ฤทธิ์ฆ่าหอยเชอรี่ Pomacea canaliculata Lamarck ของซาโปนินจากเมล็ดชา Camellia oleifera Abel.

นางสาวสุพรรษา กิจประยูร

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

Miss Supunsa Kijprayoon

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สุพรรณ ไชยวิชชาชีวะ. ฤทธิ์ยับยั้งของสารสกัด Camellia oleifera Abel. (MOLLUSCICIDAL ACTIVITY OF SAPONINS FROM TEA SEEDS Camellia oleifera Abel. ON GOLDEN APPLE SNAILS Pomacea canaliculata Lamarck.) อาจารย์ประจำเป็น: รองศาสตราจารย์ ดร. อมร เพรชมาน, อาจารย์ที่ปรึกษาร่วม: อาจารย์ วิชาศ์ โตเงิน...83... หน้า 974-14-2301-2

ได้ทำการศึกษาฤทธิ์ยับยั้งของสารสกัด Camellia oleifera Abel. พบว่าสารสกัดจากกล้วยยาวแสดงความเป็นพิษต่อยอดเชื้อพืชที่ระดับความเข้มข้น LC₅₀ ที่ 24 ชั่วโมง มีค่าเท่ากับ 0.79 มิลลิกรัมต่อตรีโอ. จากการแยกโดยการคัดตามเหลาะสังเกตุก็มีฤทธิ์ยับยั้งที่สูงในแต่ละชั้นน้ำ สามารถแยกกลุ่มของสารไปย้อย (D4) ได้โดยที่สารมีมวลต่อกลุ่มย่อยในช่วง 1226-1338 ต่อดัง และที่ระดับความเข้มข้น 0.66 มิลลิกรัมต่อตรีโอมีผลทำให้ ยอดเชื้อพืชตาย 43.33 เปอร์เซ็นต์ภายใน 24 ชั่วโมง สำหรับการวิเคราะห์วัตถุทางกลุ่มสาร D4 โดยเทคนิค RP-HPLC โดยใช้ดูดเป็นตัวตรวจวัด สารที่เหมาะสมในการวิเคราะห์คือ คอมลิมเนท C18 ขนาด 250x4.6 มม. วัฏจักรเตลเลอร์ที่ 40% น้ำอัลกอฮอล์ 0.1 % เอซิลิคอิกีดในน้ำ ที่อัตราการไหล 0.80 มิลลิลิตรต่อนาที ตรวจวัดที่ความยาวคลื่น 235 นาโนเมตร การแยกตามมาตรฐานให้ ค่าสัมประสิทธิ์ผสมผสานที่คี. มีค่ามากกว่า 0.9990 ในช่วงความเข้มข้น 10-200 มิลลิกรัมต่อตรีโอ รวมทั้งได้ทำการประมวลผลของสาร D4 จากตัวอย่างที่ได้จากถังสังเกตจากกล้วยยาว และสิ่ง ที่สำคัญจากการแยกสารที่มีจานนายเชื้อพืชผักตังค์มาloid (CU1-MeOH) 0.43 เปอร์เซ็นต์โดยน้ำหนัก ในส่วนที่มีจานนายเชื้อพืชผักตังค์มาloid (CU1-MeOH) 0.43 เปอร์เซ็นต์ โดยน้ำหนัก และที่สัมพันธ์น้ำ (CU1-H₂O) 0.25 เปอร์เซ็นต์โดยน้ำหนัก ในส่วนที่เป็นน้ำของโลหะที่สกัดเม throphanol (FPM-MeOH) 1.26 เปอร์เซ็นต์โดยน้ำหนัก และที่สัมพันธ์น้ำ (FPM-H₂O) 0.07 เปอร์เซ็นต์โดยน้ำหนัก ตามลำดับ.
SUPUNSA KIJPARYOON: MOLLUSCICIDAL ACTIVITY OF SAPONINS FROM TEA SEEDS Camellia oleifera Abel. ON GOLDEN APPLE SNAILS Pomacea canaliculata Lamarck. THESIS ADVISOR: ASSOC. PROF. AMORN PETSON, 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 LC50 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 (R²>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-H2O, FP-MeOH and FP-H2O were found to be 6.82, 0.43, 0.25 and 1.07%w/w, respectively.
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<tr>
<td>µL</td>
<td>micro-liter</td>
</tr>
<tr>
<td>µm</td>
<td>micro-metre</td>
</tr>
<tr>
<td>λ&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximum wavelength detection</td>
</tr>
<tr>
<td>Ac</td>
<td>acetyl</td>
</tr>
<tr>
<td>AcOH</td>
<td>acetic acid</td>
</tr>
<tr>
<td>Ang</td>
<td>angeloyl</td>
</tr>
<tr>
<td>CH&lt;sub&gt;3&lt;/sub&gt;OH</td>
<td>methanol</td>
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<tr>
<td>CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>methyl</td>
</tr>
<tr>
<td>CH&lt;sub&gt;3&lt;/sub&gt;CN</td>
<td>acetonitrile</td>
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<td>CHCl&lt;sub&gt;3&lt;/sub&gt;</td>
<td>chloroform</td>
</tr>
<tr>
<td>Cin</td>
<td>cinnamoyl</td>
</tr>
<tr>
<td>CU&lt;sub&gt;1&lt;/sub&gt;</td>
<td>CU-one (Tea seed cake contained 12% Saponins)</td>
</tr>
<tr>
<td>DDI</td>
<td>double de-ionized water</td>
</tr>
<tr>
<td>FP</td>
<td>Fruit pericarp</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier Transform Infrared spectrophotometer</td>
</tr>
<tr>
<td>GAS</td>
<td>golden apple snails</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>KBr</td>
<td>potassium bromide</td>
</tr>
<tr>
<td>LC&lt;sub&gt;0&lt;/sub&gt;</td>
<td>0% lethality concentration</td>
</tr>
<tr>
<td>LC&lt;sub&gt;100&lt;/sub&gt;</td>
<td>100% lethality concentration</td>
</tr>
<tr>
<td>LC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>500% lethality concentration</td>
</tr>
<tr>
<td>LC&lt;sub&gt;90&lt;/sub&gt;</td>
<td>900% lethality concentration</td>
</tr>
<tr>
<td>m/z</td>
<td>mass to charge ratio</td>
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<td>MALDI-TOF</td>
<td>matrix assisted laser desorption ionization time-of-flight</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
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<tr>
<td>mL</td>
<td>milliliter</td>
</tr>
<tr>
<td>mm</td>
<td>millimeter</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>n-BuOH</td>
<td>normal butanol</td>
</tr>
</tbody>
</table>
nm nanometer
NMR Nuclear Magnetic Resonance Spectrometer
ODS Octadecyl
ppm parts per million
$R_f$ retardation factor
RSD relative standard deviation
t $\frac{1}{2}$ 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 Endosulfan. 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 antimitation 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.](image)
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 from tea seed cake of *C. oleifera* Abel.

