ฤทธิ์ฆ่าหอยเชอร์รี่ *Pomacea canaliculata* Lamarck ของซาโปนินจากเมล็ดชา

Camellia oleifera Abel.

นางสาวสุพรรษา กิจประยูร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเคมี ภาควิชาเคมี
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2548

ISBN 974-14-2301-2

MOLLUSCICIDAL ACTIVITY OF SAPONINS FROM TEA SEEDS *Camellia oleifera* Abel. ON GOLDEN APPLE SNAILS *Pomacea canaliculata* Lamarck.

Miss Supunsa Kijprayoon

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Chemistry
Department of Chemistry
Faculty of Science
Chulalongkorn University
Academic Year 2005
ISBN 974-14-2301-2

Thesis Title	Molluscicidal activity of saponins from tea seeds *Camellia oleifera* Abel. on golden apple snails *Pomacea canaliculata* Lamarck.
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Field of Study	Chemistry
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สุพรรษา กิจประยูร: ฤทธิ์ฆ่าหอยเชอร์รี่ *Pomacea canaliculata* Lamarck. ของซาโปนินจากเมล็ดชา *Camellia oleifera* Abel. (MOLLUSCICIDAL ACTIVITY OF SAPONINS FROM TEA SEEDS *Camellia oleifera* Abel. ON GOLDEN APPLE SNAILS *Pomacea canaliculata* Lamarck.) อาจารย์ที่ปรึกษา: รองศาสตราจารย์ ดร. อมร เพชรสม, อาจารย์ที่ปรึกษาร่วม: อาจารย์ วาสนา โตเลี้ยง...83... หน้า. ISBN 974-14-2301-2

ได้ทำการศึกษาฤทธิ์ฆ่าหอยเชอร์รี่ *Pomacea canaliculata* Lamarck. จากกากเมล็ดชา *Camellia oleifera* Abel. พบว่าสารสกัดจากกากเมล็ดชาแสดงความเป็นพิษต่อหอยเชอร์รี่ที่ระดับความเข้มข้น LC_{50} ที่ 24 ชั่วโมง มีค่าเท่ากับ 6.79 มิลลิกรัมต่อลิตร จากการแยกโดยการติดตามเฉพาะสิ่งสกัดที่มีฤทธิ์สูงที่สุดในแต่ละขั้นนั้น สามารถแยกกลุ่มของซาโปนิน (D4) ได้โดยที่สารมีมวลโมเลกุลอยู่ในช่วง 1226-1338 ดัลตัล และที่ระดับความเข้มข้น 0.66 มิลลิกรัมต่อลิตรมีผลทำให้หอยเชอร์รี่ตาย 43.33 เปอร์เซ็นต์ ภายใน 24 ชั่วโมง สำหรับการวิเคราะห์ปริมาณของกลุ่มซาโปนิน D4 โดยเทคนิค RP-HPLC โดยใช้ยูวีเป็นตัวตรวจวัด สภาวะที่เหมาะสมในการวิเคราะห์คือ คอลัมน์ C18 ขนาด 250x4.6 mm วัฏภาคเคลื่อนที่คือ 40% เมทานอลต่อ 0.1 % แอซิติกแอซิดในน้ำ ที่อัตราการไหล 0.80 มิลลิลิตรต่อนาที ตรวจวัดที่ความยาวคลื่น 235 นาโนเมตร กราฟเทียบมาตรฐานให้ค่าสัมประสิทธิ์สหสัมพันธ์ที่ดี มีค่ามากกว่า 0.9990 ในช่วงความเข้มข้น 10-200 มิลลิกรัมต่อลิตร รวมทั้งได้ทำการหาปริมาณของสาร D4 จากตัวอย่างที่ได้จากสิ่งสกัดจากเปลือกเมล็ดชา และสิ่งสกัดจากกากเมล็ดชาที่มีจำหน่ายเชิงพาณิชย์ซึ่งเป็นสารฆ่าหอยเชอร์รี่ที่ใช้กันในปัจจุบัน พบว่าสาร D4 มีอยู่ใน ซาโปนินที่สกัดจากกากเมล็ดชา 6.82 เปอร์เซ็นต์โดยน้ำหนัก ในกากเมล็ดชาที่มีจำหน่ายเชิงพาณิชย์ที่สกัดด้วยเมธานอล (CU1-MeOH) 0.43 เปอร์เซ็นต์โดยน้ำหนัก และที่สกัดด้วยน้ำ (CU1-H_2O) 0.25 เปอร์เซ็นต์โดยน้ำหนัก ในส่วนที่เป็นเนื้อของผลชาที่สกัดด้วยเมธานอล (FP-MeOH) 1.26 เปอร์เซ็นต์โดยน้ำหนัก และที่สกัดด้วยน้ำ (FP-H_2O)1.07 เปอร์เซ็นต์โดยน้ำหนัก ตามลำดับ

ภาควิชา.....เคมี.....ลายมือชื่อนิสิต.....สุพรรษา กิจประยูร.....

สาขาวิชา.....เคมี.....ลายมือชื่ออาจารย์ที่ปรึกษา.....

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467 24583 23: MAJOR CHEMISTRY
KEYWORDS: MOLLUSCICIDAL, SAPONIN, TEA SEED, *Pomacea canaliculata*, *Camellia oleifera*.

SUPUNSA KIJPRAYOON: MOLLUSCICIDAL ACTIVITY OF SAPONINS FROM TEA SEEDS *Camellia oleifera* Abel. ON GOLDEN APPLE SNAILS *Pomacea canaliculata* Lamarck. THESIS ADVISOR: ASSOC. PROF. AMORN PETSOM, Ph.D., THESIS CO-ADVISOR: MRS. VASANA TOLIENG ...83... pp. ISBN 974-14-2301-2

The molluscicidal activity of tea seed extract from *Camellia oleifera* Abel. against golden apple snails *Pomacea canaliculata* Lamarck. showed the LC_{50} values at 6.79 ppm after 24 h. Bioassay-directed fractionation of *C. oleifera* Abel extract resulted in the isolation of saponins group designated D4 with molecular weight between 1226-1338 Dalton. The bioassay data revealed that at 0.66 ppm D4 had molluscicidal activity with 43.33% mortality against the golden apple snails. In addition, the quantitative analysis of D4 was performed by reversed phase high performance liquid chromatography with UV detector. Optimal chromatographic conditions are C18 column (250x4.60mm), 40% methanol-0.1% acetic acid in water as mobile phase, flow rate 0.80 mL/min, wave length detection at 235 nm. Satisfactory validation parameter was obtained: standard calibration was constructed from 10-200 ppm (R^2>0.9990). Extract of fruit pericarp from *C. oleifera* Abel. and extract of commercial tea seed granule was analyzed with HPLC method. The amount of D4 in saponin crude extract from tea seed cake, CU1-MeOH, CU1-H_2O, FP-MeOH and FP-H_2O were found to be 6.82, 0.43, 0.25 and 1.07 %w/w, respectively.

Department.....Chemistry..... Student's signature.....
Field of study.....Chemistry ..... Advisor's signature.....
Academic year.....2005..... Co-advisor's signature.....

ACKNOWLEDGEMENTS

I would like to express my appreciation to my advisor Assoc. Prof. Dr. Amorn Petsom for his kind supervision, instruction and encouragement during my research. I would also like to thank my co-advisor Mrs. Vasana Tolieng for her help and advise. I would like to thank Prof. Dr. Sophon Roengsumran, Assoc. Prof. Dr. Chaiyo Chaichantipyuth, Assist. Prof. Dr Polkit Sangvanich for their valuable suggessions and comments as committee members.

In addition, I wish to thank the Institute of Biotechnology and Genetic Engineering for all facilities and grant. Appreciation is also extended to the Graduate School of Chulalongkorn University for granting partial support to conduct this research.

The samples of tea seed cake form S. T. Seeds Co. Ltd., Siriwat Products Co. Ltd. and Ekyongwong Co. Ltd. are highly appreciate.

Special thanks also give to all members of Institute of Biotechnology and Genetic Engineering for valuable friendship and helpfulness as well as all of my friends for encouragement.

Finally, I wish to express my deep sense of appreciation to my beloved family for their inspiration, understanding, great support and encouragement throughout my study.

CONTENTS

Page

Page

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

Page

LIST OF SCHEMES

Scheme Page

LIST OF ABBREVIATIONS AND SYMBOLS

μL	micro-liter
μm	micro-metre
λ_{max}	Maximun wavelength detection
Ac	acetyl
AcOH	acetic acid
Ang	angeloyl
CH_3OH	methanol
CH_3	methyl
CH_3CN	acetronitrile
$CHCl_3$	chloroform
Cin	cinnamoyl
CU1	CU-one (Tea seed cake contained 12 % Saponins)
DDI	double de-ionized water
FP	Fruit pericarp
FT-IR	Fourier Transform Infrared spectrophotometer
GAS	golden apple snails
HPLC	high performance liquid chromatography
KBr	potassium bromide
LC_0	0 % lethality concentration
LC_{100}	100 % lethality concentration
LC_{50}	500 % lethality concentration
LC_{90}	900 % lethality concentration
m/z	mass to charge ratio
MALDI-TOF	matrix assisted laser desorption ionization time-of-flight
MeOH	methanol
mg	milligram (s)
MHz	mega-hertz
mL	milliliter
mm	millimeter
MS	mass spectrometry
n-BuOH	normal butanol

nm	nanometer
NMR	Nuclear Magnetic Resonance Spectrometer
ODS	Octadodecyl
ppm	parts per million
R_f	retardation factor
RSD	relative standard deviation
$t_{½}$	time half-life
t	tonnes
Tig	tigloyl
TLC	thin layer chromatography
UV	ultraviolet
w/w	weight by weight
WHO	World health organization

CHAPTER I

INTRODUCTION

1.1 State of problem

Thailand is an agricultural country where rice is one of the most important crops. Thai farmers have two major agricultural problems which are weeds and pests such as crabs, birds, rats and especially snails [Chanyapate, 1997].

The golden apple snail *Pomacea canaliculata* (Lamarck) is found in the new world; South America and Central America. It was illegally introduced into Thailand for economic farmed animals and the fish trading for cleaning fish aquaria because of its ability to consume several kinds of aquatic plant and algae. The golden apple snail was first discovered naturally in Thailand in 1984 [Keawjam, 1990]. Unfortunately the introduction of *P. canaliculata* Lamarck was done in haste without prior studies on its ecological impact or market information. When market demand for snails was poor, many snails-farming projects were abandoned and in many instances the snails escaped and subsequently became a major problem for crops, mainly rice because the snails are able to eat almost everything available in their environment.

According to the recent report, the escaped snails quickly spread through waterways and irrigated rice fields. In 1995, it was estimated that 403,896 Rai of rice fields in 27 provinces were devastated by the snails. and 459,229 Rai of rice fields in 30 provinces were devastated in 1996 [Chanyapate, 1999].

Several techniques were used to control snails, including biological, cultural and chemical controls such as Niclosamide, Metaldehyde, Copper Sulfate and Endosalfan. These chemicals are toxic to human and other species such as fish, shrimp and are hazard to public health including skin problems, blurring vision and blindness. There are also significant and long-lasting downstream effects on natural ecosystems. The types of chemicals generally used are persistent and accumulated in

sediment. No chemicals are currently known to be safe, effective and cheap enough to use on a large scale, although various ones are in use, including ones which have been banned in many countries such as Japan, Taiwan and the Philippines.

Many alternative golden apple snails controls have been proposed, such as biological control. Natural plant molluscicides are gradually gaining attention, because natural products have low toxicity and are considered safe and ecologically more suitable for snail control.

The residues from tea oil processing has been used to formulate pesticides, feeds, and fertilizers. The triterpenoid saponins from camellia has been shown to improve immune function, enhance antibacterial and antiviral activities, and to have antimutation and antioxidation properties in humans and animals (Zhan, 1999). Tea seed residues have been used for active control of the following pests: rice blast, rice hopper and snails (Shan and Ying 1982). Extracts of the seed cake left over after processing are known to deter larval development in insects. In the late 1980s and early 1990s the annual production of tea oil in China was about 150,000 tonnes (t)/year (Tang *et al.*,1993). For this reasons, the possibility of developing new biologically based molluscicides is possible.

Figure. 1.1 The oil product from *C. oleifera* Abel.

In several literature reviews and searching for appropriate molluscicidal agents [Toshiaki *et al*., 1989; Lemmich *et al*., 1995 and Yang *et al*., 2001], it was found that the saponins extract from tea seed *Camellia oleifera* Abel. has potential of molluscicidal activity against golden apple snails. Phosawad (2000) reported (molluscicidal activity of) tea seed cake extracted with distilled waster at 12 hour maceration on three sizes of golden apple snail and also their eggs. The tea seed cake extract was effective in controlling golden apple snail but had no effect on the hatching of their eggs. [นันทิยา, 2543]

However, there is no report concerning the isolation, structure determination and quantitative analysis of the molluscicidal activity of saponins from this plant. Therefore, the quantitative determination of saponin is the most important factor when screening for different batch or kind of the plant [slacanin *et al*., 1988]. Thus, this research will emphasize on the molluscicidal activity of saponins from tea seed cake (*C. oleifera* Abel.) on golden apple snails *P. canaliculata*, fractionation and isolation of saponins from tea seed, and quantitative analysis of saponins by spectroscopic method.

1.2 Objectives of study

1.2.1 To study the chemical constituents form tea seed cake of *C. oleifera* Abel.

12.2 To study the molluscicidal activity on golden apple snails *P. canaliculata* Lamarck.

1.3 Scope of study

1.3.1 Fractionation and isolation of saponins with molluscicidal activity from tea seed cake

1.3.2 Structure determination and quantitative analysis of saponins by spectroscopic method

1.3.3 Molluscicidal activity of saponins on golden apple snail

CHAPTER II

LITERATURE REVIEWS

2.1 Golden Apple Snails

Golden apple snails *Pomacea canaliculata* Lamarck. are tropical and sub-tropical freshwater snails from the family Ampullaridae**.** They have an appearance like an apple. They are commonly known as golden apple snails, because of their large size and round shape. This species comes in different colors from brown to albino or yellow and even blue, with or without banding. The body of these snails also shows great variation from black to yellow and gray. When taken good care of some apple snail species can reach a large size. Apple snails are in fact the biggest living freshwater snails on earth.[จรรยาเพศ และ อาชวาคม, 2541]

Figure 2.1. *Pomacea canaliculata* Lamarck.

The identification of species is not exactly. There are three species of *Pomacea* that devastated the rice fields in Thailand as follow, *P. canaliculata* Lamarck., *P. insularus* and *P. spp.* [Chanyapate, 1997]

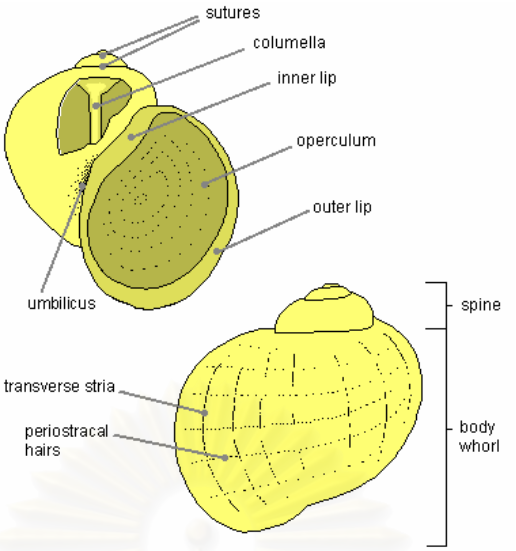


Figure 2.2 The structure of the shell of *P. canaliculata* Lamarck**.** [online]

Available from : http://applesnail.net

2.1.2 The life cycle of golden apple snails

The apple snail is found in the wild floodplain and swamps. Snails are well adapted to live in alternating wetland and dry land habitats such as seasonal swamp or rice fields.

Eggs stage : To lay eggs, adult females crawl out of the water in early morning and evening and lay 25-500 eggs in bright pink batches on rice tillers, sedges, rice field dikes, or other firm substrata protruding from water. Within 1-2 weeks after deposition, the eggs in the egg masses gradually lighten and hatching commences. Hatch ability was highly variable, ranking from 7% to 90%

Pre-adult stage : The newly hatched neonated snail drops into the water and soon starts moving about, feeding on algae and detrital aggregates. The abundance of juvenile snails is negatively correlated with the density of adult snails. When grown to shell height of about 1.5 cm, juveniles start consuming plant material.

Adult stage : The golden apple snail has separated sexes, which can be morphologically distinguished by curve of the operculum. Under favorable conditions, females reach maturity within 60-85 days after hatching and may spawn at weekly intervals throughout the year.

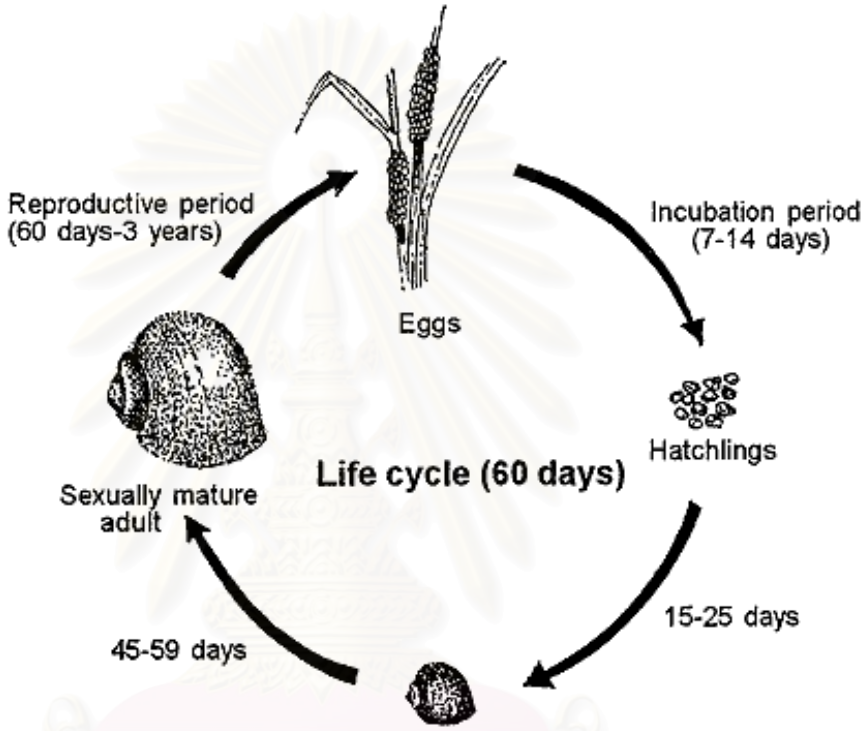


Figure 2.3 The life cycle of golden apple snails [Online] Available from : http://applesnail.net

2.2 *Camellia* Genus

The plants in *Camellia* genus (Theaceae family) have distant origins, as can be seen from the fairly primitive structure of the flower, and is found naturally in vast area of the East and the South East Asia. According to the most recent research, there are about 250 species. The Camellia have been grown for many centuries for decorative purposes and for food.

2.2.1 The Classification of *Camellia oleifera* Abel.

Kingdom	Plantae (Plants)
Subkingdom	Tracheobionta (Vascular plants)
Superdivision	Spermatophyta (Seed plants)
Division	Magnoliophyta (Flowering plants)
Class	Magnoliopsida (Dicotyledons)
Subclass	Dilleniidae
Order	Theales
Family	Theaceae (Tea family)
Genus	*Camellia* L. (camellia)
Species	*Camellia oleifera Abel.* (tea oil plant)

2.2.2 General characterization of *Camellia oleifera* Abel.

Tree : *Camellia oleifera* Abel. or the tea oil plant is a big shrub, 8m tall with many virgate stems arising from the base of the plant near the ground.

Leaf : Leaf hard, thick and leathery; marginal veins indistinct and appear sunken in lamina. Blade elliptic with obtuse or broadly obtuse apex; base cuneate, margin bluntly serrulate to sinuate-serrulate with more or less incurved teeth, glabrous above and villose below when young, becoming sparsely villose as the leaf ages. Ultimately becoming glabrous. Young leaves garnet-brown through ox-blood to

purple in colour. Petiole short, 3-7 mm long, stout, usually giving the leaf an erect pose.

Flowers : Flowers perulate, terminal, solitary or in pairs, fragrant, white with 5-7 petals. Flowers usually 5-6 cm across but can reach 8-9 cm in some specimens.

Fruit : Round shape, 0.5-1 inch length, hard or dry fruit.

Seed : Containing 1 spherical seeds, 15-20 mm in diameter.

Figure 2.4 The general characterization of *C. oleifera* Abel.

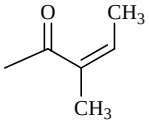
[http:www.tea \ *Camellia oleifera* Abel. - definition of *C. oleifera* Abel.

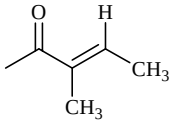
in Encyclopedia_files]

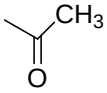
2.2.3 Constituents of *Camellia* spp.

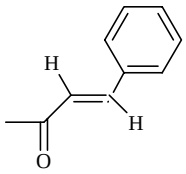
Table 2.1 Chemical constituents found in *Camellia* spp.

