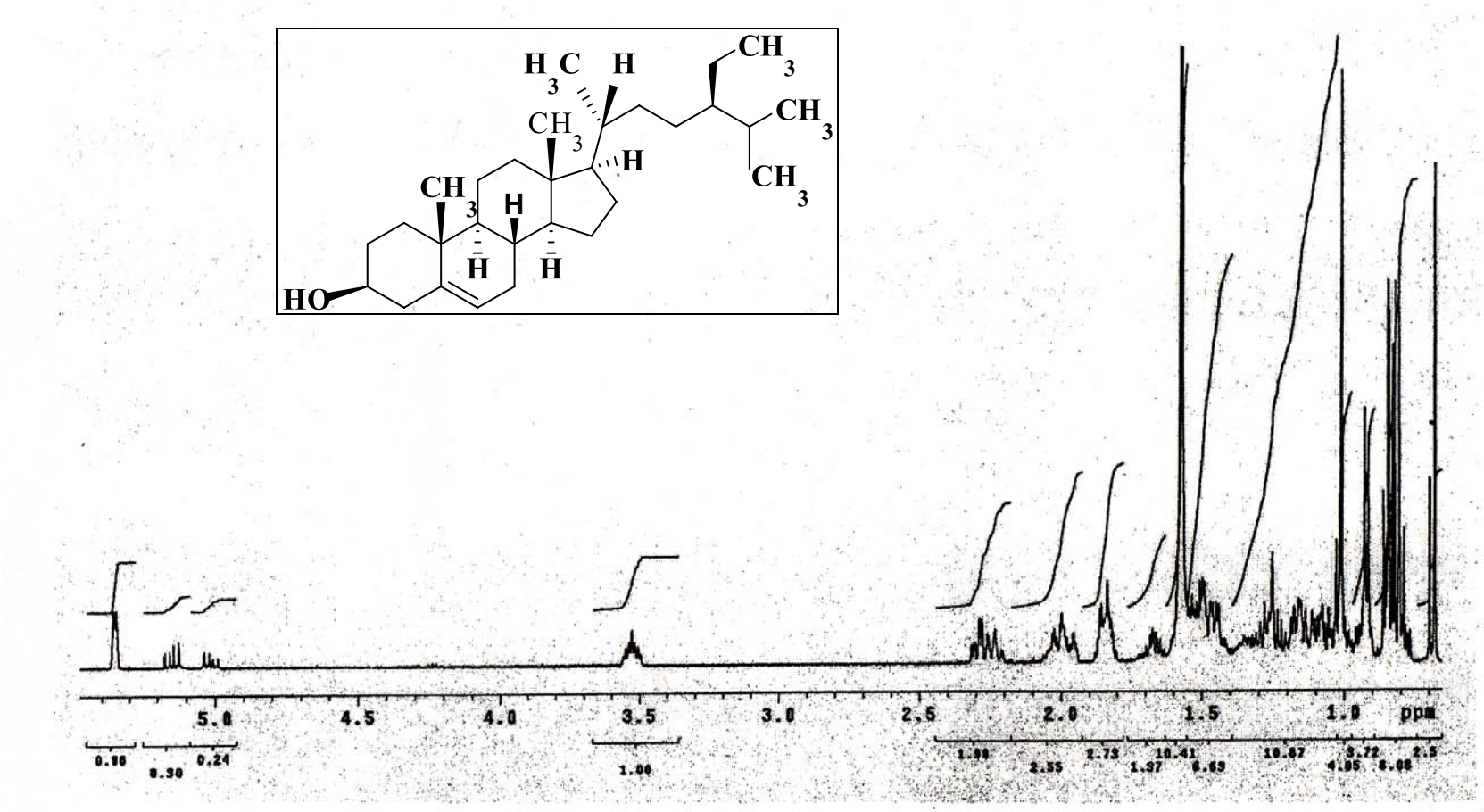
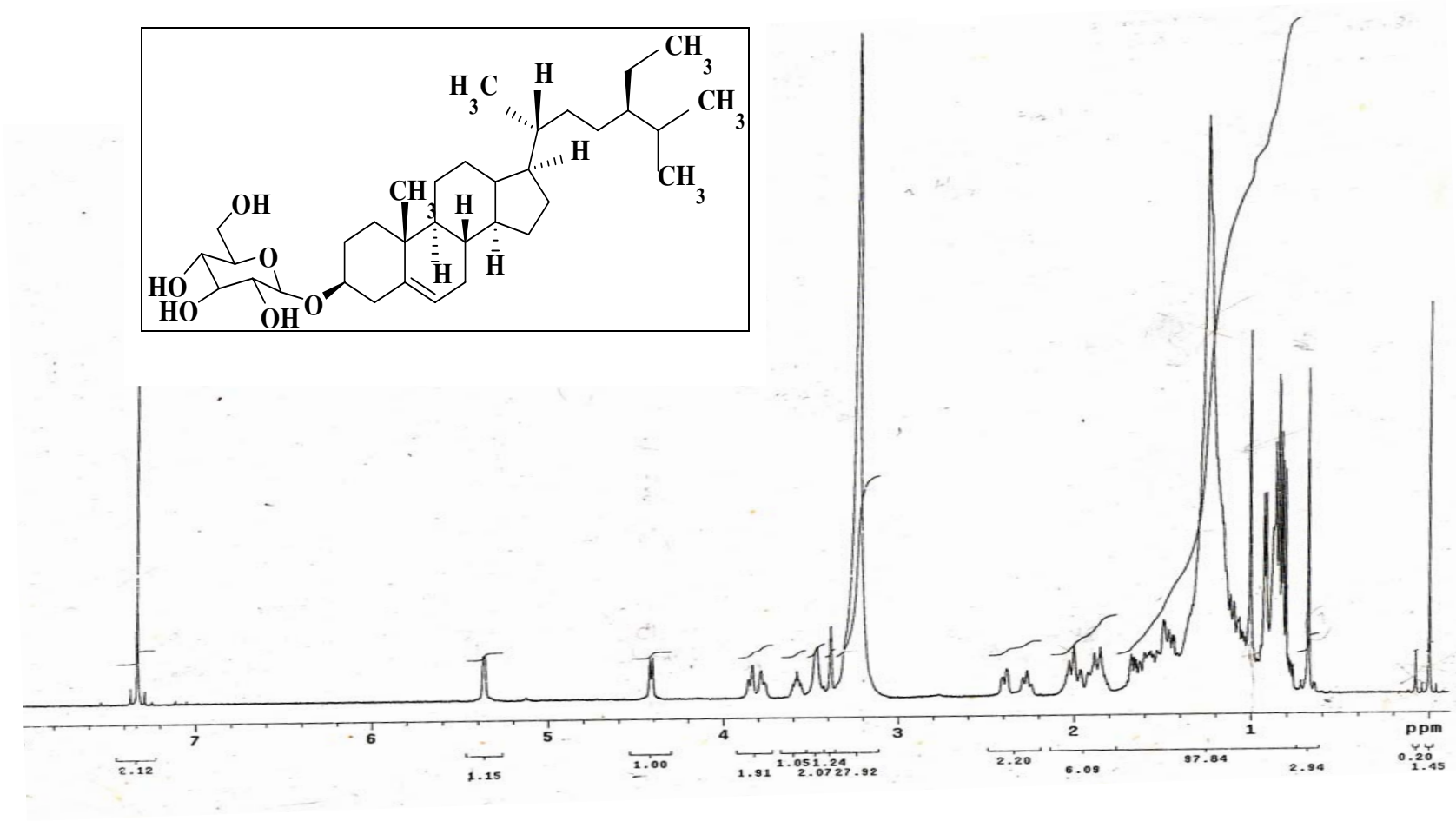
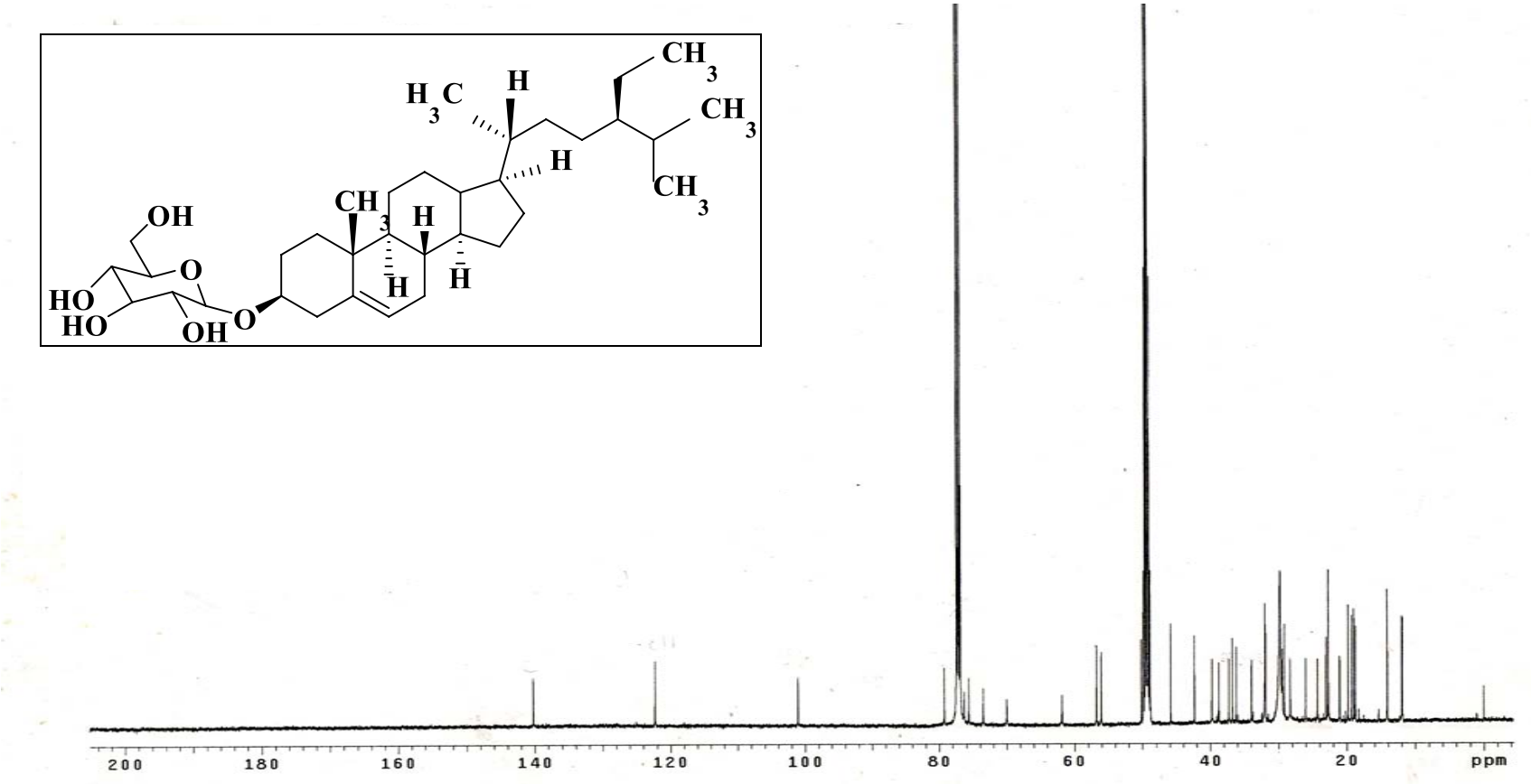
APPENDIX
DATA FROM COMPOUND



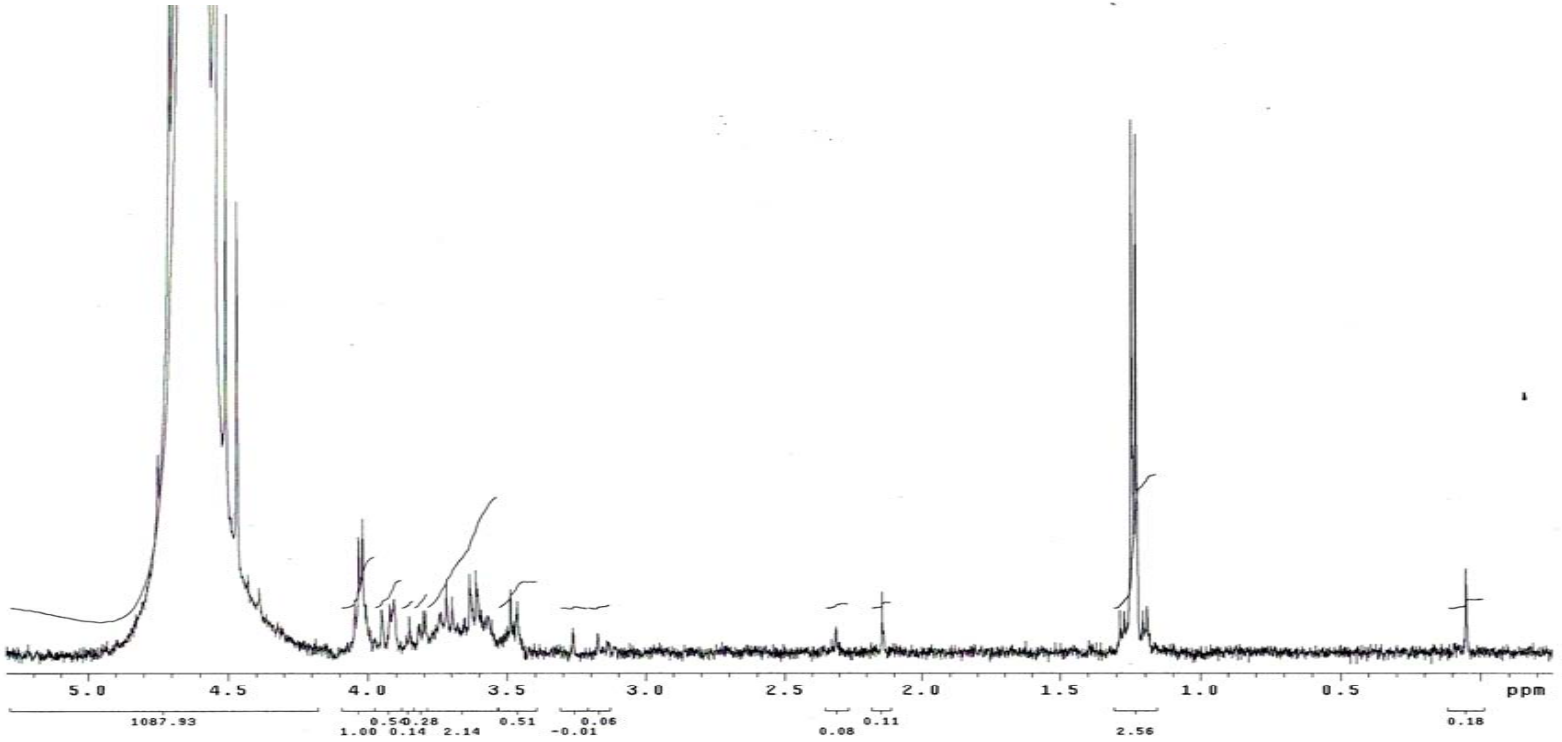