1.2.2 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 subtropical freshwater snails from the family Ampullariidae. 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.[จรรยาเพศและอาชวาคม, 2541]

![Figure 2.1. *Pomacea canaliculata* Lamarck.](image)

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]
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.

![The life cycle of golden apple snails](http://applesnail.net)

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

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Plantae (Plants)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subkingdom</td>
<td>Tracheobionta (Vascular plants)</td>
</tr>
<tr>
<td>Superdivision</td>
<td>Spermatophyta (Seed plants)</td>
</tr>
<tr>
<td>Division</td>
<td>Magnoliophyta (Flowering plants)</td>
</tr>
<tr>
<td>Class</td>
<td>Magnoliopsida (Dicotyledons)</td>
</tr>
<tr>
<td>Subclass</td>
<td>Dilleniidae</td>
</tr>
<tr>
<td>Order</td>
<td>Theales</td>
</tr>
<tr>
<td>Family</td>
<td>Theaceae (Tea family)</td>
</tr>
<tr>
<td>Genus</td>
<td>Camellia L. (camellia)</td>
</tr>
<tr>
<td>Species</td>
<td><em>Camellia oleifera</em> Abel. (tea oil plant)</td>
</tr>
</tbody>
</table>

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. japonica* | Seed      | Camelliasaponins A1- A2 (16-17)                                                        | Yoshikawa *et al.*, 1994                      |
|               | Flower    | Camelliadin I (26), Camelliadin II (27)                                                |                                              |
|               | Leaf      | Camelliasaponin I (28)                                                                 |                                              |
| *C. oleifera* | Seed      | Sesamine (34), Kaempferol (35), Theasapogenoal A (31), E(35)                          | Bin and Yongming *et al.*, 2003; Lee and Yen *et al.*, 2006 |
|               |           | Camelliagenin A (43)                                                                   |                                               |
|               |           | Barringtogenol C (44)                                                                  |                                               |
Figure 2.5 Saponins found in *Camellia sinensis* var *assamica*
Figure 2.5 (Cont)
Figure 2.5 (Cont)

Figure 2.6 Saponins found in *Camellia japonica* L.