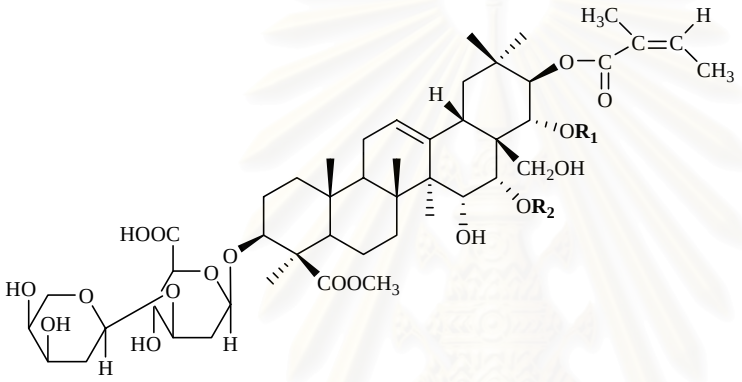
Species	Part	Compounds	Ref.
C. *assamica*	Root	TR-saponin A-C(1-3)	Lu *et al.*,2000.
	Leaf	Assamsaponins J (13)	Uemura *et al*., 1994.
	Seed	Assamsaponins A-J (4-13) Theasaponins E_1 - E_2 (14-15) Camelliasaponins B_1(18)	Murakami *et al*., 1999a; Kitagawa *et al*.,1998 ; Murakami *et al*.,1999b
C. *japonica* L.	Seed	Camelliasaponins A_1- A_2 (16-17) Camelliasaponins B_1- B_2 (18-19) Camelliasaponins C_1- C_2 (20-21	Yoshikawa *et al*.,1994
	Flower	Camelliosides A-D (22-25)	Nagata *et al*.,1998; Yoshikawa *et al*.,2001
	Leaf	Camelliadin I (26), Camelliadin II (27)	Nishio *et al*.,1986 ; Yoshikawa *et al*., 1996
C. *sinensis* L.	seed	Camelliasaponin 1 (28) Theasapogenols A-E(29-33) Theasaponins E_1 - E_2 (14-15)	Sagesaka *et al.*, 1994, Kitagawa *et al*.,1998 ; Nagata *et al*.,1985
C. *oleifera* Abel.	Seed	Sesamine (34), Kaemferol (35), Theasapogenaol A (31), E(35) Camelliagenin A (43) Barringtogenol C (44)	Bin and Yongming *et al*.,2003; Lee and Yen *et al*., 2006

Ang : angeloyl 

Tig : tigloyl 

Ac : acetyl 

Cin : cinnamoyl 



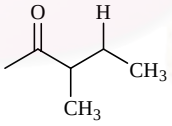
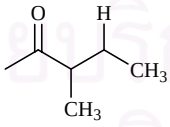
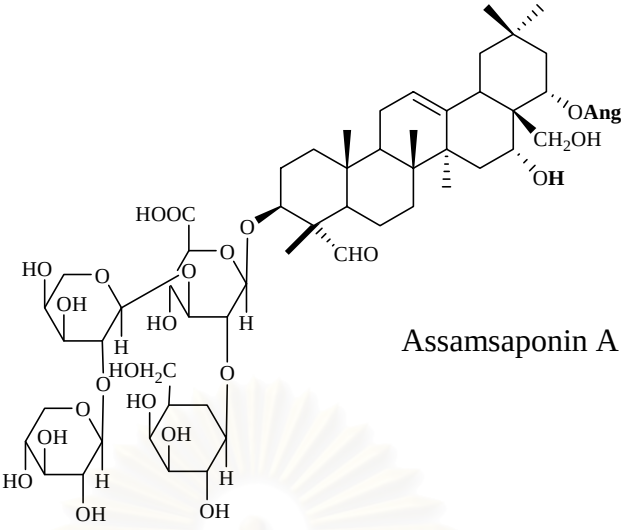
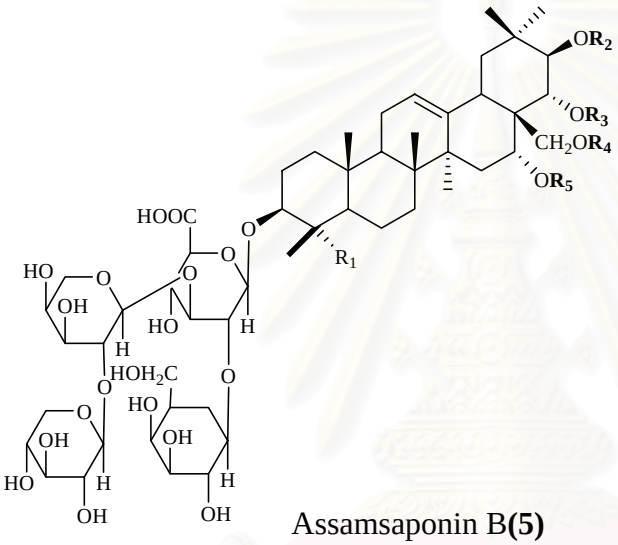
	R1	R2
TR-saponin A **(1)**	Ang	H
TR-saponin B **(2)**		H
TR-saponin C **(3)**		COCH3

Figure2.5 Saponins found in *Camellia sinensis* var *assamica*

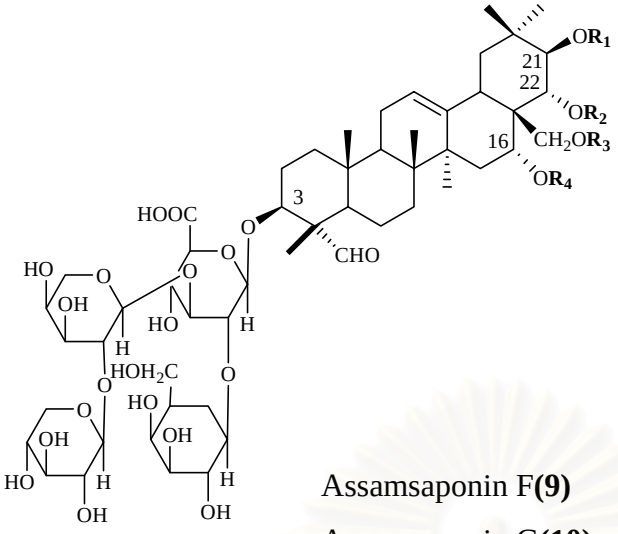


Assamsaponin A **(4)**



	R_1	R_2	R_3	R_4	R_5
Assamsaponin B**(5)**	CHO	Ang	Ac	H	Ac
Assamsaponin C**(6)**	CHO	Tig	H	Ac	H
Assamsaponin D**(7)**	CH_2OH	Ang	Ac	H	H
Assamsaponin E**(8)**	CH_3	Tig	H	Ac	H
Theasaponin E1**(14)**	CHO	Ang	Ac	H	H
Theasaponin E2**(15)**	CHO	Ang	H	Ac	H

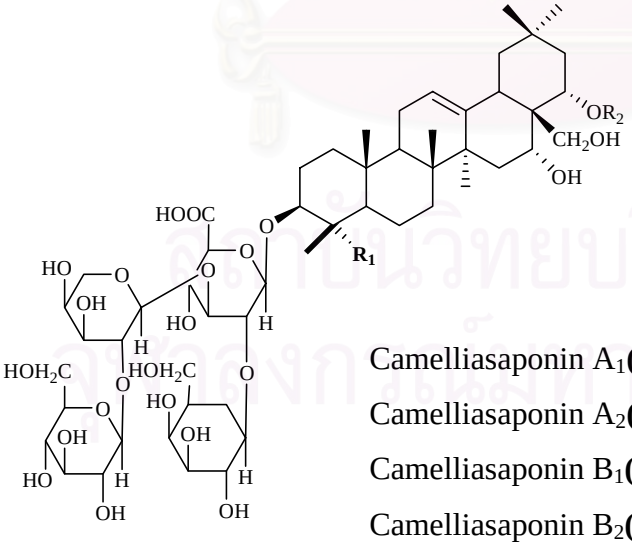
Figure2.5 (Cont)



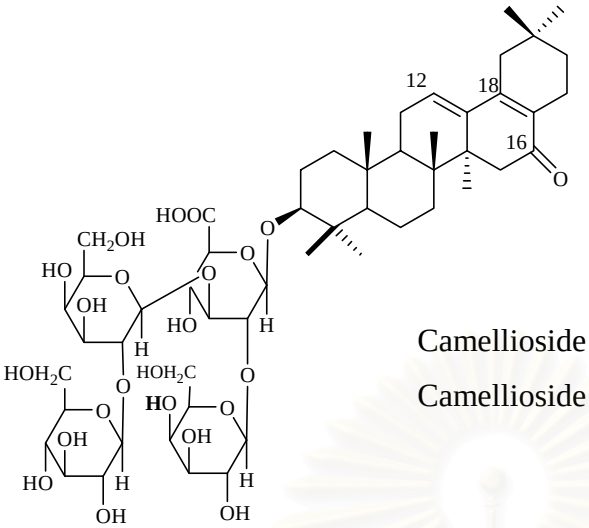
	R_1	R_2	R_3	R_4
Assamsaponin F**(9)**	Ang	Ac	H	Ac
Assamsaponin G**(10)**	Ang	Ac	H	H
Assamsaponin H**(11)**	Ang	H	Ac	H
Assamsaponin I**(12)**	Tig	H	Ac	H
Assamsaponin J**(13)**	Ac	Cin	H	Ac

Figure2.5 (Cont)

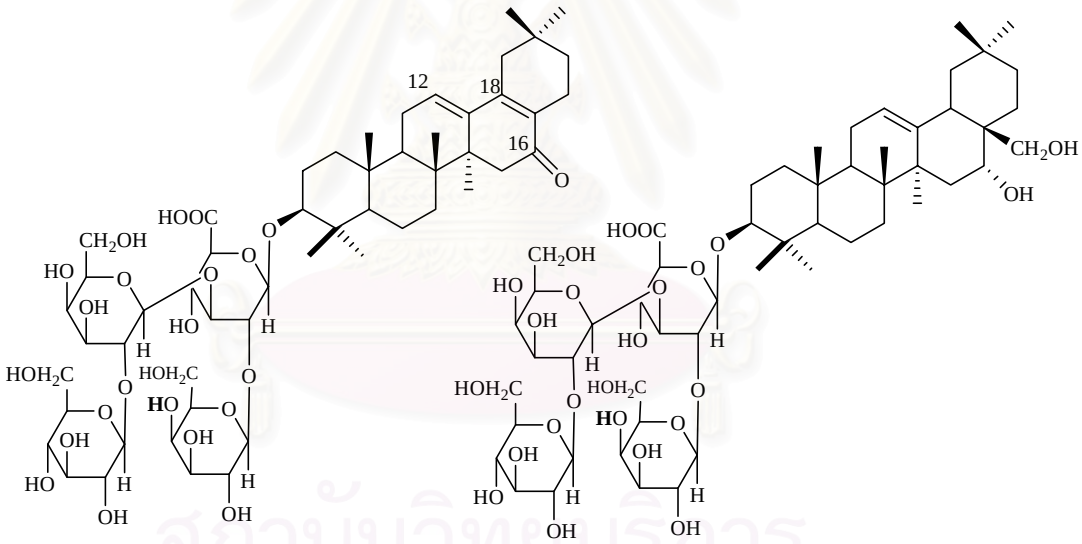
Figure2.6 Saponins found in *Camellia japonica* L.



	R_1	R_2
Camelliasaponin A_1**(18)**	CH_3	Ang
Camelliasaponin A_2**(19)**	CH_3	Tig
Camelliasaponin B_1**(20)**	CHO	Ang
Camelliasaponin B_2**(21)**	CHO	Tig
Camelliasaponin C_1**(22)**	CH_2OH	Ang
Camelliasaponin C_2**(23)**	CH_2OH	Tig

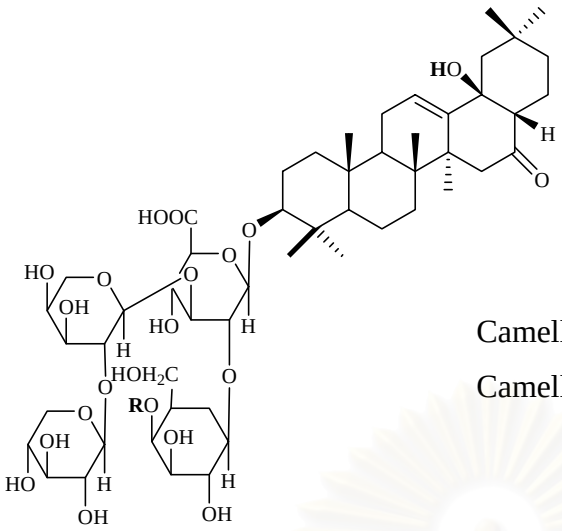


	R
Camellioside A**(22)**	H
Camellioside B**(23)**	Ac



Camellioside C**(24)** Camellioside D**(25)**

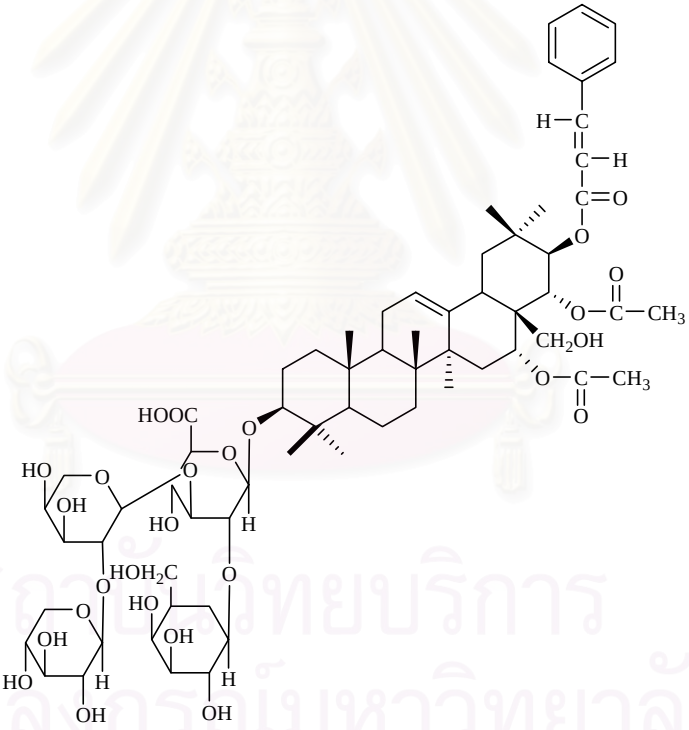
Figure2.6 (Cont)



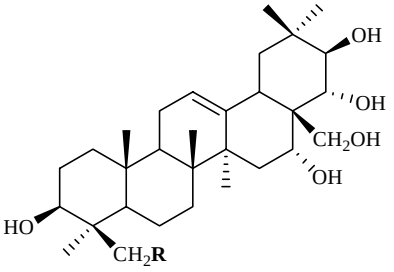
	R
Camellidin I**(26)**	Ac
Camellidin II**(27)**	H

Figure2.6 (Cont)

Figure2.7 Saponins found in *Camellia sinensis* L.

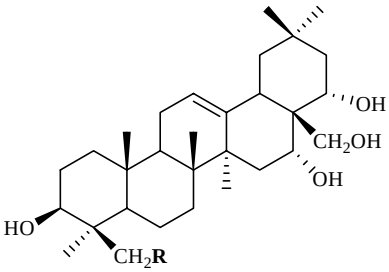


Camelliasaponin 1 **(28)**



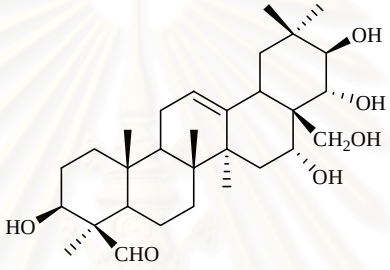
Theasapogenol A **(29)** : R=OH

Theasapogenol B **(31)** : R=H



Theasapogenol C **(30)** : R=OH

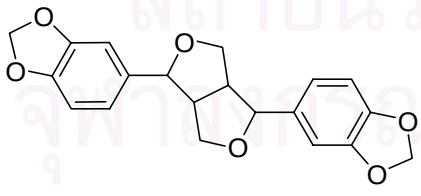
Theasapogenol D **(32)** : R=H



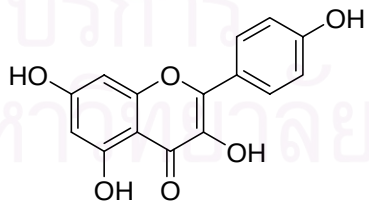
Theasapogenol E **(33)**

Figure2.7 (Cont)

Figure2.8 Compounds found in *Camellia oleifera* Abel.



Sesamine **(34**)



Kaemferol **(35)**

2.3 Saponins

2.3.1 Definitions

The aglycone or non-saccharide portion of the saponins molecule is called the genin or sapogenin. Depending on the type of genin present, the saponins can be divided into three major classes:

1) Triterpene glycosides
2) Steroid glycosides
3) Steroid alkaloid glycosides.

The genins of these three classes can be depicted as shown in figure 2.9 The aglycones are normally hydroxylated at C-3 and certain methyl groups are frequently oxidized to hydroxymethyl, aldehyde or carboxyl functionalities. When an acid moiety is esterified to aglycone, the term ester saponin is often used for the respective glycosides.

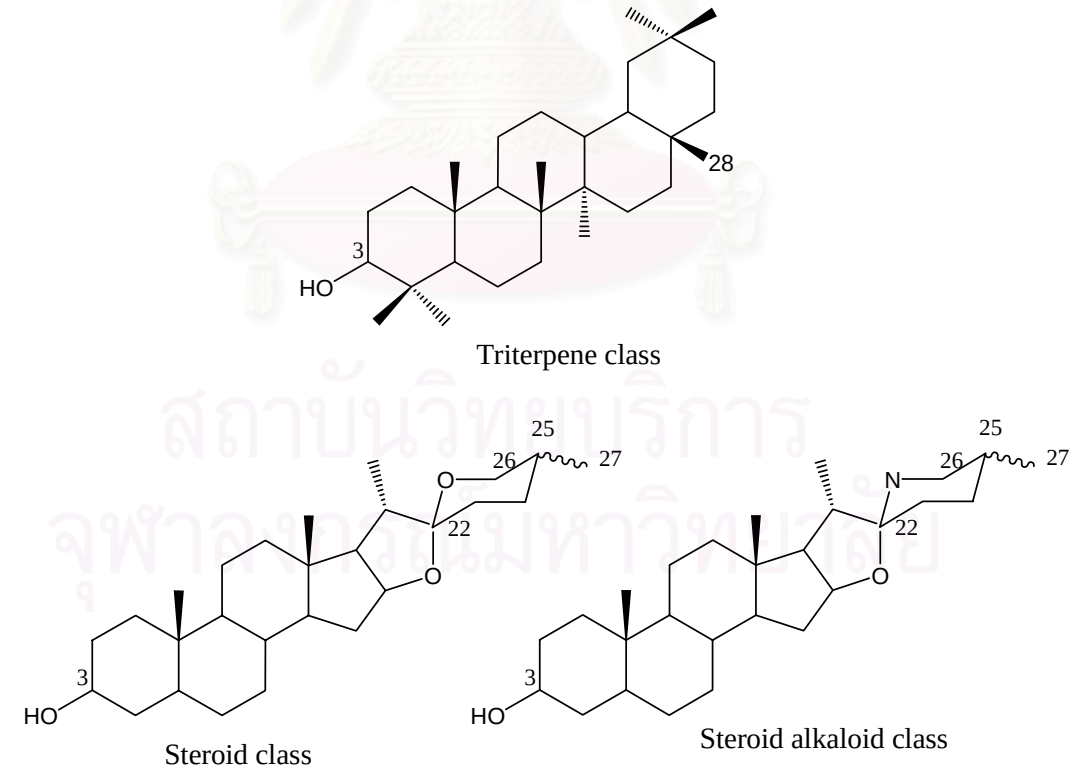


Figure 2.9 Skeletal types of genin found in the three principal classes of saponin.

All saponins have in common the attachment of one or more sugar chains to the aglycone.

Monodesmosidic saponins have a single sugar chain, normally attached at C-3.

Bidesmosidic saponins have two sugar chains, often with one attach through an ether linkage at C-3 and one attach through an ester linkage (acyl glycoside) at C-28 (triterpene saponins).

Tridesmosidic saponins have three sugar chains and are seldom found.

2.4 Literature Review

2.4.1 Literature review on the analysis and quantitative determination of saponin by HPLC method.

HPLC method is the most powerful and the most frequently used for saponins and sapogenins determination because of its speed, sensitivity and adaptability to non-volatile, highly polar compounds. Most of work has been performed on reversed phase C-8 and C-18 column, but other modified silica gel supported including NH_2 and DIOL are occasionally used. If difference is not too great within a series of saponins under test (only small changes in the sugar chain), isocratic elution is possible [Domon *et al*., 1984] and more polar bidesmosidic saponins eluted much faster than monodesmosidic saponins.

Slacanin (1998) report the quantitative determination of monodesmosidic saponins from *P. dodecandra*. Treatment of oleanoic acid glycosides with 4-bromophenacyl bromide in the presence of potassium bicarbonate and crown ether results in the formation of the bromophenylacyl derivatives. The bromophenylacyl strongly absorb at 254 nm and detection can be performed at this wave length without interference from solvent.