^{1}H-NMR spectrum of compound 1 (500 Hz, $CDCl_3$)



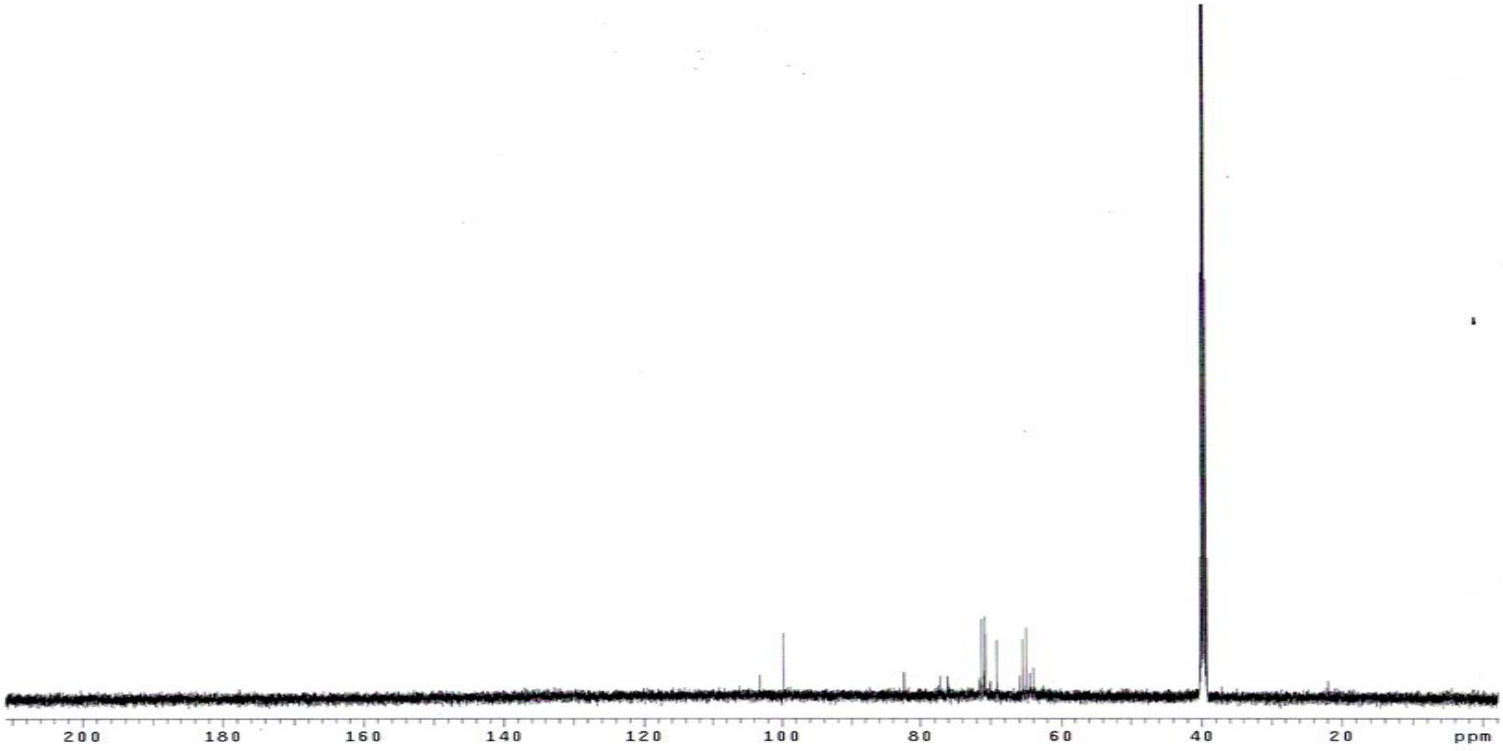
[1]H-NMR spectrum of compound 2 (500 Hz, $CDCl_3$: CD_3OD)



^{13}C-NMR spectrum of compound 2 (500 Hz, $CDCl_3$: CD_3OD)



^{1}H-NMR spectrum of compound 3 (500 Hz, DMSO-d_6+D_2O)



^{13}C-NMR spectrum of compound 3 (500 Hz, DMSO-d_6+D_2O)

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Kanchana Jeenchookaew, Chalermkiat Songkram, Lawan Chanhome, Pannipa Julasukuln & Jindaporn Puripattanavong. Screening Of *Cinnamomum Iners* With Snake Venom Antagonistic Activity And Antihaemorrhagic Necrosis Of Snake Venom From *Naja Kaouthia* And *Calloselasma Rhodostoma*. The sixth regional IMT-GT uninet conference 2008. The Gurney Resort Hotel & Residences, PENANG, MALAYSIA.