<table>
<thead>
<tr>
<th>Saponin</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>R₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assamsaponin F(9)</td>
<td>Ang</td>
<td>Ac</td>
<td>H</td>
<td>Ac</td>
</tr>
<tr>
<td>Assamsaponin G(10)</td>
<td>Ang</td>
<td>Ac</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Assamsaponin H(11)</td>
<td>Ang</td>
<td>H</td>
<td>Ac</td>
<td>H</td>
</tr>
<tr>
<td>Assamsaponin I(12)</td>
<td>Tig</td>
<td>H</td>
<td>Ac</td>
<td>H</td>
</tr>
<tr>
<td>Assamsaponin J(13)</td>
<td>Ac</td>
<td>Cin</td>
<td>H</td>
<td>Ac</td>
</tr>
</tbody>
</table>

Camelliasaponin A₁(18) | R₁  | R₂  |
Camelliasaponin A₂(19) | CH₃ | Tig |
Camelliasaponin B₁(20) | CHO | Ang |
Camelliasaponin B₂(21) | CHO | Tig |
Camelliasaponin C₁(22) | CH₂OH| Ang |
Camelliasaponin C₂(23) | CH₂OH| Tig |
Figure 2.6 (Cont)

Camellioside A(22)
Camellioside B(23)
Camellioside C(24)
Camellioside D(25)

R

H
Ac
Figure 2.6 (Cont)

Camellidin I (26) $R_{Ac}$

Camellidin II (27) $H$

Figure 2.7 Saponins found in *Camellia sinensis* L.

Camelliasaponin I (28)
Theasapogenol A (29) : R=OH
Theasapogenol B (31) : R=H
Theasapogenol C (30) : R=OH
Theasapogenol D (32) : R=H

Figure 2.7 (Cont) 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 oleanic 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
For glycoalkaloids, sample preparation with Sep-Pak 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 broadening is to be avoided [Henry et al., 1989]. This can be achieved 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$_g_1$, 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$_3$CN-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 mild 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/min 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 an increase in their permeability and a subsequent loss of important physiological electrolytes. Studies on the mode of action, however, have 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 Hostettmann, 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.

Somkasetrin (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 ($t_{1/2}$ = 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 weight 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 )
Guard Column : ODS C$_{18}$
Pre-column : Sep-Pak® C$_{18}$
Software : Chromquest
Fraction collector : Foxy Jr.

3.4 Chemicals

3.4.1 Solvents for column chromatography

All solvents used for column chromatography such as methanol, ethanol and dichloromethane were commercial grade.
3.4.2 Solvent for High Performance Liquid Chromatography (HPLC)

MeOH and CH$_2$CN 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 chromatography.

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 (0.82 g), n-butanol extract (0.4 g) and aqueous extract (3.6 g). The partition procedure is shown in the scheme 3.1.
saponins crude extract of the tea seed cake of *C. oleifera* Abel. 4.82g /H₂O 100 ml

**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 molluscidal 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 H₂O, 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<sub>18</sub> 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<sup>2</sup>).

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 -H2O.

**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-H2O.

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 (LC100) and the highest concentration that killed none of the snails (LC0). 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 LC50 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 H₂O and CHCl₃, and then H₂O 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](image)

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.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Appearance</th>
<th>Weight (g)</th>
<th>% w/w of powder (4.82g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHCl₃</td>
<td>Yellowish powder</td>
<td>0.82</td>
<td>17</td>
</tr>
<tr>
<td>n-BuOH</td>
<td>Brown powder</td>
<td>0.35</td>
<td>7</td>
</tr>
<tr>
<td>H₂O</td>
<td>Dark Brown powder</td>
<td>3.65</td>
<td>76</td>
</tr>
</tbody>
</table>

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 Table 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 LC₅₀ and LC₉₀ 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.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>LC$<em>{50}$ (ppm) (95% confidence limits of LC$</em>{50}$)</th>
<th>LC$<em>{90}$ (ppm) (95% confidence limits of LC$</em>{90}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical Controlled</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Niclosamide</td>
<td>0.27</td>
<td>1.12</td>
</tr>
<tr>
<td>Metaldehyde</td>
<td>34.98</td>
<td>89.57</td>
</tr>
<tr>
<td>CU1</td>
<td>60.32</td>
<td>99.32</td>
</tr>
<tr>
<td>CU1 NO. 2</td>
<td>60.56</td>
<td>108.18</td>
</tr>
<tr>
<td>Bionine</td>
<td>0.2 *</td>
<td>0.68 *</td>
</tr>
<tr>
<td>Partition- fraction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saponin crude extract of tea seed cake</td>
<td>9.20</td>
<td>40.88</td>
</tr>
<tr>
<td>H$_2$O- fraction</td>
<td>14.01</td>
<td>44.35</td>
</tr>
<tr>
<td>n-BuOH- fraction</td>
<td>6.79</td>
<td>14.43</td>
</tr>
<tr>
<td>CHCl$_3$- fraction</td>
<td>7.43</td>
<td>31.36</td>
</tr>
</tbody>
</table>

*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 its 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$_2$O-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 mollusccicides and partition-fraction from MeOH extract of tea seed cake of C. oleifera Abel against P. canaliculata Lamarck.](image-url)
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 H2O, 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.