In order to remove interfering (often highly UV-absorbing) material, a pre-purification step may be necessary. This can take the from of a clean-up on Sep-Pak

$^{R}C_{18}$ [Pieta *et al*.,1986; Ireland and Dziedzic, 1986b] For glycoalkaloids, sample preparation with Sep-Pak^{R} C_{18} and Sep-Pak ^{R} NH_2 cartridges has been reported [Saito *et al*., 1990 a].

In the case of ionic compounds (those containing a free carboxyl group on the aglycone or glucoronic acid moieties, for example), some method of expressing ion formation is required if peak broadending is to be avoid [Henry *et al.*, 1989]. This can be achieve by addition of a low UV-adsorbing acid to the eluent, such as phosphoric acid [Wagner *et al*., 1985] or trifluoroacetic acid [Apers *et. Al.*, 1998].

A reversed phase high performance liquid chromatographic determination of ginsenosides Rb_1, Rb_2, Rb_C, Rb_d, Re, Rb_{g1}, Gypenoside XVII, pseudoginsenoside-F11. and an indirect determination of the malonyl-ginsenosides Rb_1, Rb_2, Rb_C, Rb_d in *Panax quinquefolium* was developed. Ground ginseng samples were extracted with aqueous methanol in an ultrasonic bath. Separations were achieved with a phosphate buffer-acetonitrile gradient system using a C_{18} reverse phase column. [Court *et al*., 1996]

Camelliasaponin A_1-A_2, B_1-B_2, C_1-C_2 were isolated from tea plants (*Camellia japonica*) by HPLC method, using MeOH-0.1 % aqueous AcOH and CH_3CN-0.1% aqueous AcOH as mobile phase [Yoshikawa *et al*., 1996]. In the same mobile phase, Assamsaponin J was isolated from leaves of *Camellia sinensis* var *assamica* PIERRE [Murakami *et al*.,2000]

Li et al. (2005) reported the quantitative analysis of saikosaponins from *Radix Bupleuri* in China by high performance liquid chromatography. Saikosaponin-a and –d were converted completely into saikosaponin-b_1 and –b_2 by mind acid treatment. Distinctive measuring of these converted diene- saponins provided a rapid and selective method for the determination of saikosaponin-a and –d in commercial samples of *Radix Bupleuri*. The HPLC analysis was performed on ODS-C18 column with flow rate of 1.0 mL/mn and detection wavelength of 250 nm. Well resolved chromatogram of saikosaponin-b_1 and –b_2 were obtained with an isocratic elution of acetonitrile : 1% formic acid water (37.5 : 62.5). [Li *et al*., 2005]

2.4.2 Literature review on molluscicidal activity of saponins

Saponins have a pronounced action on mollusks and organisms which use gills for breathing, such as frogs, and fish the mechanism of this toxicity most likely involves the binding of the saponins to the gills membranes, resulting in and increase in their permeability and a subsequent loss of important physiological electrolytes. Studies on the mode of action, however, have a produced conflicting results. Although toxic to cold-blood species, if taken orally by warm-blooded species, saponins have only a weak toxicity [Bruneton, 1995], which is probably attributed to low absorption rates.

Molluscicidal activity has so far only been observed for monodesmosidic saponins [Domon and Hostettmann, 1984], with bidesmosidic saponins requiring basic or enzymatic hydrolysis to the corresponding monodesmosides before the induction of activity. Saponins of oleanolic acid or hederagenin are the most active, while the corresponding aglycones are inactive [Marston and Hosttetmann, 1985]. Other factors which are important for the activity are :

- The nature of sugar chains
- The sequence of the monosaccharide
- The interglycosidic linkages

Lauhachinda (1996) reported, neem seed extract to control the snail directly revealed that the concentration at 2-3 ppm caused 73-100 % of the tested animals to die at 72 h after application. Field test of neem seed extract on various size of snails demonstrated that concentration at 6 ppm can caused 70-80 % death of all size snail at 72 h.

Somkasettrin (1999) reported that neem extracts at concentration 3 ppm caused 100% mortality to small size snails (20-30mm) at 48 h while the medium (30-40mm) and large size (50-60mm) snails reach 100% mortality at 72 h.

Derris root and tea seed cake extracted by distilled water at 12 h maceration were tested on three sizes (5-20, 21-35 and 36-50 mm) of golden apple snail and also

tested against their eggs. The LC_{50} value of tea seed cake extract was 21.49, 42.10 and 48.79 mg/L for three sizes of snail, respectively. The LC_{50} value of derris root extract was 25.20, 45.26 and 77.00 mg/L for three sizes of snail, respectively. The hatching of eggs showed that there was no effect with the extracts in all concentration [นันทิยา, 2543].

Somrudee (2002) studied the toxicity of indigenous plant extract to golden apple snail. The result showed that strong activity was observed in aqueous extracts of *Bougainvillea spectabilis*, *Calotropis gigantean* and *Croton tiglium* and many types of plant in ethanolic extract. [Somrudee, 2002]

Phytolacca dodecandra and *P. icosandra* L. berries contain saponins with highly molluscicidal activity [Treyvaud *et al.,* 2000]. Aqueous extract (25 ppm) of *P. icosandra* L. had very high molluscicidal activity against *Biomphalaria glabrata* snails. According to Treyvaud (2000), the activity can be attributed to the presence of monodesmosidic saponins of serjanic and spergulagenic acids.

Molgaard (2000) investigated the biodegradability of molluscicidal fraction of water-extract saponins from the berries of *Phytolacca dodecandra*. Results showed that the saponins in an aqueous extract of *Phytolacca dodecandra* readily biodegraded ($t_{½}$ = 15.8 h). The saponins were completely consumed within 10 days which indicates their abilities to degrade in aquatic environments under aerobic conditions. [Molgaard *et al*., 2000]

Apers (2001) tested 10 saponins isolated from the leaves of *Maesa lanceolata* for molluscicidal activity against *Biomphalaria glabrata* snails. The LC_{50} values of the saponins mixture was 1.25 mg/mL. However, it was concluded that one of saponins, maesasaponin VI_2 is responsible for a large part of the activity of the mixture. This saponin had LC_{50} value of 0.5 mg/ml in its isolated form. [Apers *et al.,* 2001]

Triterpenoid hederagenin saponins isolated from *Sapindus mukorossi* Garetn. (*Sapindaceae*) had molluscicidal effects against the golden apple snail, *Pomacea canaliculata*, which have become major pests of rice and other aquatic

crops throughout Taiwan and other parts of Asia [Huang *et al*.,2003]. Seven isolated hederagenin saponins including one new hederagenin saponins, resulted in 70%-100% mortality at 10 ppm against the golden apple snails. Hederagenin saponins with three sugar moieties had higher molluscicidal activity than triterpene saponins with one sugar moiety.

CHAPTER III

EXPERIMENTAL

3.1 Plant Material.

The tea seed cake of *Camellia oleifera* Abel. originated from China was obtained locally.

3.2 Tested animals

The golden apple snails *Pomacea canaliculata* (Lamarck) with 3.5-5.0 cm. shell length and average weigh of about 12-15 grams were used for molluscicidal test. The snails were collected from Amphoe Wang Noi, Ayuthaya Province, Thailand, in 2005.

Figure 3.1 The golden apple snails *Pomacea canaliculata* (Lamarck) with 3.5-5.0 cm. shell length. were used for molluscicidal test.

3.3 Instruments and Equipments.

3.3.1 Fourier Transform Infrared spectrophotometer (FT-IR).

The FT-IR spectra were recorded on a Nicolet Impact 410 Spectrophotometer. Spectra of solid samples were recorded as KBr pellets.

3.3.2 Nuclear Magnetic Resonance Spectrometer (NMR)

The ^{1}H and ^{13}C Nuclear Magnetic Resonance Spectra were recorded at 500 MHz on Varian Mercury +500 NMR spectrophotometer. Chemical shifts are expressed in part per million (ppm) using residual protonated solvents as reference.

3.3.3 Mass Spectrometer (MS)

The mass spectra were recorded on a MALDI-TOF mode

3.3.4 High Performance Liquid Chromatography (HPLC)

HPLC separations were performed on a Thermofinigan apparatus with

Pump : Thermofinigan P4000

Detector : SpectraSYSTEM® UV-detector (Thermo Finigan UV 6000LP)

Analytical Column : Phenomenex ® Luna 5 μm C18 250x4.60 mm.

Semi-Prep Column : Hyperprep® HS C18 (250 x 10 mm. i.d. 12μ) (Thremo Hypersil)

Guard Column : ODS C_{18}

Pre-column : Sep-Pak ^{R} C_{18}

Software : Chromquest

Fraction collector : Foxy Jr.

3.4 Chemicals

3.4.1 Solvents for column chromatrography

All solvents used for column chromatrography such as methanol, ethanol and dichloromethane were commercial grade.

3.4.2 Solvent for High Performance Liquid Chromatography (HPLC)

MeOH and CH_3CN were HPLC grade from Merck Co.

3.4.3 Other chemicals.

3.4.3.1 Diaion HP-20 (Mitsubishi Chemical Co., Tokyo, Japan) was used as adsorbent for column chromatrography.

3.4.3.2 Precoated silica gel (Merck 60 F-254) plates were used for TLC to identify the identical fractions.

3.4.3.3 *p*-anisaldehyde (Merck) was used as detecting agent for detection of the steroid or triterpenoid compounds on TLC plates

3.4.3.4 Merck's silica gel 60 Art. 1.07734.100 (70-230 mesh ASTM) was used as adsorbent for column chromatography.

3.4.3.5 Niclosamide 70 % wettable powder (WP)(Bayerthai,Co.Th), Bionene (Cothtech. Co. Th.), Metaldehyde 80 % WP(Sotus International. Co. Th.) and CU-1 (tea seed granule with 12 % saponin) (Siriwat Product. Co.) were used as the positive control chemicals.

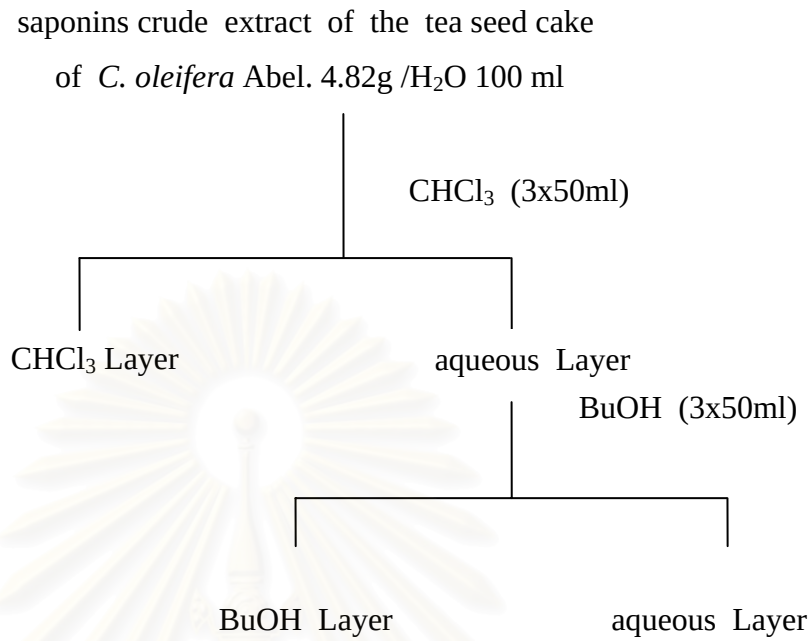
3.4.4 Chemical Test

***p*-anisaldehyde reagent**

The reagent is consisted of 15 ml MeOH, 2 ml acetic acid, 10 ml sulfuric acid and 0.2 ml *p*-anisaldehyde. Reagent was sprayed to the spots on TLC plates for detection of the spots of steroid or triterpenoid compounds. If the spots change to dark blue or greenish blue, the sample may contain steroid or triterpenoid compounds.

3.4.5 Extraction and isolation.

The saponins crude extract from tea seed cake of *Camellia oleifera* Abel. (4.82 g) was suspended in 100 ml water and partitioned with chloroform (3x50 ml) and n-butanol (3x50 ml) at room temperature, respectively. Each layer was evaporated under reduced pressure until dry to obtain $CHCl_3$ extract (0.82g), n-butanol extract (0.4 g) and aqueous extract (3.6 g). The partition procedure is shown in the scheme 3.1.



Scheme 3.1 The partition procedure of saponins crude extract of the tea seed cake of *C. oleifera* Abel.

3.5 Separation of the BuOH extract of the tea seed cake of *C. oleifera* Abel.

The n-BuOH extract, the most active fraction for molluscicidal activity testing, was obtained as a brown powder after evaporation. Preliminary purification of BuOH extract was made by using column chromatography over 4.5 x 25 cm high Diaion HP-20 porous polymer resin column. The column was eluted with H_2O, 30, 50, 70 and 100 % MeOH, successively. Each fraction was analyzed by TLC and spots were detected with *p*-anisaldehyde solutions. The result of preliminary purification of n-BuOH extract using Diaion HP-20 column chromatography is shown in Table 4.3.

3.6 Separation and quantitative analysis of the saponins extract by High Performance Liquid Chromatography (HPLC)

3.6.1 The optimization of HPLC separation

An HPLC equipped with a C_{18} column and 0.1 % acetic acid-water **(A)** and methanol **(B)** as a mobile phase was used to develop optimum separation. The injection volume was 10 μL and the detector was UV-Visible Type. The optimization condition was determined by varying the mobile phase strength and flow rate. The optimal HPLC condition is summarized and shown in Table 4.5.

3.6.2 The construction of standard calibration curve

1. Preparation of 500 ppm standard stock solutions

Saponins standard was weighed accurately to the nearest 0.0050 g and dissolved in 50 % MeOH in 10.00 mL volumetric flask.

2. Standard calibration curves was constructed by

2.1 Series of 10, 50, 100, 150 and 200 ppm standard solutions were prepared from 500 ppm standard stock solutions

2.2 Each of standard solutions in step 2.1 was analyzed three times by HPLC and area under standard peak was averaged.

2.3 Peak area of standards were recorded and regressed with corresponding concentrations to obtain regression lines and corresponding correlation coefficients (R^2).

3.7 Samples preparation

Fruit pericarp of *C. oleifera* Abel. samples

3.7.1 MeOH-extracted

Dried and powdered fruit pericarp of *C. oleifera* Abel. (3 g) was extracted with MeOH by soaking at room temperature for 1 days. The brown solution was filtered and solvent was removed under vacuum to obtain a brown MeOH-extracted crude sample designated as FP-MeOH.

3.7.2 Water-extracted

The water extracted of fruit pericarp was done in parallel as the same procedure as in 3.7.1, excepted the solvent was changed from MeOH to water. A dark brown crude extract was obtained after evaporation and referred as water-extracted sample designated as FP -H_2O.

CU1 Samples

3.7.3 MeOH-extracted

Dried and powdered of CU1(3 g) was extracted with MeOH by soaking at room temperature for 1 days. The brown solution was filtered and solvent was removed under vacuum to furnish a brown MeOH-extract of CU1 designated as CU1-MeOH.

3.7.4 Water-extracted

The water extract of CU1 was done in parallel as the same procedure as in 3.7.3, excepted the solvent was changed from MeOH to water. A dark brown crude extract was obtained after evaporation and referred as water-extracted sample designated as CU1-H_2O.

3.8 Molluscicidal activity testing [Huang , 2003]

3.8.1 Preparation of tested snails.

The snails used for this study was the golden apple snails *Pomacea canaliculata* (Lamarck). For preparation of dechlorinated water for golden apple snails, tap water was left at room temperature at least 24 h before used. The snails were collected from Amphoe Wang Noi, Ayuthaya Province and were acclimated to laboratory conditions before testing (at least 3 days) by feeding them with water mimosa and banana leafs. Before Molluscicidal testing, the snails were selected by size of shell length at about 3.5-5.0 cm.

3.8.2 Molluscicidal test

The experiment was carried out in two steps that were preliminary molluscicidal test and definitive molluscicidal test. For preliminary molluscicidal test, the range finding test was guided to find out the range which was defined as the interval between the lowest concentration that killed all snails (LC_{100}) and the highest concentration that killed none of the snails (LC_0). The experiment in preliminary molluscicidal range finding test consisted of six concentrations of crude extract (from tea seed cake, of *C. oleifera* Abel) and extracting solvent. The amount of mortal snails of range finding test was recorded and these data were then used to establish a more narrow concentration range for the definitive molluscicidal tests.

The experiment in definitive molluscicidal tests consisted of five different concentrations for each compound with three replicates. The control was done in parallel excepted the snails were submerged in the dechlorinated water without any chemicals addition.

For comparison, Niclosamide [70 % wettable powder (WP)], Bionene, Metaldehyde [80 % WP] and CU1 (tea seed cake containing 12 % saponins) were done as same condition as the test experiment. Five different concentrations for each compound with three replicated (10 snails for each) were kept in a 1-L plastic jar containing 300 ml of solution. Snails were submerged in the respective test compound solution in a jar covered with net to prevent them from escaping. The snails were fed for 24 h. and checked for dead snails by probing with a needle to detected their response and the number of dead snails was count after 24 h.

Figure 3.2 The experiment in molluscicidal tests consisted of six different concentrations for each compound with three replicate (300 mL).

Figure 3.3 The snails were submerged in the test solution (10 snails for each).

Figure 3.4 Snails were submerged in the respective test compound solution in jars covered with net to prevent them from escaping.

Figure 3.5 The snails were left for 24 h. and checked for dead snails.

3.8.3 Statistical procedure

Percentage mortality was corrected by Abbott's formula [Abbott 1925]. If the control replication had dead snail between 5-20 %, the mortality percentage was used the Abbott's formula as follows:

$$\% \text{ Mortality} = \frac{\% \text{ test mortality} - \% \text{ control mortality}}{100 - \% \text{ control mortality}} \times 100$$

% test mortality is percentage of total dead snail in extract and chemicals solution at 24 h.

% control mortality is percentage of total dead snail in dechlorinated water at 24 h.

If the control replication had more than 20% dead snail, the experiment was cancelled and had to be repeated it again.

Data on accumulated mortality of snails after 24 h were analyzed by Probit Analysis [Meeposom, 2004; Finney, D. J., 1925] that was done by SPSS computer software (Version13). The 95% confidence intervals of the LC_{50} were determined as a measure of the toxicity. The details of Probit Analysis is shown in appendix D.

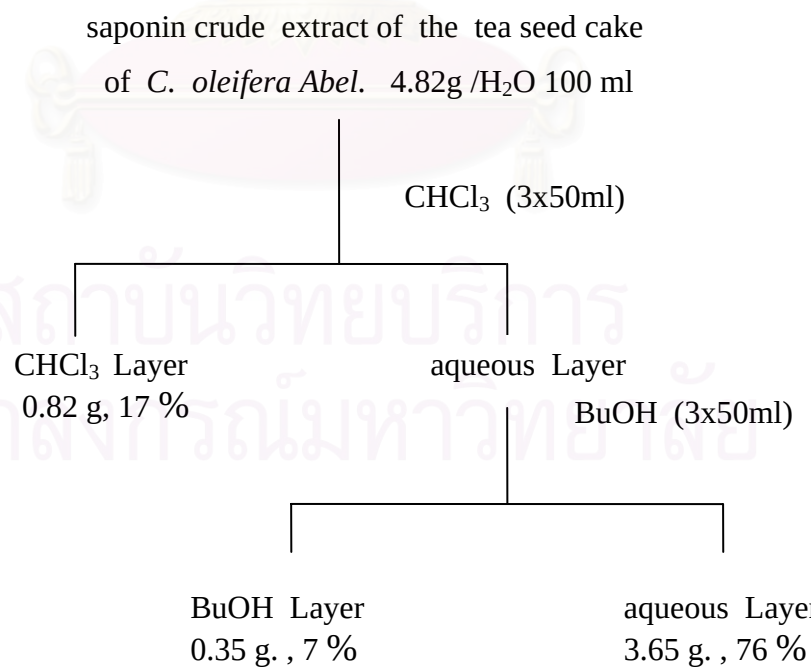
CHAPTER IV

RESULTS AND DISCUSSION

According to the preliminary investigation the MeOH extracted of *Camellia* seed exhibited high molluscicidal activity against *P. canaliculata* Lamarck. (Golden apple snails).

4.1 The results of extraction

The methanolic extract of the tea seed cake of *C. oleifera* Abel. (4.82g) was partitioned between H_2O and $CHCl_3$, and then H_2O and n-BuOH. The result according to the procedure described in Chapter III are shown in Scheme 4.1. Weight of each crude extract and percentage yield (w/w of dried plant material) are summarized as shown in Table 4.1.



Scheme 4.1 The partition procedure of saponin crude extract of the tea seed cake of *C. oleifera* Abel.

Table 4.1 The result from partition of saponin crude extract of the tea seed cake of *C. oleifera* Abel.

Solvent	Appearance	Weight (g)	% w/w of powder (4.82g)
$CHCl_3$	Yellowish powder	0.82	17
n-BuOH	Brown powder	0.35	7
H_2O	Dark Brown powder	3.65	76

According to molluscicidal activity guide, each partition-layer was subjected to molluscicidal testing. The result indicated that the n-BuOH fraction has the highest molluscicidal activity.

4.2 Molluscicidal activity testing of tea seed extract, partition-fraction and chemical control

This molluscicidal activity testing was performed following the procedure described in Chapter III. Each partition-fraction as referred in Tabel 4.1 and chemical controlled samples were subjected for molluscicidal activity testing against *P. canaliculata* Lamarck. The snails death were observed, at 24 h. intervals, and used for calculation of mortality. The results of molluscicidal testing are shown in Table C1 and C2 (Appendix C).

The results of molluscicidal activity test with LC_{50} and LC_{90} are displayed in Table 4.2 and Figure 4.1-4.3.

Figure 4.1 The golden apple snails before saponins exposure.

Figure 4.2 The dead snails after saponin exposure.

Table. 4.2 The results of molluscicidal activity testing against *P. canaliculata* Lamarck at 24 h. intervals.

Compounds	**LC_{50} (ppm) (95 % confidence limits of LC_{50})**	**LC_{90} (ppm) (95 % confidence limits of LC_{90})**
Chemical Controlled		
Niclosamide	0.27	1.12
Metaldehyde	34.98	89.57
CU1	60.32	99.32
CU1 NO. 2	60.56	108.18
Bionine	0.2 *	0.68 *
Partition- fraction		
Saponin crude extract of tea seed cake	9.20	40.88
H_2O- fraction	14.01	44.35
n-BuOH- fraction	6.79	14.43
$CHCl_3$- fraction	7.43	31.36

*LC_{50} and LC_{90} value in mL/L

The W.H.O. quantitates toxicity by means of LC_{90} values but LC_{50} values and 100 % snail kill values, all in ppm are also currently used [Marston and Hostettmann, 1985].

The results of LC_{50} molluscicidal activity test of saponins crude extract and it's partition-fraction with chemical controlled substances showed high molluscicidal activity. Niclosamide showed the highest activity level with LC_{50} value of 0.27 ppm but this pesticide is banned in many countries. Schnorbach (1995) reported that the LC_{50} value of Niclosamide was 0.40 ppm. The rapid mortality, probably due to an acute toxic effect, is desirable, as it reduces the possibility of escaping behavior by the mollusc.

CU1 and CU1 NO.2 tea seed cake contained 12 % saponins) showed medium activity level with LC_{50} value of 60.30 and 60.56 ppm, respectively. The result was comparable to that reported by Nantiya [นันทิยา, 2543], in which the LC_{50} value of tea

seed cake extract against golden apple snails with 3.5-5.0 cm.shell length was 48.79 ppm.

Among partition- fraction, the n-BuOH- fraction displayed the highest molluscicidal activity level with LC_{50} value of 6.79 ppm. The $CHCl_3$- fraction had higher activity than that of H_2O- fraction at LC_{50} value of 7.43 and 14.01 ppm, respectively.

The molluscicidal activity results revealed that the n-BuOH- fraction had potential for further investigation. Based upon the information from molluscicidal activity guide, the n-BuOH- fraction was selected for further isolation.

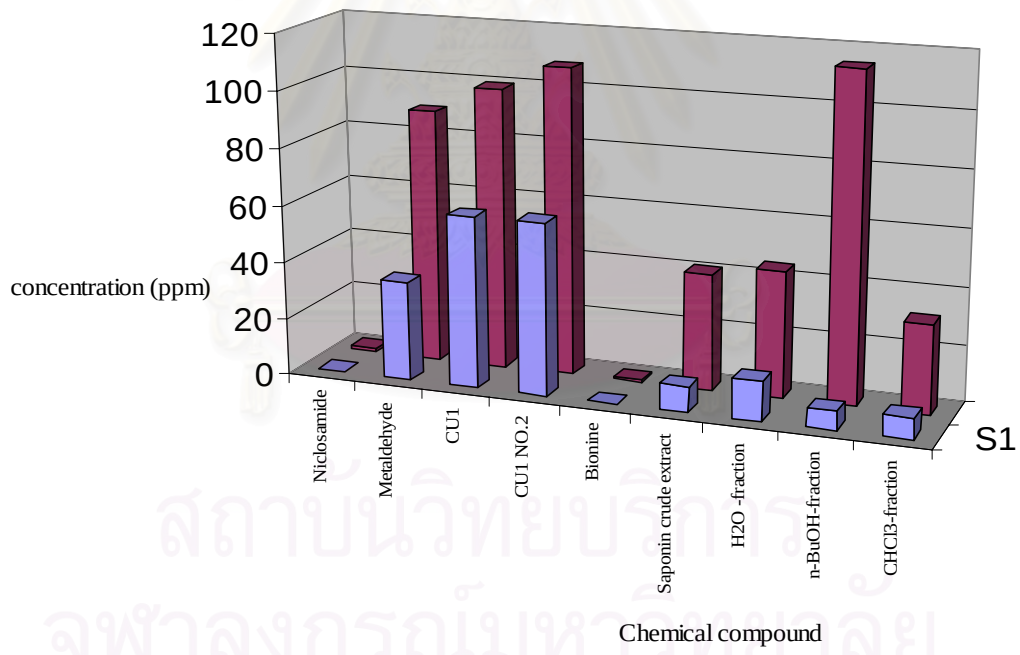


Fig.4.3 LC_{50} values from molluscicidal activity testing of commercial molluscicides and partition- fraction from MeOH extract of tea seed cake of *C. oleifera* Abel against *P. canaliculata* Lamarck.

4.3 The results of preliminary purification of the n-BuOH extract of the tea seed cake

The n-BuOH extract, the most active fraction for molluscicidal activity testing, was obtained as a brown powder after evaporation. Preliminary purification of n-BuOH extract (0.90g) was made by using column chromatography over 4.5 x 25 cm. high Diaion HP-20 porous polymer resin column. The column was eluted with H_2O, 30, 50, 70 and 100 % MeOH, successively (approximately 25 ml per fraction) for 110 fractions. Each fraction was analyzed by TLC using *p*-anisaldehyde solutions as detecting agent. The separation of n-BuOH extract with Diaion HP-20 resin gave 5 group of compounds as shown in Table 4.3.

Table 4.3 The results of preliminary purification of n-BuOH extract was made by using Diaion HP-20 column chromatography.

Group of compounds	Fraction Number	Appearance	% w/w of n-BuOH-fraction
A	1-35	Brown Solid	16.7
B	35-47	Bright Yellow Powder	13.3
C	48-77	Brown Powder	14.4
D	77-99	Brown Powder	21.1
E	99-110	Brown Powder	25.6

According to molluscicidal activity guide, each group of compounds A-E was subjected for molluscicidal testing. The MALDI-TOF data of group D and E exhibited the m/z between 1226-1338. The preliminary literature review showed that saponins compound from *Camellia* spp. have a molecular weight between 1100-1400 [Kitagawa *et al*., 1998; Nagata and Tsudhida, 1985]. From this reason, group D and E could be saponins containing fractions.

4.4 Molluscicidal activity testing of the compounds A-E

This molluscicidal activity testing was performed following the procedure described in Chapter III. The group of compounds A-E were subjected for molluscicidal activity testing against *P. canaliculata* Lamarck. The snails death were observed, at 24 hours intervals, and used for calculation of mortality. The results of molluscicidal testing are shown in Table B1, B2 (Appendix B).

The results molluscicidal activity level with LC_{50} and LC_{90} are displayed in Table 4.4.

Table 4.4 The results of molluscicidal activity testing against *P. canaliculata* Lamarck at 24 hours intervals.

Compound	**LC_{50} (ppm) (95 % confidence Limits of LC_{50})**	**LC_{90} (ppm) (95 % confidence Limits of LC_{90})**
A	>100	>100
B	>100	>100
C	>100	>100
D	6.52	19.57
E	20.92	43.39

From groups A-E, group D displayed the highest molluscicidal activity level with LC_{50} value of 6.52 ppm. These promising results prompted us to further purify bioactive constituents from this fraction.

4.5 Separation and quantitative analysis of the saponins extract by High Performance Liquid Chromatography (HPLC)

HPLC is the most powerful and the most frequently used technique for saponins determination due to the fact that it can be very effective with non-volatile, highly polar compound. HPLC optimization for separation of tea seed cake was carried out by varying acid concentration, ratio of eluent and flow rate. The separations were performed usually on reverse-phase C8 or C18 column., of which C18 has been definitely preferred. Separation of crude extracts of tea seed cake (*C. oleifera* Abel.) was first test on Phenomenex®Luna C18 250x4.60mm, 5 μm column using water/acetonitrile as mobile phase but could not achieve good separation. Adjustment of gradient elution or using different condition did not improve the separation. However, changing organic mobile phase from acetonitrile to methanol could achieve good separation. Therefore, C18 column and methanol/water were selected for analysis of saponin in this work.

4.5.1 Effect of acid

The chromatogram of tea seed cake extract using 40% MeOH/water as mobile phase is illustrated in figure 4.4a which exhibited broadening peak. Hence, 0.1% acetic acid was added to the mobile phase resulting in better separation as shown in figure 4.4b.

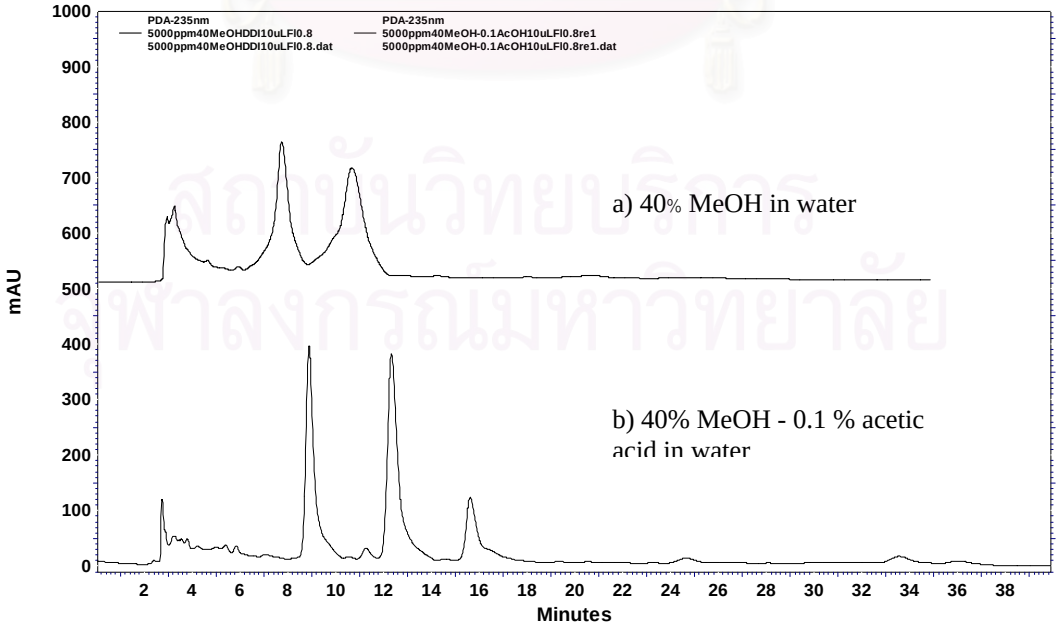


Figure 4.4 The chromatogram of saponins crude extract using different mobile phase a) 40% MeOH in water b) 40% MeOH - 0.1 % acetic acid in water.

4.5.2 Effect of solvent strength

The ratio of methanol in water is one of the important factors affecting separation. Various ratio of methanol, 35, 40, 45, 50 and 55 were used as eluent for separation. The resulting chromatograms are shown in figure 4.5 a-e. An increase in methanol ratio resulted in decrease retention time but could not achieve separation as shown in chromatogram in figure 4.5 a and b. But a decrease in methanol ratio, Figure 4.5, resulted in to long retention time of separation. Moreover, the selection condition was guide by the requirement for obtaining chromatograms with good resolution of adjacent peaks within a short time. Therefore, in this work, 40% methanol-0.1 % acetic acid in aqueous solution was chosen as a mobile phase.

4.5.3 Effect of flow rate.

The chromatogram was carried out using the different flow rate in order to produce good resolution and reduced analysis time. From figure 4.6, an increased in flow rate resulted in a decrease in retention time but it produced good resolution. The suitable flow rate use in this work was 0.8 ml/min as shown in figure 4.6c.

4.5.4 Detection wavelength

The UV detection was scanned from 192-798nm, and photodiode array was monitored and found to absorb UV light with maximum absorbance at wave length 235 nm.

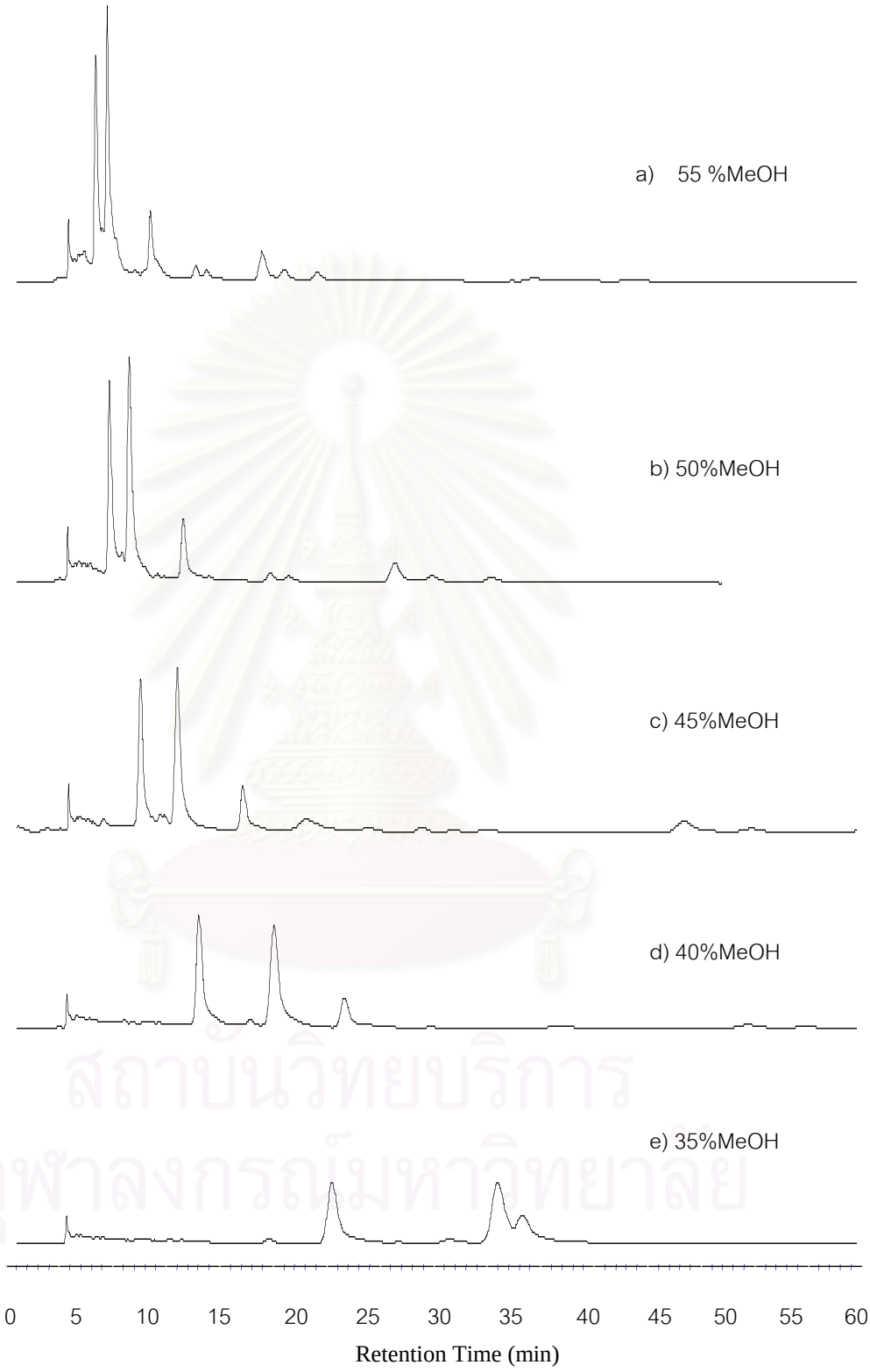


Figure 4.5 The chromatogram of saponins crude extract using different mobile phase with flow rate at 1.0 mL/min a) 55% MeOH - 0.1 % acetic acid in water b) 50% MeOH - 0.1 % acetic acid in water c) 45% MeOH - 0.1 % acetic acid in water d) 40% MeOH - 0.1 % acetic acid in water e) 35% MeOH - 0.1 % acetic acid in water

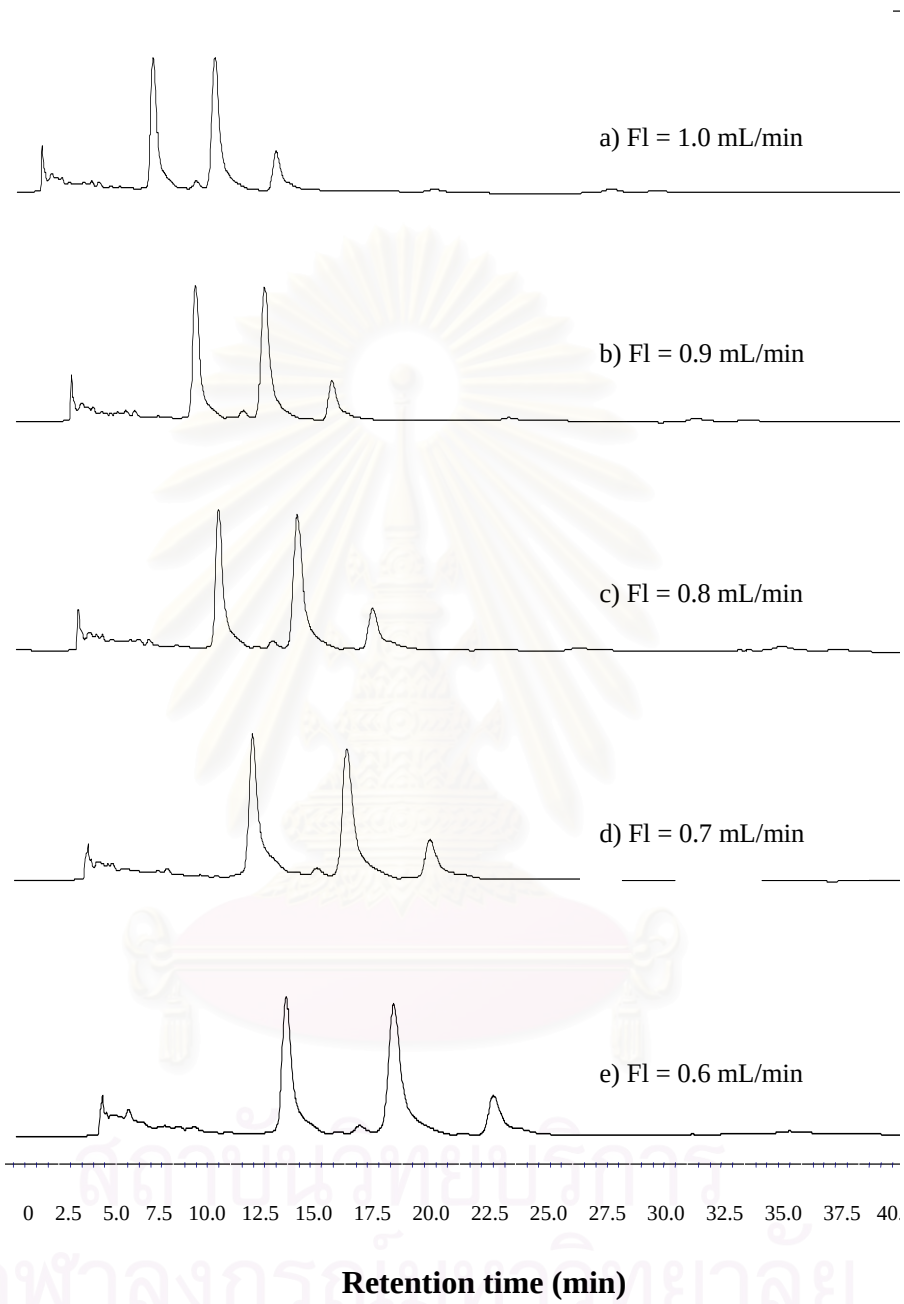


Figure 4.6 The chromatogram of saponins crude extract using 40% MeOH - 0.1 % acetic acid in water as mobile phase with differrent flow rate a) 1.0 mL/min b) 0.9 mL/min c) 0.8 mL/min d) 0.7 mL/min e) 0.6 mL/min.

The HPLC method was developed and the optimum condition is reported in Table 4.5 The chromatogram of saponins crude extract (*C. oleifera* Abel) is shown in Figure 4.7.

Table 4.5 High Performance Liquid Chromatographic conditions.

HPLC Parameter	HPLC Conditions
Analytical Column	Phenomenex®Luna 5 μm C18 250x4.60mm
Mobile phase	40% MeOH - 0.1 % Acetiac acid
Flow rate	0.8 mL/min
Detector	UV-detector
Wave length detection	235 nm

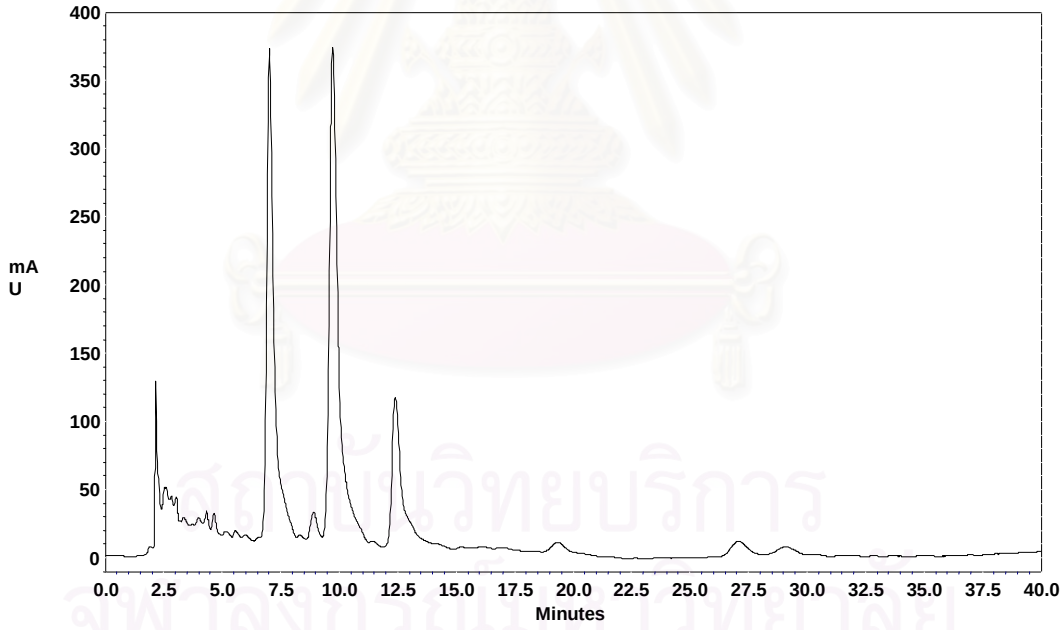


Figure 4.7 The chromatogram of tea seed cake extract using 40% MeOH - 0.1 % acetic acid in water with flow rate 1.0 mL/min.

4.6 The results of preliminary purification of fraction D by High Performance Liquid Chromatography (HPLC)

Fraction D, a group of saponins, was further isolated by HPLC analysis. Compound D4 was obtained from the separation of fraction D by using HPLC condition as shown in Table. 4.6 and chromatogram of fraction D is shown in Figure 4.8. After fractionation by HPLC using condition in Table 4.6 compound D4 was obtained by using fraction collector with retention time at 20.40 min as shown in the chromatogram in Figure 4.9.

Table. 4.6 High Performance Liquid Chromatographic conditions

HPLC Parameters	HPLC Conditions
Semi-Preparative Column	Hyperprep® HS C18 (250 x 10 mm. i.d. 12μ) (Thremo Hypersil)
Mobile phase	40% MeOH - 0.1 % Acetic acid
Flow rate	1.4 mL/min
Detector	UV-detector
Wave length detection	235 nm
Sample Concentration	0.1g/ ml
Volume injection	100 uL

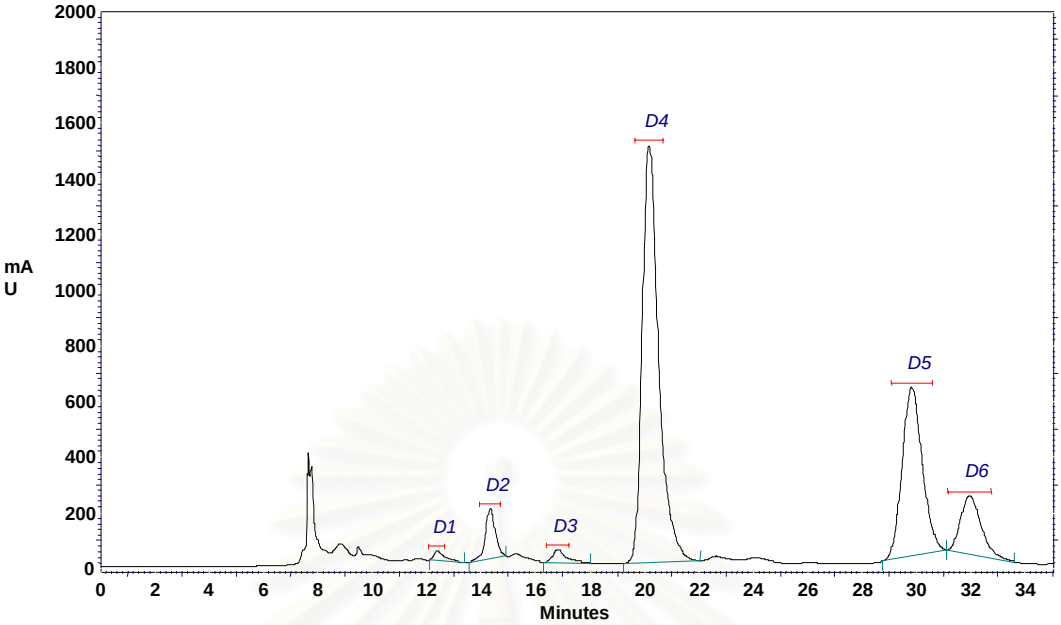


Figure4.8 The chromatogram of fraction D using HPLC condition in Table 4.6

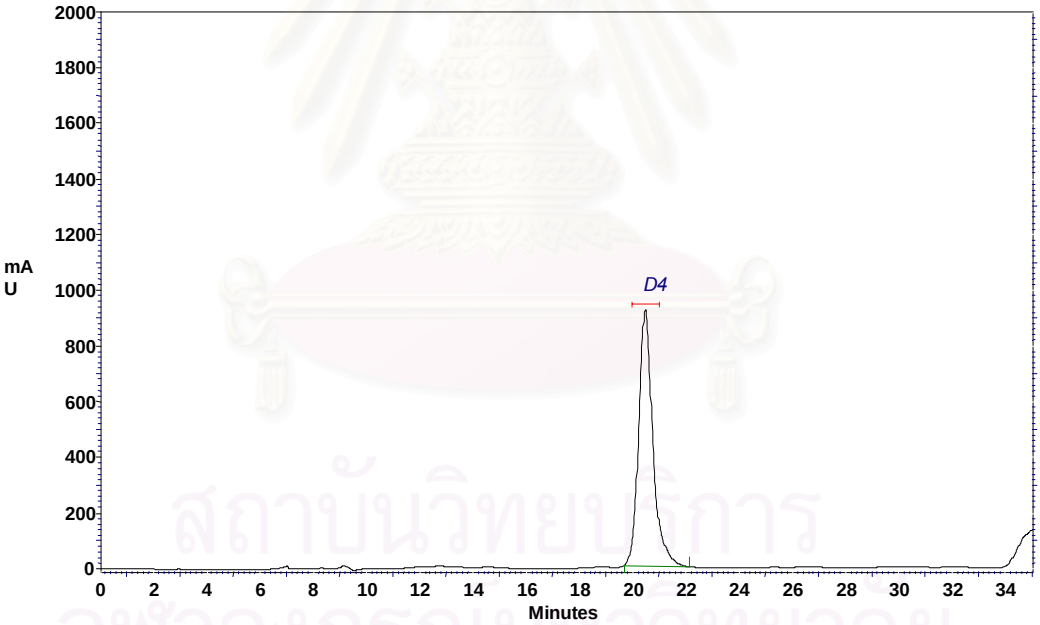


Figure4.9 The chromatogram of compound D4 using HPLC condition in Table 4.6

4.6.1 Properties and spectroscopic data of compound D4

The white powder (6 mg) was isolated as compound D4 of saponins fraction with retention time of 20.05 min by HPLC using condition in Table 4.6. It showed only dark purple spot on TLC after spraying with *p*-anisaldehyde reagent on TLC plates with Rf value of 3.8 (6:2:1 ratio of ethyl acetate : methanol : water).

The bioassay data revealed that D4 at 0.66 ppm exhibited 43 % mortality against the golden apple snails.

The MALDI-TOF MS techniques was used to study the molecular weight of this compound. Figure A1 showed the MALDI-TOF MS spectrum of compound D4 which have molecular weight between 1226-1338. From literature review, it was found that saponins in *Camellia* spp. have molecular weight between 1100-1400 [Kitagawa *et al*., 1998; Sagesaka *et al.*, 1994; Kitagawa *et al*., 1998 ; Nagata *et* Murakami *et al*., 1999b]. Thus, D4 was saponins in *Camellia* spp.

The FT-IR spectrum (Figure A2) of this compound showed a major absorption band of hydroxy group at 3134 cm^{-1} Other signals were tentatively assigned as shown in Table 4.7.

Table 4.7. The IR absorption band assignments of compound D4.

Wave number (cm^{-1})	Intensity	Tentative assignments
3134	Strong	O-H stretching vibration of hydroxy group
3047	Strong	=C-H stretching for sp^2
2823	Medium	C-H stretching vibration of CH_3
1761	Medium	C-O stretching
1403	Strong	C-H bending of methylene group
1082	Medium	C-O stretching vibration

From the comparison of the MALDI-TOF MS spectrum of compound D4 with literature review of saponins in *Camellia* genus, it was found that the first component with m/z at 1306.63 had molecular formular $C_{65}H_{94}O_{27}$. This component is possibly camelliasaponin 1(MW 1306.60). The second component showed m/z at 1228.60 indicated molecular formular of $C_{59}H_{90}O_{27}$. this component is possibly Theasaponin E1 and Theasaponin E2 (MW 1230.10). Thus compound D4 may contain camelliasaponin 1, Theasaponin E1 and Theasaponin E2 as shown in Figure 4.10-4.11.

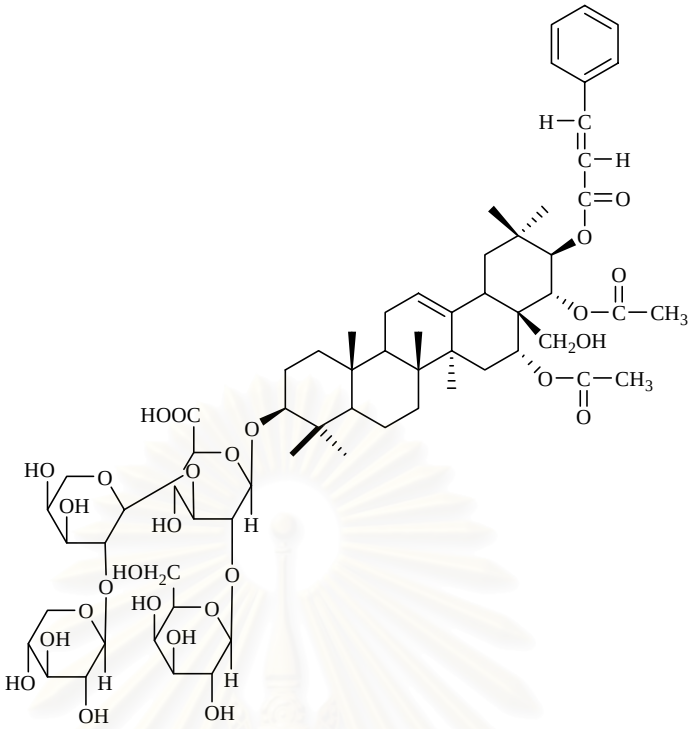
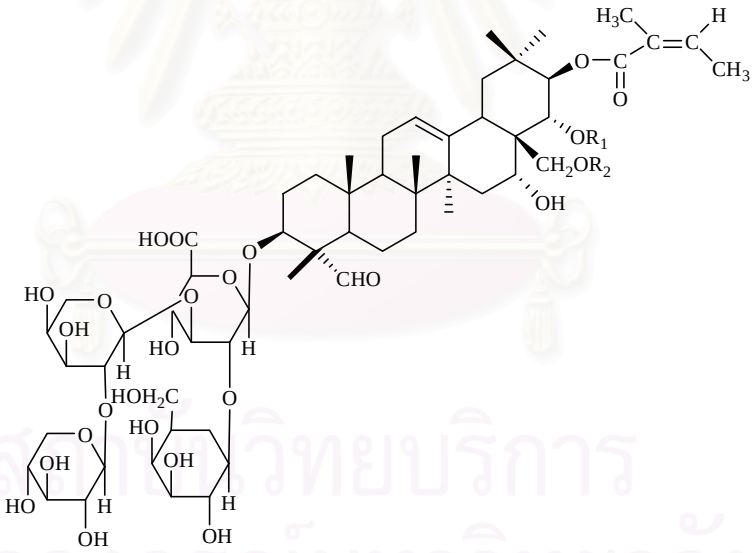


Figure. 4.10 The structure of Camelliasaponin 1 (MW. 1306.60)



	R_1	R_2	R_3	R_4	R_5
Theasaponin E1 (MW. 1230.10)	CHO	Ang	Ac	H	H
Theasaponin E2 (MW. 1230.10)	CHO	Ang	H	Ac	H

Figure. 4.11 The structure of Theasaponin E1 and Theasaponin E2

4.7 Quantitative analysis of the saponins extract by High Performance Liquid Chromatography (HPLC)

The calibration curve of each standard saponin was constructed between the peak area (y) and the quantity of D4 Standard (x, ppm) using standard working solution. Triplicate injections were performed to obtain the absorption plots ranged from 10 to 200 ppm. As shown in the table 4.8, the resulted of regression analysis revealed that the calibration curve of D4 compound had a correlation coefficient very close to 1. Regression plots of relation and peak area are shown in figure 4.12.

Table 4.8 Linear regression results of compound D4 using HPLC condition in Table 4.5.

Concentration	Area			Mean area	% RSD
	Rep 1	Rep 2	Rep3		
10	121872	120171	128737	123593.33	3.6
50	545948	516498	535413	532619.67	2.8
100	1071283	1143613	1120826	1111907.33	3.3
150	1657487	1674592	1622899	1651659.33	1.59
200	2182396	2189129	2188500	2186675	0.17

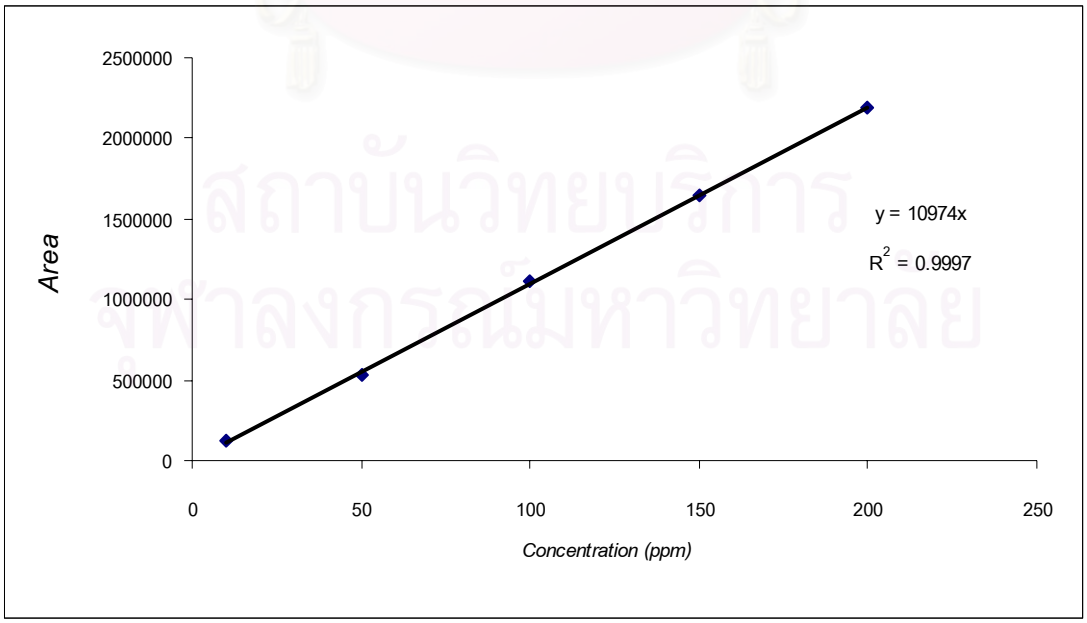


Figure 4.12. Linear regression line of peak area vs. concentration of compound D4 obtained from HPLC

Regression plots of relation and peak area in Figure 4.12 showed R^2 –values approaching 1.0 indicating very high linear detector responses with this concentration range.

Table. 4.9 The concentration of compound D4 in each sample using HPLC condition in Table 4.5.

Samples	Concentration (ppm)			Mean	% RSD
	Rep 1	Rep 2	Rep3		
Saponins crude extract of tea seed cake	167.91	171.56	172.26	170.58	3.6
CU1-MeOH	43.38	42.46	44.78	43.87	1.79
CU1-H_2O	26.61	23.54	25.30	25.15	6.1
FP-MeOH	99.71	99.29	101.82	100.27	1.30
FP-H_2O	52.55	54.10	54.19	53.60	1.72

Table. 4.10 The concentration of compound D4 in each sample.

Sample	Concentration (ppm) ± S.D.	% w/w± S.D	mg /100 g .± S.D
Saponin crude extracts of tea seed cake	170.58±2.34	6.82±0.09	6823±93
CU1-MeOH	43.87±1.23	0.43±0.01	430±8
CU1-H_2O	25.15±1.54	0.25±0.02	250±15
FP-MeOH	100.27±1.04	1.26±0.02	1260±17
FP-H_2O	53.60±0.64	1.07±0.02	1070±17

From the above data, the comparison between methanol versus aqueous extractions at room temperature indicated that compound D4 was more readily extracted with methanol.

Each sample was extracted with methanol and water which were commonly used for extraction of saponins [Domon, B. and Hostettmann, K., 1984]. The amount of compound D4 in saponins crude extract from tea seed cake, CU1-MeOH, CU1-H_2O, FP-MeOH and FP-H_2O using methanol and water were compared. It was found that the amount of saponins extracted with methanol, CU1-MeOH (0.43 %w/w) and FP-MeOH (1.26%w/w), was higher than the amount extracted with water, CU1- H_2O (0.25%w/w) and FP-H_2O (1.07%w/w). The difference was also statically significant. Therefore, methanol was the preferred solvent for the extraction and quantification of saponins.

The amount of compound D4 in fruit pericarp (*C. oleifera* Abel) extract and CU1 were also compared and the result is presented in Table 4.10 It was found that the amount of D4 in FP-MeOH and FP-H_2O, were 3 fold higher than that in CU1-MeOH. Thus fruit pericarp which is the by-product of tea seed oil industry could be a good source of saponins for molluscicide as well.

CHAPTER V

CONCLUSION AND PROPOSE FOR THE FUTURE WORK

In our search for molluscicidal compounds from *C. oleifera* Abel., saponins crude extracts from tea seed cake was partitioned between H_2O and $CHCl_3$, and then between H_2O and n-BuOH. According to molluscicidal activity guide, each partition–layer and chemical controlled samples were subjected to molluscicidal testing. The n-BuOH-layer exhibited the highest molluscicidal activity against *P. canaliculata* by submersion method after 24 h treatment at LC_{50} 6.79 ppm.

The molluscicidal activity results revealed that the n-BuOH was selected for further isolation. After preliminary purification, the n-BuOH was separated by column chromatography with Diaion HP-20 porous polymer resin to give 5 groups of compounds A-E. Each compound was subjected to molluscicidal testing. The results revealed that fraction D displayed the highest molluscicidal activity level with LC_{50} value of 6.52 ppm. These promising results prompted us to further purified bioactive constituents from this fraction.

Fraction D was further isolated by semi-preparation HPLC analysis. The white powder was isolated as compound D4 which has molecular weight between 1226-1338. This compound was subjected to molluscicidal assay. The bioassay data showed that compound D4 had 43% mortality at a concentration of 0.66 ppm against *P. canaliculata* after 24 h treatment.

The quantitative analysis of compound D4 was performed by reversed phase high performance liquid chromatography with UV detector. Optimal chromatographic conditions are C18 column (250x4.60mm), 40% methanol-0.1% acetic acid in water, mobile phase flow rate 0.80 mL/min, wave length detection at 235 nm. The standard

calibration curves revealed good linear relationships with R^2 >0.9990 from 10-200 ppm indicating very high linear detector responses with this concentration range.

The amount of compound D4 in saponins crude extract from tea seed cake, CU1-MeOH, CU1-H2O, FP-MeOH and FP- H_2O were 6.82, 0.43, 0.25, 1.07 and 1.26 %w/w, respectively.

In conclusion, methanol was the preferred solvent for the extraction and quantification of saponins. Moreover, fruit pericarp which is the waste of tea seed oil industry could be a good source of saponins for molluscicide as well.

Propose for the future work

Further purification of compound D4 could be improved by HPLC using acetonitrile/0.1 % acetic acid in water as mobile phase. Alteration of the temperature column during chromatography also improved the separation of component which were difficult to separate. Another aspect that preliminary research for molluscicidal activity of H_2O-fraction showed good activity. The possible future study on chemical constituent from this fraction should be carried out. In addition, the quantitative analysis data showed that methanol was the preferred solvent for the extraction and quantification of saponins. In stead of methanol, hot water and sonication may be used for extraction in order to minimize cost of organics solvent.

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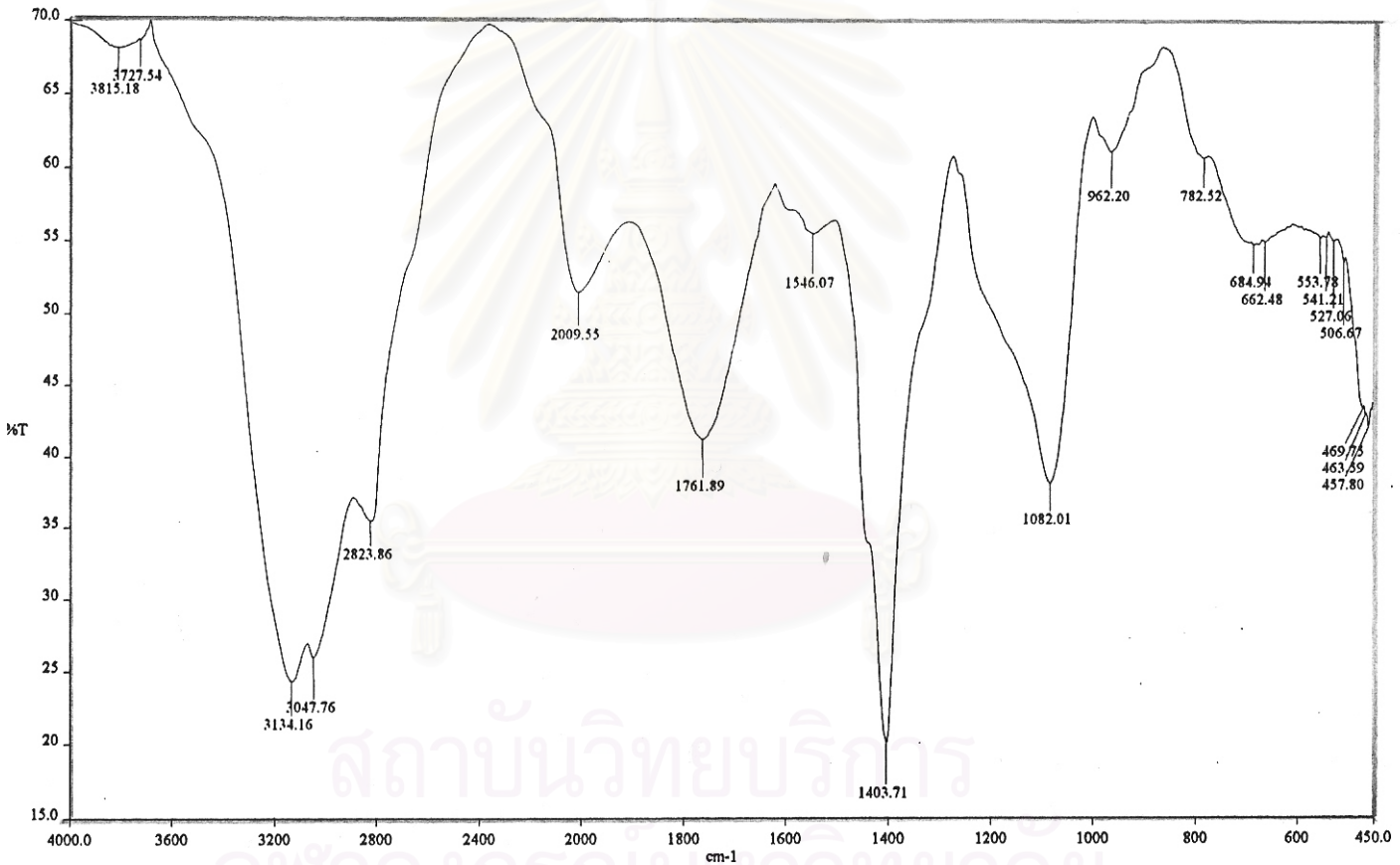
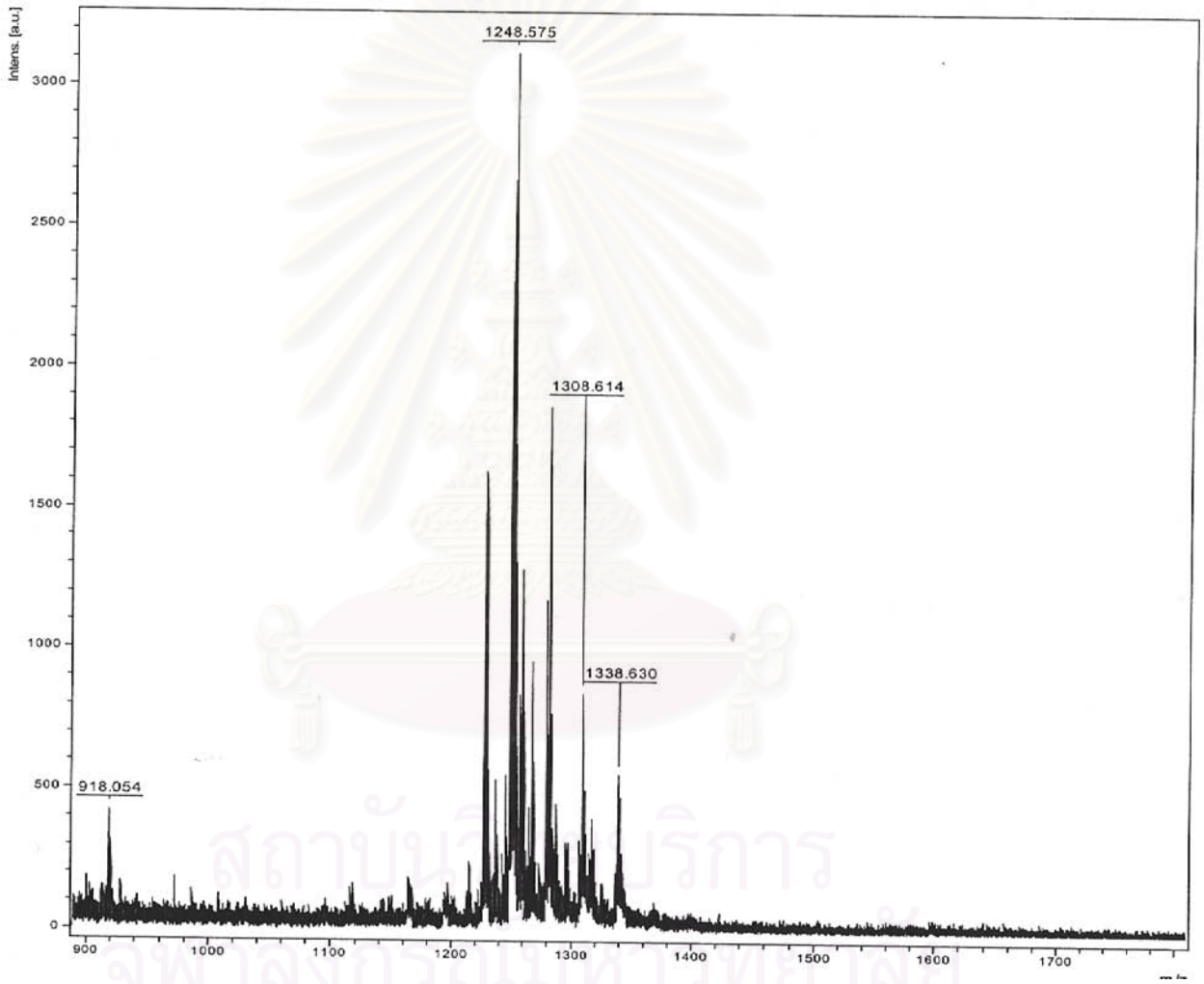
APPENDICES

Scientific and Technological Research Equipment Centre
Chulalongkorn University

Fourier Transform Infrared Spectrometer, PerkinElmer (Spectrum One)





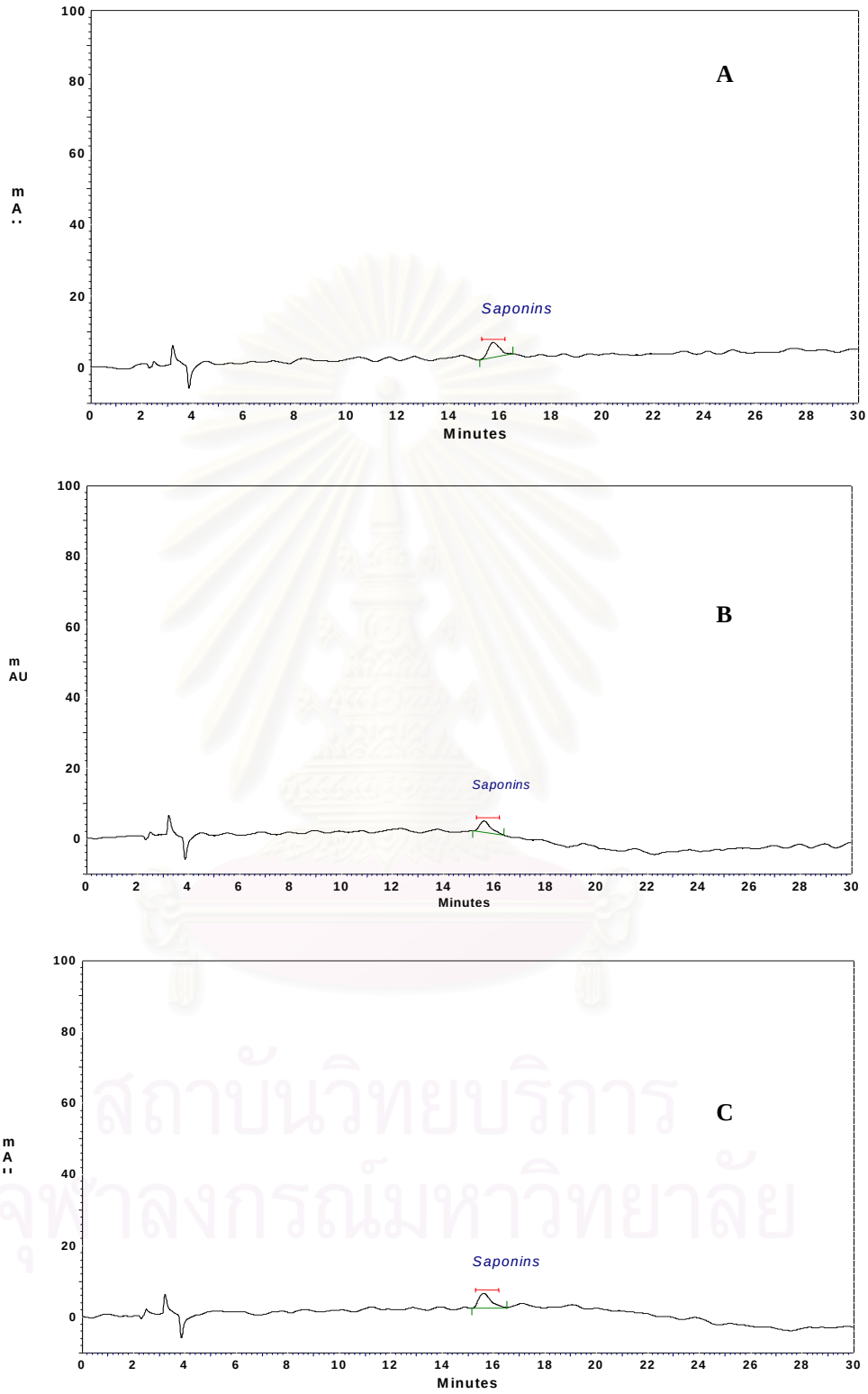


Fig. A3 HPLC chromatograms of 10 ppm compound D4 using condition in Table. 4.5. Triplicate analyses of homogenized sample.
A : 1st analysis, B : 2nd Analysis, C : 3th Analysis

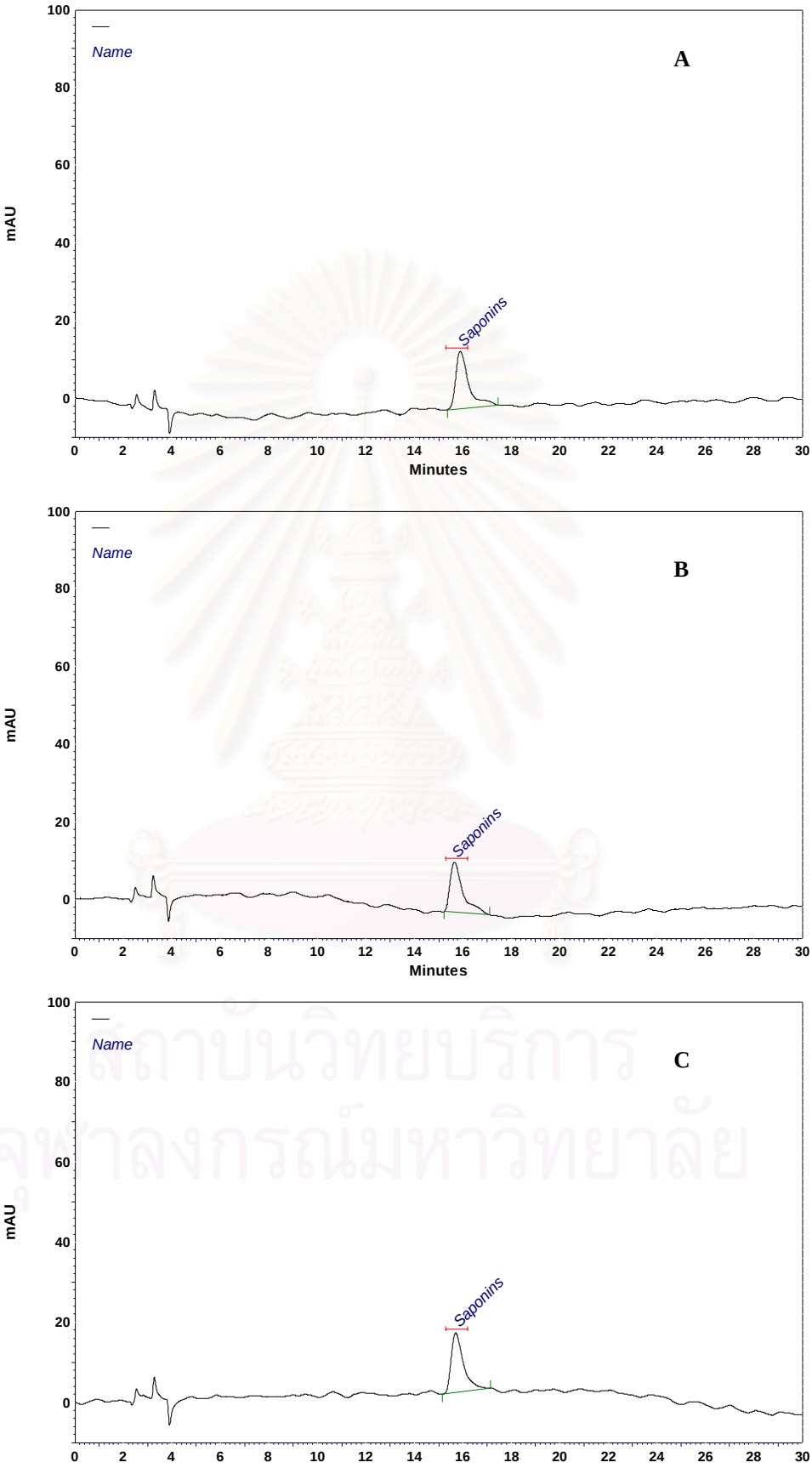


Fig.A4 HPLC chromatograms 50 ppm compound D4 using condition in Table. 4.5. Triplicate analyses of homogenized sample.
A : 1st analysis, B : 2nd Analysis, C : 3th Analysis

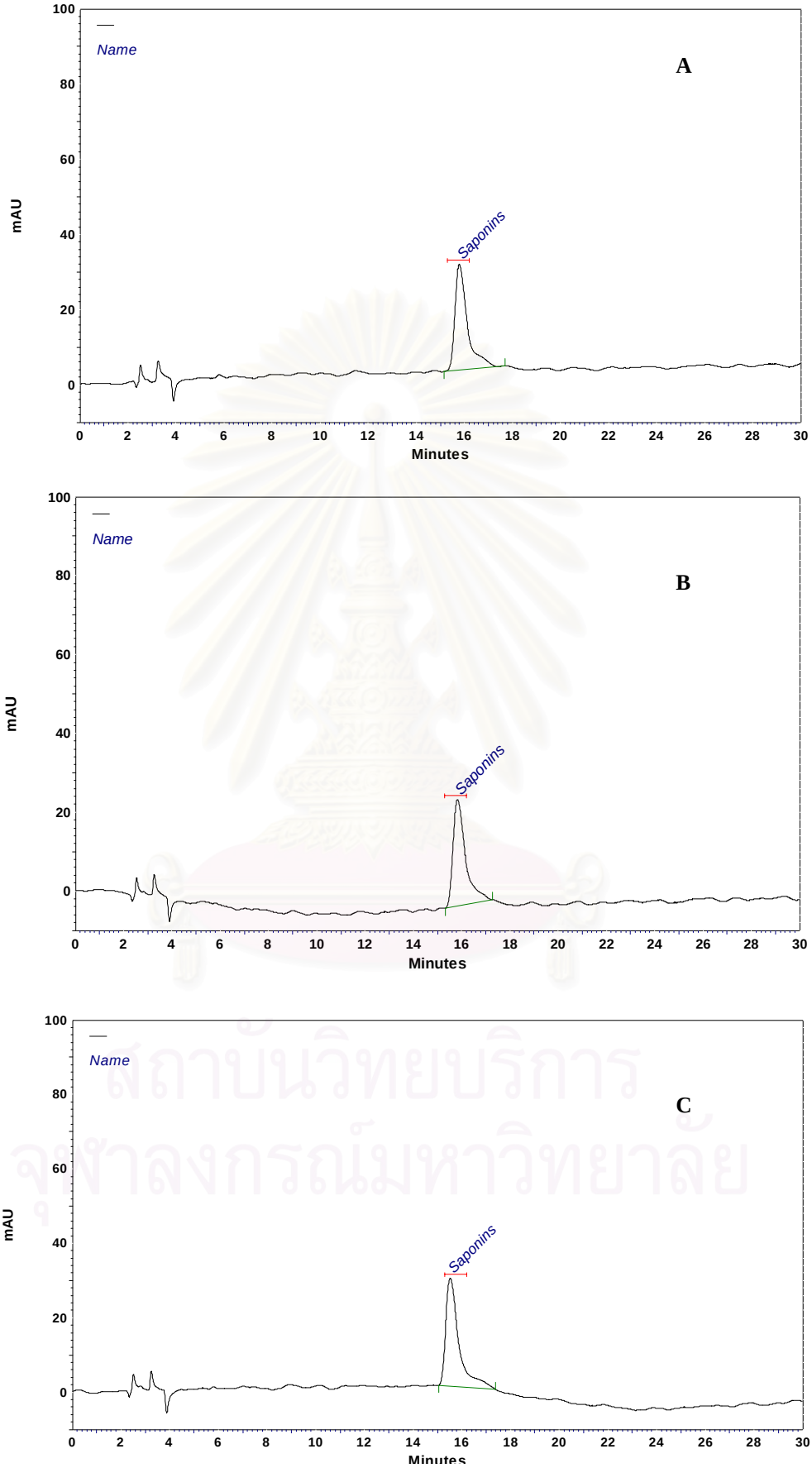


Fig.A5 HPLC chromatograms 100 ppm compound D4 using condition in Table. 4.5. Triplicate analyses of homogenized sample.
A : 1st analysis, B : 2nd Analysis, C : 3th Analysis

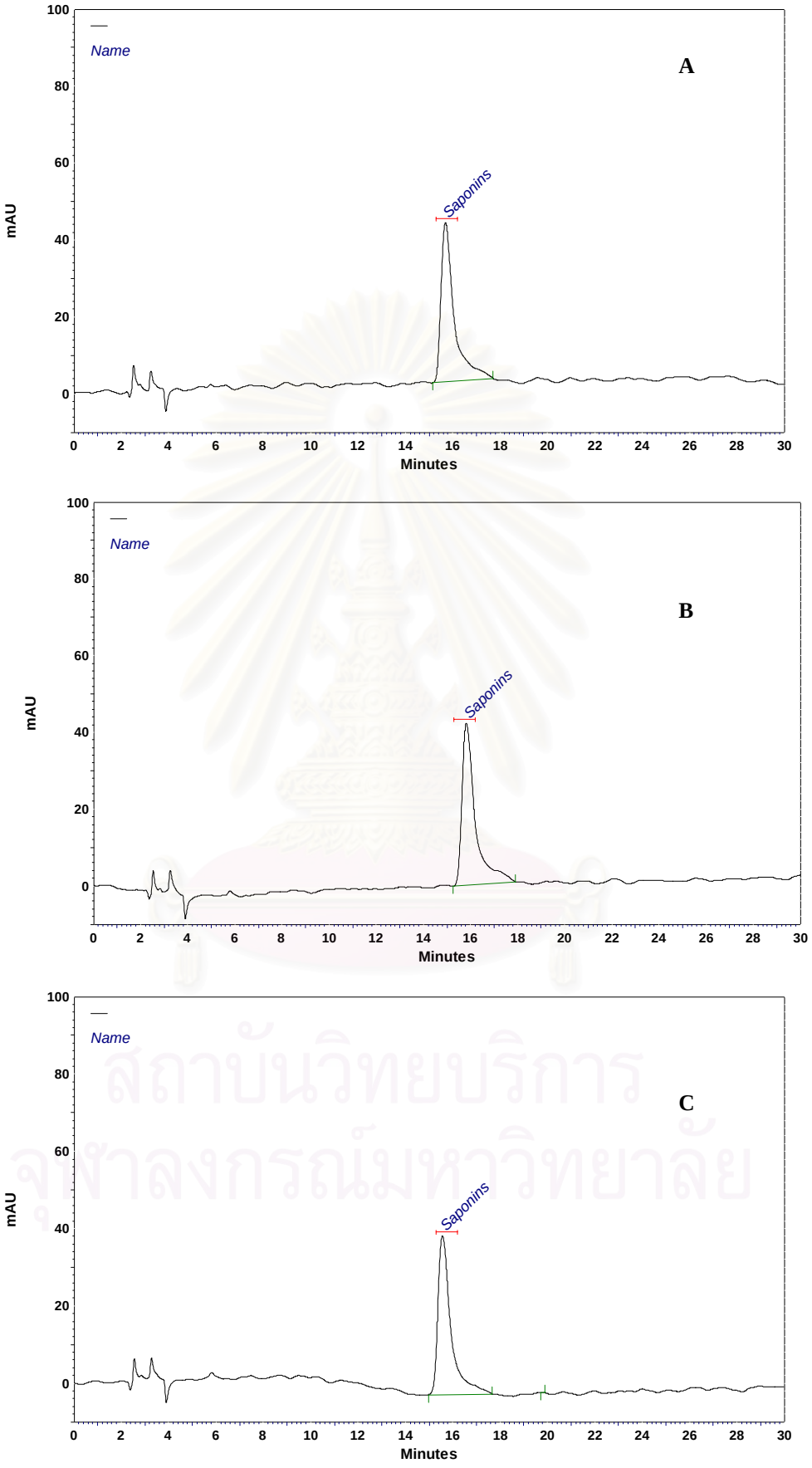


Fig.A6 HPLC chromatograms 150 ppm compound D4 using condition in Table. 4.5.Triplicate analyses of homogenized sample.
A : 1st analysis, B : 2nd Analysis, C : 3th Analysis

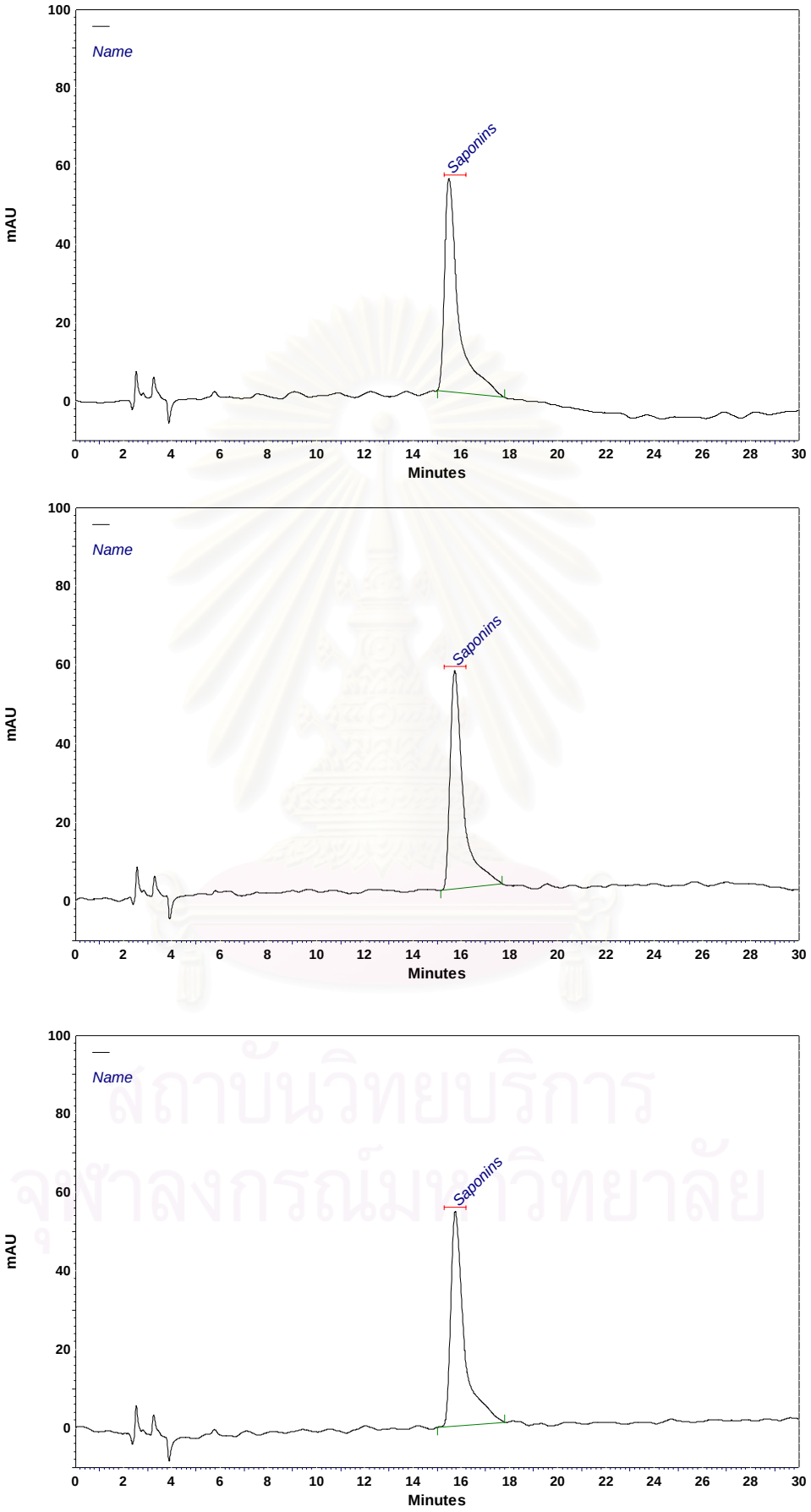


Fig.A7 HPLC chromatograms 200ppm compound D4 using condition in Table. 4.5.Triplicate analyses of homogenized sample.
A : 1st analysis, B : 2nd Analysis, C : 3th Analysis

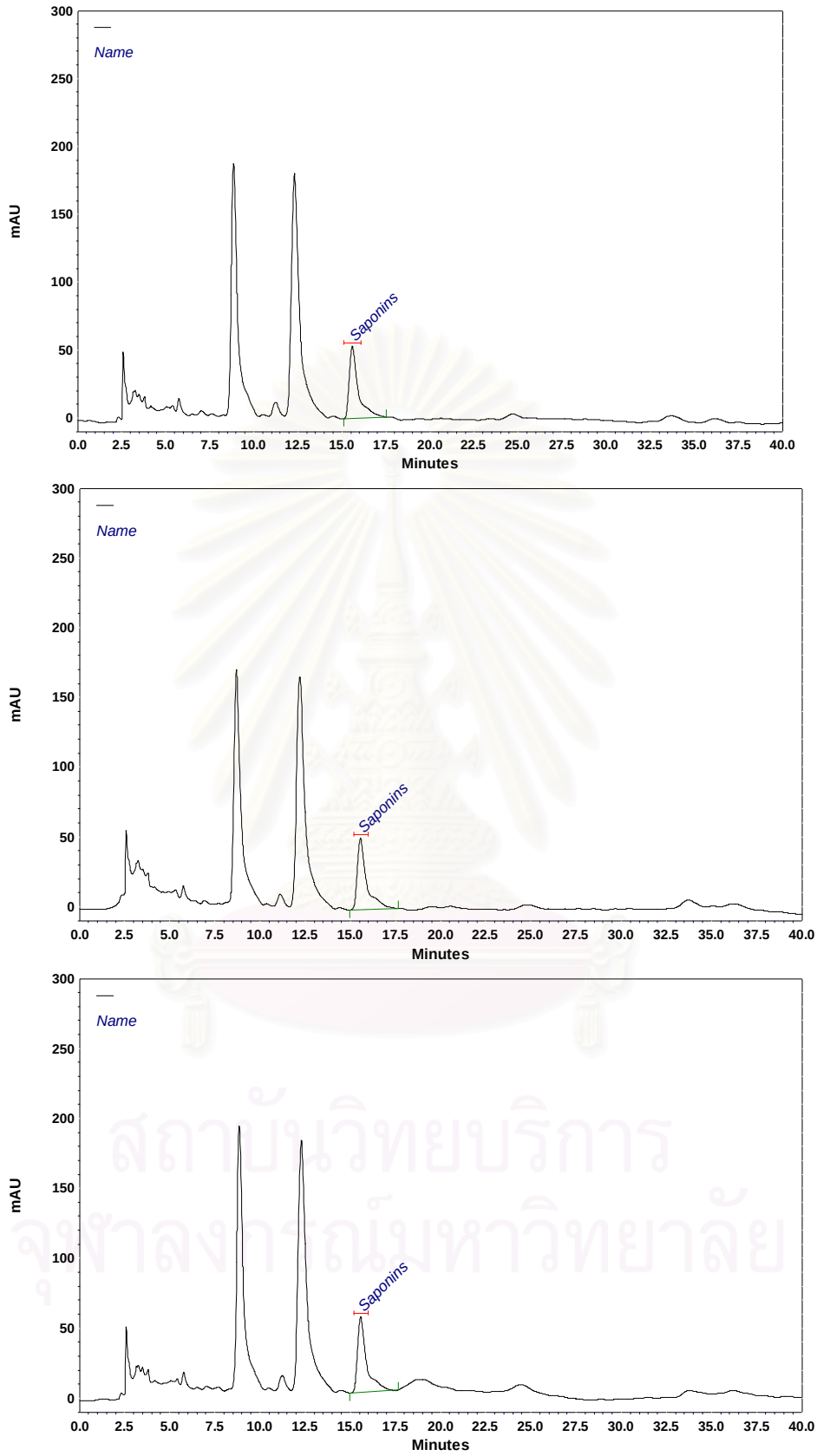


Fig. A8 HPLC chromatograms of $5x10^3$ ppm Tea seed cake (*Camellia oleifera* Abel) using condition in Table. 4.5. Triplicate analyses of homogenized sample. A : 1st analysis, B : 2nd Analysis, C : 3th Analysis

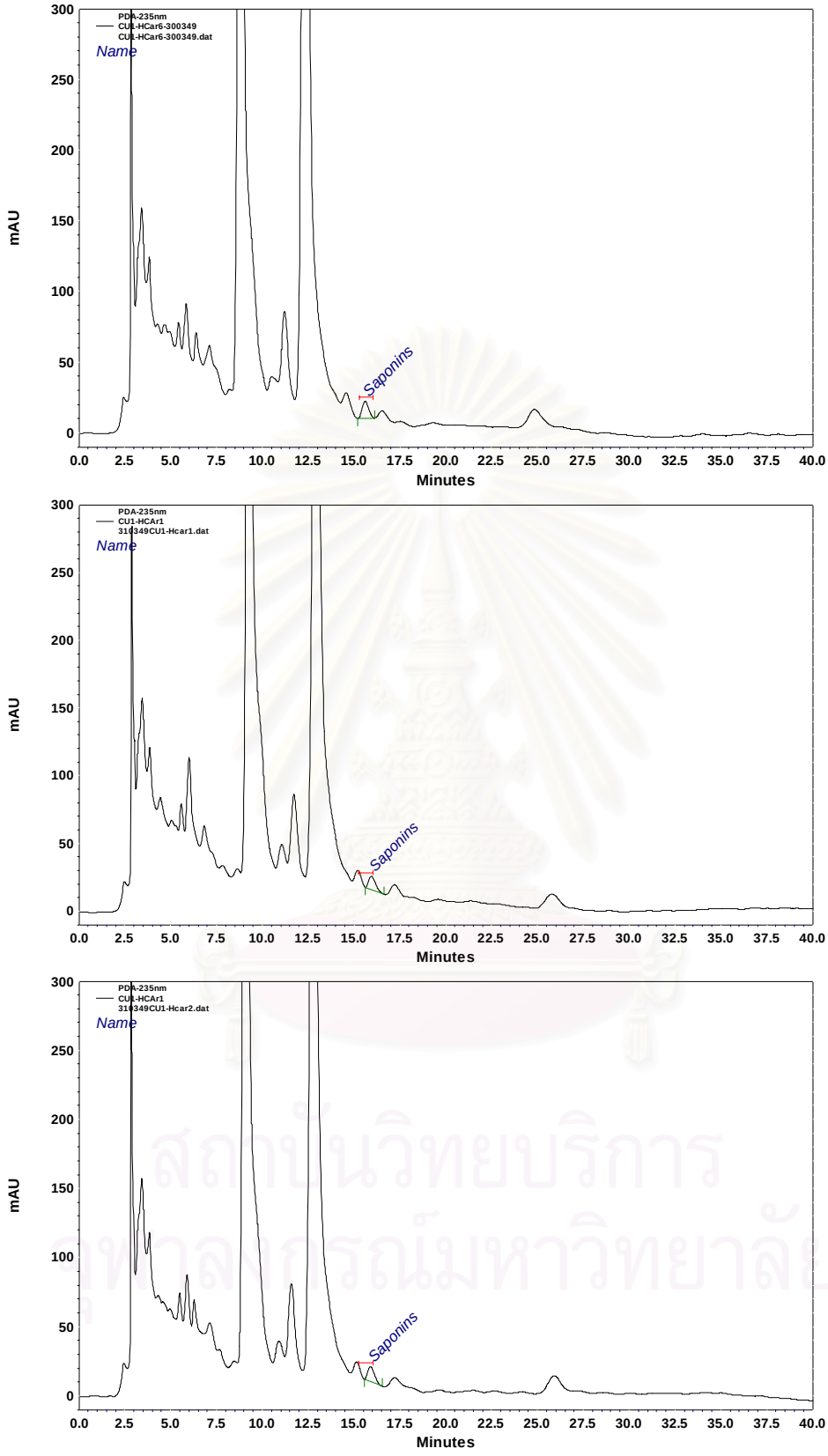


Fig. A9 HPLC chromatograms of $8x10^3$ ppm CU1-H_2O extract condition in Tabel. 4.5. Triplicate analyses of homogenized sample.
A : 1st analysis, B : 2nd Analysis, C : 3th Analysis

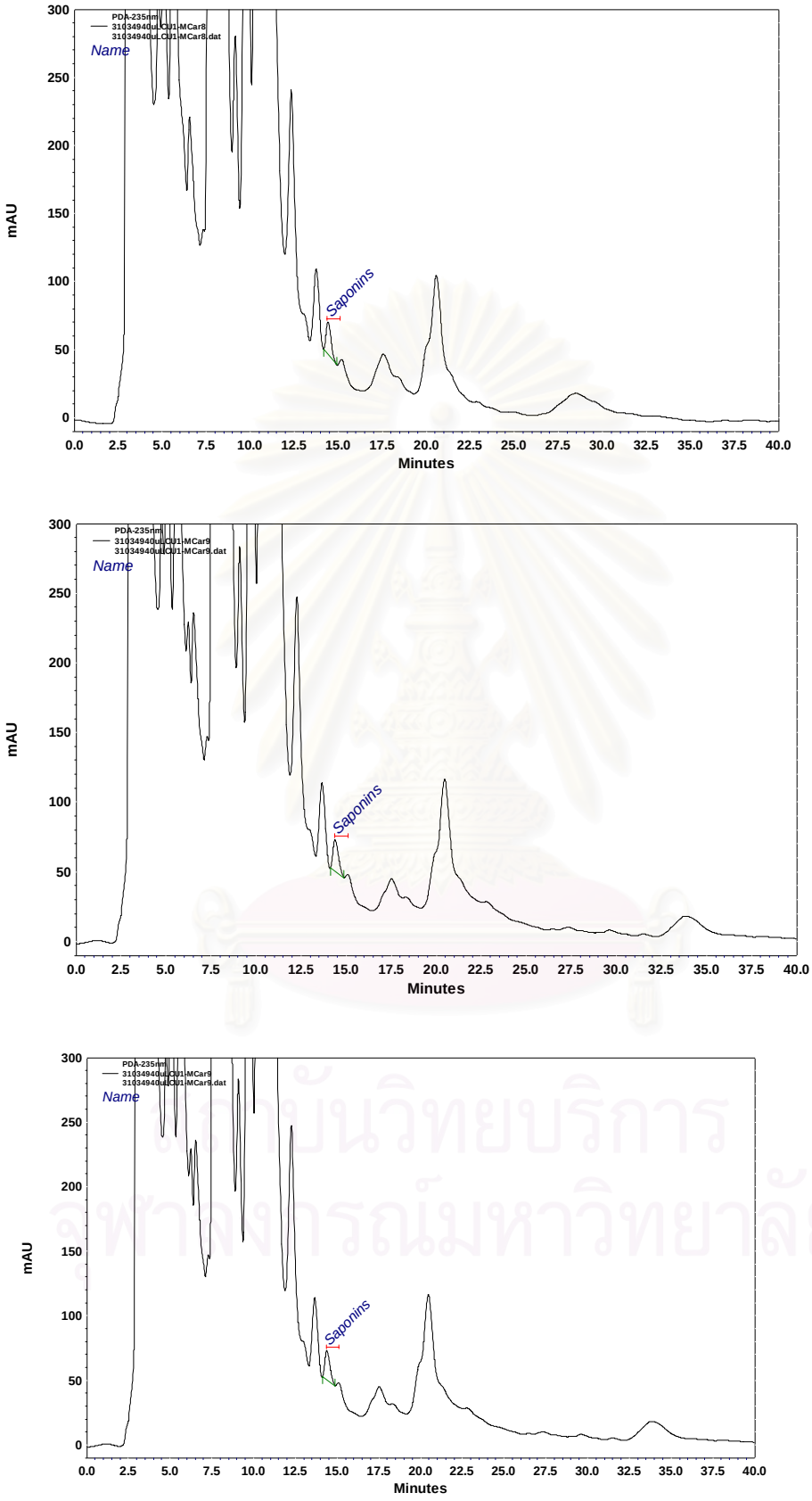


Fig. A10HPLC chromatograms of $8x10^3$ ppm CU1-MeOH extract using condition in Table. 4.5. Triplicate analyses of homogenized sample. A : 1st analysis, B : 2nd Analysis, C : 3th Analysis

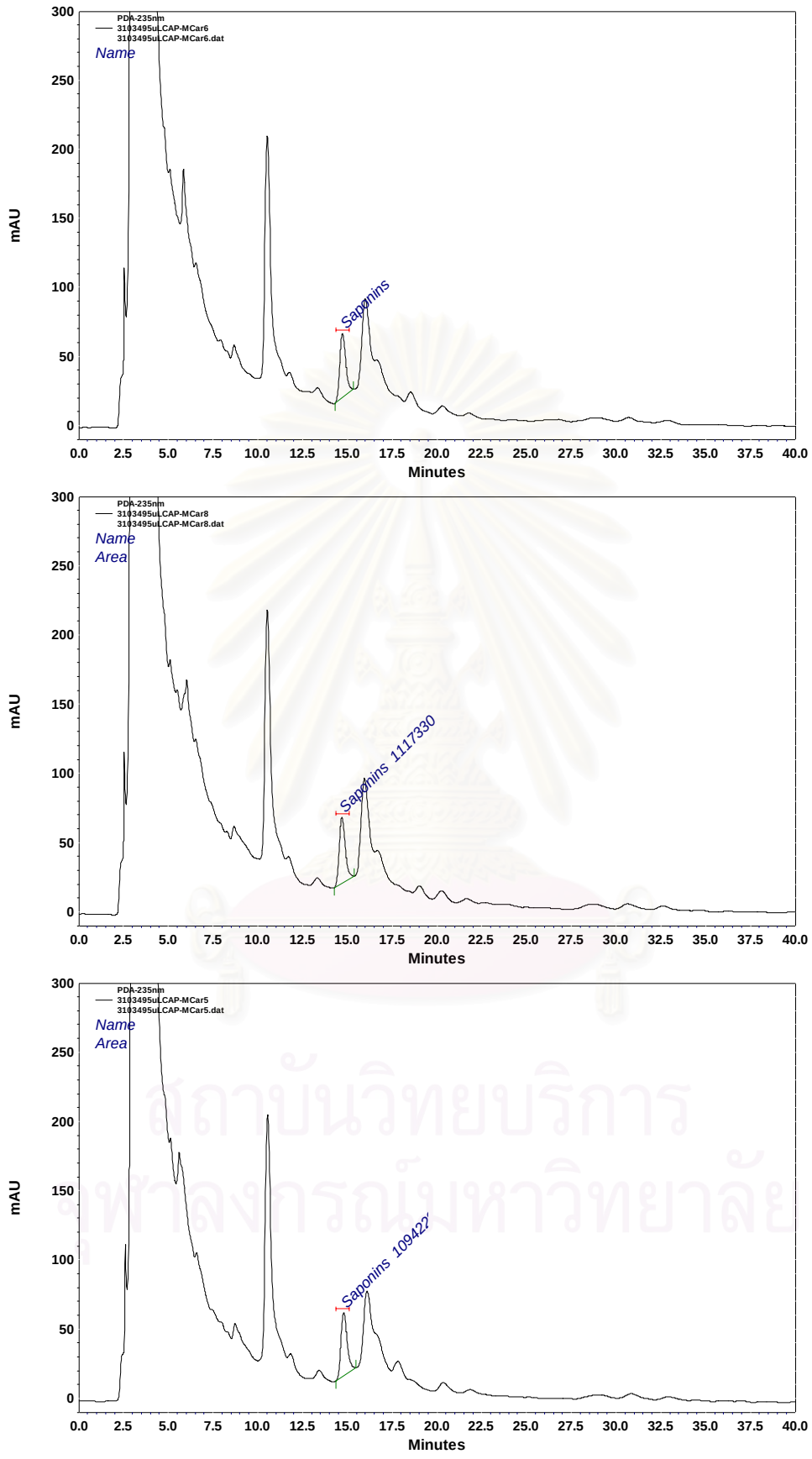


Fig.. A11 HPLC chromatograms of FP-MeOH extract using condition in Table. 4.5. Triplicate analyses of homogenized sample.
A : 1st analysis, B : 2nd Analysis, C : 3th Analysis

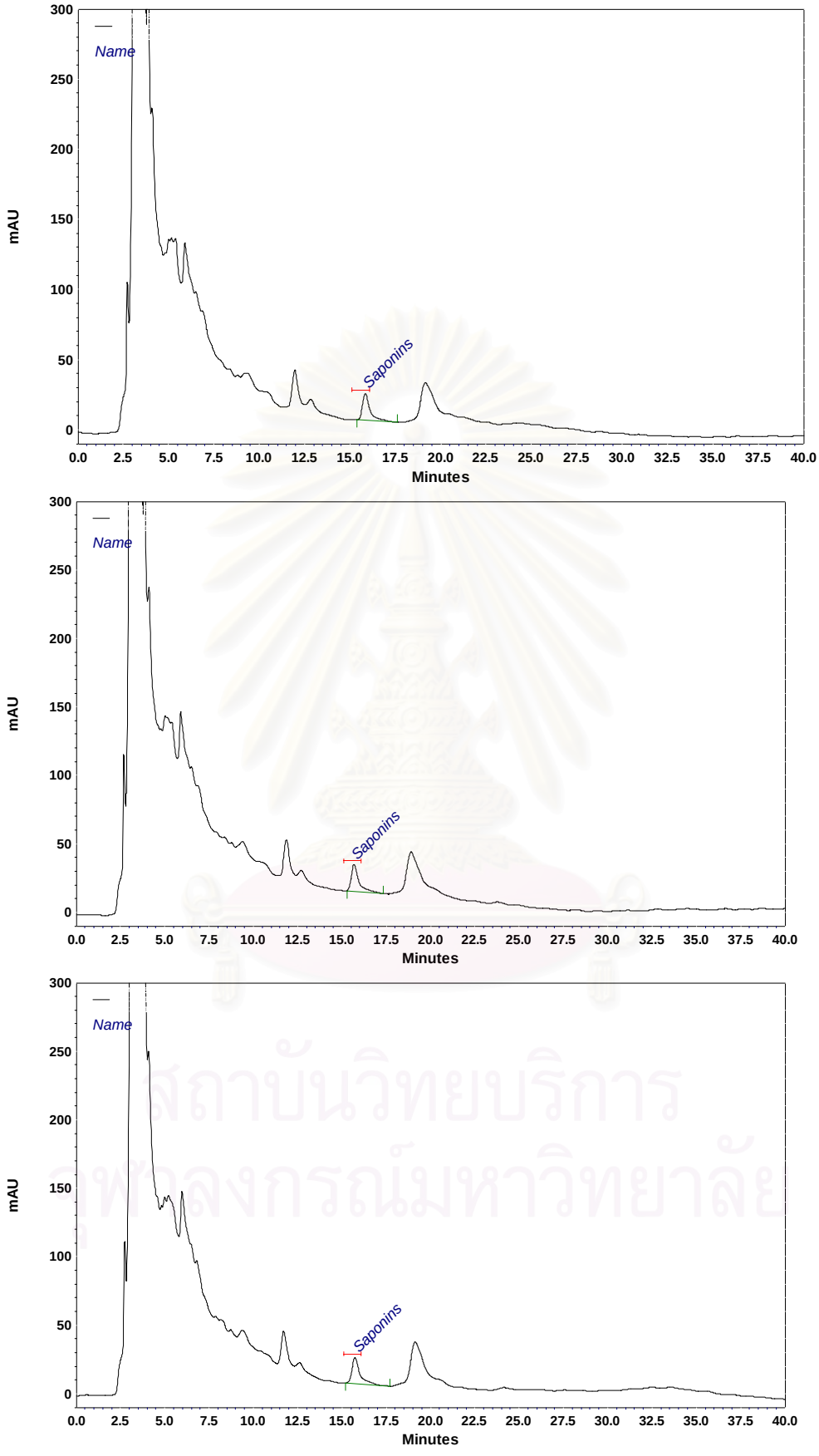


Fig. A12 HPLC chromatograms of FP-H_2O extract using condition in Table.4.5. Triplicate analyses of homogenized sample.
A : 1st analysis, B : 2nd Analysis, C : 3th Analysis

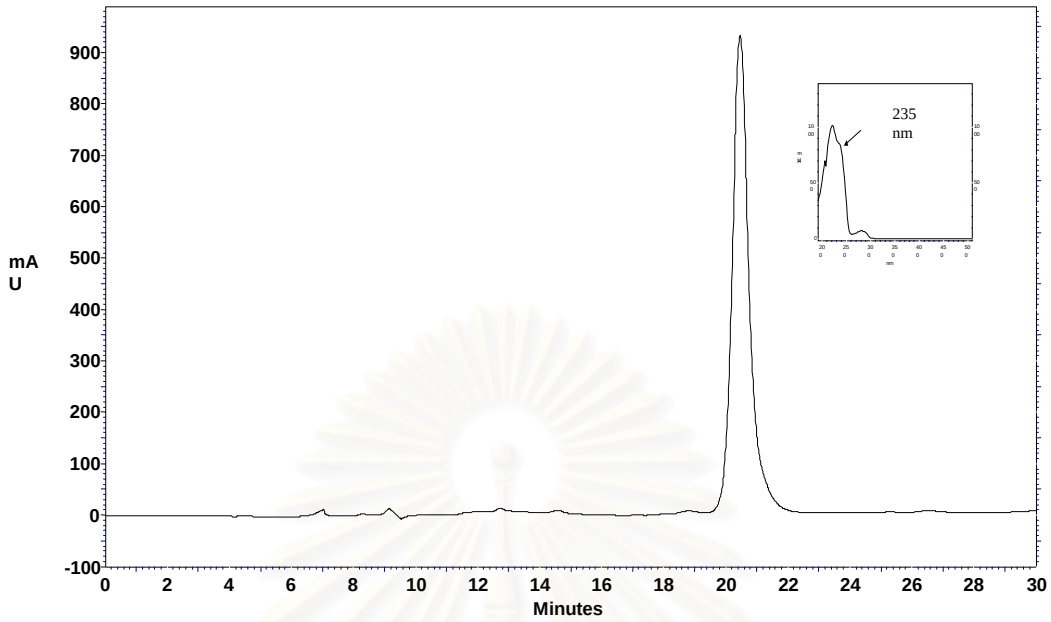


Figure A13 HPLC chromatogram of compound D4 using 40% MeOH - 0.1% acetic acid in water as mobile phase with flow rate at 1.4 mL/ min.

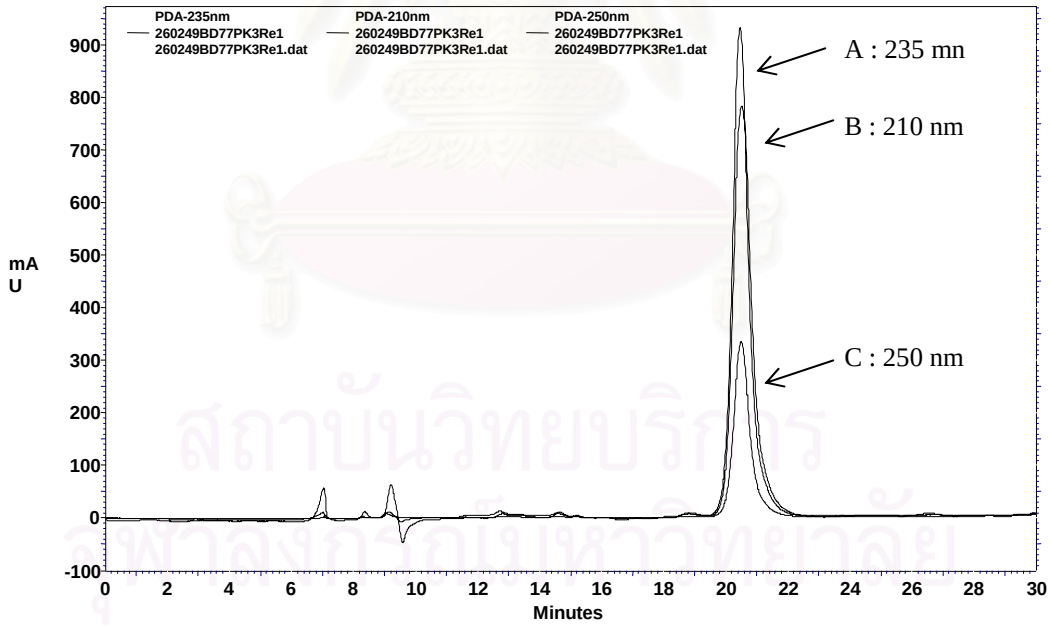


Figure. A14 HPLC chromatogram of saponins fraction D using 40% MeOH - 0.1 % acetic acid in water as mobile phase with flow rate at 1.4 mL/min A) wave length detection at 235 nm, area: 33913335 B) wave length detection at 210 nm, area: 33435803 C) wave length detection at 250 nm, area: 11008832.

APPENDIX B

Molluscicidal activity test

The results of molluscicidal testing of saponins crude extract of tea seed cake against golden apple snails with 3.5-5.0 shell length

Table C1 The results of % mortality at 24, 48 and 72 h against *P. canaliculata* Lamarck

Concentration (ppm)	% mortality at 24 h	% mortality at 48 h	% mortality at 72 h
1	0	0	0
2	13.33	13.33	13.33
5	26.67	30.00	30.00
10	66.67	66.67	66.67
25	73.33	76.66	76.66
50	100.00	100	100
0 (control)	0.00	0.00	0.00

Table C2 The results of molluscicidal activity testing of saponins crude extract against *P. canaliculata* Lamarck at 24 hours intervals.

Concentration (ppm)	Total snails	Death snails	% mean of mortality	LC_{50} (ppm)	LC_{90} (ppm)
1.00	30	0.00	0±0.00	9.21 (7.96-10.68)	36.19 (29.18-48-31)
2.00	30	4.00	13.33±3.33		
5.00	30	8.00	26.67±6.67		
10.00	30	20.00	66.67±3.33		
25.00	30	22.00	73.33±3.33		
50.00	30	30.00	100.00±0.00		
0 (control)	30	0.00	0.00		

95 % confidence Limits of LC_{50} and LC_{90}
Mean±S.E., calculated from Triplicate (each per 10 snails)

The results of molluscicidal testing of Niclosamide against golden apple snails with 3.5-5.0 shell length

Table C3 The results of % mortality at 24, 48 and 72 h against *P. canaliculata* Lamarck.

Concentration (ppm)	% mortality at 24 h	% mortality at 48 h	% mortality at 72 h
0.05	0.00	0.00	0.00
0.10	26.67	26.67	26.67
0.25	53.33	53.33	53.33
0.50	66.67	66.67	70.00
1.00	83.33	83.33	83.33
3.00	100.00	100.00	100.00
0 (control)	0.00	0.00	0.00

Table C4 The results of molluscicidal activity testing of Niclosamide against *P. canaliculata* Lamarck at 24 hours intervals.

Concentration (ppm)	Total snails	Death snails	% mean of mortality	LC_{50} (ppm)	LC_{90} (ppm)
0.05	30	0.00	0±0.00	0.27 (0.20-0.35)	1.12 (0.78-1.92)
0.10	30	8.00	13.33±3.33		
0.25	30	16.00	26.67±3.33		
0.50	30	20.00	66.67±3.33		
1.00	30	25.00	73.33±3.33		
3.00	30	30.00	100.00±0.00		
0 (control)	30	0.00	0.00		

95 % confidence Limits of LC_{50} and LC_{90}
Mean±S.E., calculated from Triplicate (each per 10 snails)
The results of molluscicidal testing of n-BuOH fraction against golden apple snails with 3.5-5.0 shell length

Table C5 The results of % mortality at 24, 48 and 72 h against *P. canaliculata* Lamarck

Concentration (ppm)	% mortality at 24 h	% mortality at 48 h	% mortality at 72 h
0.05	0	0	0
0.10	26.67	26.67	26.67
0.25	53.33	53.33	53.33
0.50	66.67	66.67	70.00
1.00	83.33	83.33	83.33
3.00	100.00	100	100
0 (control)	0.00	0.00	0.00

Table C6 The results of molluscicidal activity testing of n-BuOH fraction against *P. canaliculata* Lamarck at 24 hours intervals.

Concentration (ppm)	Total snails	Death snails	% mean of mortality	LC_{50} (ppm)	LC_{90} (ppm)
0.05	30	0.00	0±0.00	0.27 (0.20-0.35)	1.12 (0.78-1.92)
0.10	30	8.00	13.33±3.33		
0.25	30	16.00	26.67±3.33		
0.50	30	20.00	66.67±3.33		
1.00	30	25.00	73.33±3.33		
3.00	30	30.00	100.00±0.00		
0 (control)	30	0.00	0.00		

95 % confidence Limits of LC_{50} and LC_{90}
Mean±S.E., calculated from Triplicate (each per 10 snails)

APPENDIX C

STATISTICS ANALYSIS

Different in concentration of tea seed cake was evaluated by one-way-ANOVA. When significant differences were observed, the Fisher's Least Significant Difference (LSD), multi-comparison test was applied to the ANOVA to determine which means were significantly different.

One-way ANOVA

ANOVA

Response

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1960.00	5	392.000	112.000	.000
	42.000	12	3.500		
Total	2002.00	17			

Multiple Comparisons

Dependent Variable: Response

LSD

(I) Tea	(J) Tea	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.0000	2.0000	-4.00000*	1.52753	.022	-7.328192	-.671808
	5.0000	-8.00000*	1.52753	.000	-11.328192	-4.671808
	10.0000	-16.0000*	1.52753	.000	-19.328192	-12.671808
	30.0000	-22.0000*	1.52753	.000	-25.328192	-18.671808
	50.0000	-30.0000*	1.52753	.000	-33.328192	-26.671808
2.0000	1.0000	4.0000000*	1.52753	.022	.671808	7.328192
	5.0000	-4.00000*	1.52753	.022	-7.328192	-.671808
	10.0000	-12.0000*	1.52753	.000	-15.328192	-8.671808
	30.0000	-18.0000*	1.52753	.000	-21.328192	-14.671808
	50.0000	-26.0000*	1.52753	.000	-29.328192	-22.671808
5.0000	1.0000	8.0000000*	1.52753	.000	4.671808	11.328192
	2.0000	4.0000000*	1.52753	.022	.671808	7.328192
	10.0000	-8.00000*	1.52753	.000	-11.328192	-4.671808
	30.0000	-14.0000*	1.52753	.000	-17.328192	-10.671808
	50.0000	-22.0000*	1.52753	.000	-25.328192	-18.671808
10.0000	1.0000	16.00000*	1.52753	.000	12.671808	19.328192
	2.0000	12.00000*	1.52753	.000	8.671808	15.328192
	5.0000	8.0000000*	1.52753	.000	4.671808	11.328192
	30.0000	-6.00000*	1.52753	.002	-9.328192	-2.671808
	50.0000	-14.0000*	1.52753	.000	-17.328192	-10.671808
30.0000	1.0000	22.00000*	1.52753	.000	18.671808	25.328192
	2.0000	18.00000*	1.52753	.000	14.671808	21.328192
	5.0000	14.00000*	1.52753	.000	10.671808	17.328192
	10.0000	6.0000000*	1.52753	.002	2.671808	9.328192
	50.0000	-8.00000*	1.52753	.000	-11.328192	-4.671808
50.0000	1.0000	30.00000*	1.52753	.000	26.671808	33.328192
	2.0000	26.00000*	1.52753	.000	22.671808	29.328192
	5.0000	22.00000*	1.52753	.000	18.671808	25.328192
	10.0000	14.00000*	1.52753	.000	10.671808	17.328192
	30.0000	8.0000000*	1.52753	.000	4.671808	11.328192

*. The mean difference is significant at the .05 level.

APPENDIX D

PROBIT ANALYSIS

The LC_{50} value was determined by Probit Analysis using SPSS version 13 for window at 95 % confidence interval. This is the example of the result from probit analysis for LC_{50} (24h) of tea seed cake in small snails (3.5-5.0 cm.)

* * * * * * * * * * * * P R O B I T A N A L Y S I S * * * * * * * * * * * *

DATA Information

18 unweighted cases accepted.
0 cases rejected because of missing data.
3 cases are in the control group.
0 cases rejected because LOG-transform can't be done.

MODEL Information

ONLY Normal Sigmoid is requested.

* * * * * * * * * * * * P R O B I T A N A L Y S I S * * * * * * * * * * * *

Parameter estimates converged after 15 iterations.
Optimal solution found.

Parameter Estimates (PROBIT model: (PROBIT(p)) = Intercept + BX):

	Regression Coeff.	Standard Error	Coeff./S.E.
Tea	2.14261	.15167	14.12694

Intercept	Standard Error	Intercept/S.E.
-2.06575	.15743	-13.12168

Pearson Goodness-of-Fit Chi Square = 24.752 DF = 16 P = .074

Since Goodness-of-Fit Chi square is significant, a heterogeneity factor is used in the calculation of confidence limits

* * * * * * * * * * * * PROBIT ANALYSIS * * * * * * * * * * * *

Observed and Expected Frequencies

Tea	Number of Subjects	Observed Responses	Expected Responses	Residual	Prob
1.70	30.0	30.0	28.269	1.731	.94231
1.70	30.0	30.0	28.269	1.731	.94231
1.70	30.0	30.0	28.269	1.731	.94231
1.40	30.0	21.0	24.710	-3.710	.82368
1.40	30.0	21.0	24.710	-3.710	.82368
1.40	30.0	24.0	24.710	-.710	.82368
1.00	30.0	18.0	15.919	2.081	.53063
1.00	30.0	18.0	15.919	2.081	.53063
1.00	30.0	12.0	15.919	-3.919	.53063
.70	30.0	9.0	8.549	.451	.28497
.70	30.0	6.0	8.549	-2.549	.28497
.70	30.0	9.0	8.549	.451	.28497
.30	30.0	3.0	2.331	.669	.07769
.30	30.0	3.0	2.331	.669	.07769
.30	30.0	6.0	2.331	3.669	.07769
.00	30.0	.0	.583	-.583	.01943
.00	30.0	.0	.583	-.583	.01943
.00	30.0	.0	.583	-.583	.01943

* * * * * * * * * * * * PROBIT ANALYSIS * * * * * * * * * * * *

Confidence Limits for Effective Tea

		95% Confidence Limits	
Prob	Tea	Lower	Upper
.01	.75574	.41573	1.15295
.02	1.01298	.59300	1.48324
.03	1.21990	.74238	1.74144
.04	1.40297	.87870	1.96574
.05	1.57196	1.00752	2.17004
.06	1.73175	1.13163	2.36121
.07	1.88516	1.25269	2.54321
.08	2.03401	1.37176	2.71859
.09	2.17957	1.48956	2.88912
.10	2.32275	1.60665	3.05605
.15	3.02276	2.19310	3.86442
.20	3.72672	2.79951	4.67165
.25	4.45996	3.44122	5.51413
.30	5.24059	4.12916	6.41925
.35	6.08544	4.87326	7.41371
.40	7.01272	5.68416	8.52755
.45	8.04415	6.57463	9.79734
.50	9.20723	7.56117	11.26994
.55	10.53847	8.66634	13.00786
.60	12.08846	9.92217	15.09816
.65	13.93047	11.37570	17.66803
.70	16.17622	13.09899	20.91400
.75	19.00758	15.20867	25.16169
.80	22.74733	17.90933	31.00202
.85	28.04486	21.60557	39.65633
.90	36.49681	27.26998	54.23191
.91	38.89433	28.83543	58.51550
.92	41.67774	30.63338	63.56308
.93	44.96870	32.73450	69.62884
.94	48.95230	35.24532	77.10455
.95	53.92812	38.33651	86.63394
.96	60.42399	42.30536	99.37089
.97	69.49175	47.73600	117.66293
.98	83.68642	56.02227	147.36540
.99	112.17148	72.03403	210.30889

VITA

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