<table>
<thead>
<tr>
<th>Group of compounds</th>
<th>Fraction Number</th>
<th>Appearance</th>
<th>% W/W of n-BuOH-fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1-35</td>
<td>Brown Solid</td>
<td>16.7</td>
</tr>
<tr>
<td>B</td>
<td>35-47</td>
<td>Bright Yellow Powder</td>
<td>13.3</td>
</tr>
<tr>
<td>C</td>
<td>48-77</td>
<td>Brown Powder</td>
<td>14.4</td>
</tr>
<tr>
<td>D</td>
<td>77-99</td>
<td>Brown Powder</td>
<td>21.1</td>
</tr>
<tr>
<td>E</td>
<td>99-110</td>
<td>Brown Powder</td>
<td>25.6</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>Compound</th>
<th>LC$_{50}$ (ppm)</th>
<th>LC$_{90}$ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(95% confidence Limits of LC$_{50}$)</td>
<td>(95% confidence Limits of LC$_{90}$)</td>
</tr>
<tr>
<td>A</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>B</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>C</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>D</td>
<td>6.52</td>
<td>19.57</td>
</tr>
<tr>
<td>E</td>
<td>20.92</td>
<td>43.39</td>
</tr>
</tbody>
</table>

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](image-url)

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 different 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.

<table>
<thead>
<tr>
<th>HPLC Parameter</th>
<th>HPLC Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analytical Column</td>
<td>Phenomenex®Luna 5 μm C18 250x4.60mm</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>40% MeOH - 0.1 % Acetic acid</td>
</tr>
<tr>
<td>Flow rate</td>
<td>0.8 mL/min</td>
</tr>
<tr>
<td>Detector</td>
<td>UV-detector</td>
</tr>
<tr>
<td>Wave length detection</td>
<td>235 nm</td>
</tr>
</tbody>
</table>

**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

<table>
<thead>
<tr>
<th>HPLC Parameters</th>
<th>HPLC Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Semi-Preparative Column</td>
<td>Hyperprep® HS C18 (250 x 10 mm, i.d. 12μ) (Thremo Hypersil)</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>40% MeOH - 0.1 % Acetic acid</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1.4 mL/min</td>
</tr>
<tr>
<td>Detector</td>
<td>UV-detector</td>
</tr>
<tr>
<td>Wave length detection</td>
<td>235 nm</td>
</tr>
<tr>
<td>Sample Concentration</td>
<td>0.1 g/ml</td>
</tr>
<tr>
<td>Volume injection</td>
<td>100 μL</td>
</tr>
</tbody>
</table>
Figure 4.8 The chromatogram of fraction D using HPLC condition in Table 4.6

Figure 4.9 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.

<table>
<thead>
<tr>
<th>Wave number (cm$^{-1}$)</th>
<th>Intensity</th>
<th>Tentative assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>3134</td>
<td>Strong</td>
<td>O-H stretching vibration of hydroxy group</td>
</tr>
<tr>
<td>3047</td>
<td>Strong</td>
<td>=C-H stretching for sp$^2$</td>
</tr>
<tr>
<td>2823</td>
<td>Medium</td>
<td>C-H stretching vibration of CH$_3$</td>
</tr>
<tr>
<td>1761</td>
<td>Medium</td>
<td>C-O stretching</td>
</tr>
<tr>
<td>1403</td>
<td>Strong</td>
<td>C-H bending of methylene group</td>
</tr>
<tr>
<td>1082</td>
<td>Medium</td>
<td>C-O stretching vibration</td>
</tr>
</tbody>
</table>
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 formula \( \text{C}_{65}\text{H}_{94}\text{O}_{27} \). This component is possibly camelliasaponin 1 (MW 1306.60). The second component showed m/z at 1228.60 indicated molecular formula \( \text{C}_{59}\text{H}_{90}\text{O}_{27} \). 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)

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.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Area Rep 1</th>
<th>Area Rep 2</th>
<th>Area Rep 3</th>
<th>Mean area</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>121872</td>
<td>120171</td>
<td>128737</td>
<td>123593.33</td>
<td>3.6</td>
</tr>
<tr>
<td>50</td>
<td>545948</td>
<td>516498</td>
<td>535413</td>
<td>532619.67</td>
<td>2.8</td>
</tr>
<tr>
<td>100</td>
<td>1071283</td>
<td>1143613</td>
<td>1120826</td>
<td>1111907.33</td>
<td>3.3</td>
</tr>
<tr>
<td>150</td>
<td>1657487</td>
<td>1674592</td>
<td>1622899</td>
<td>1651659.33</td>
<td>1.59</td>
</tr>
<tr>
<td>200</td>
<td>2182396</td>
<td>2189129</td>
<td>2188500</td>
<td>2186675</td>
<td>0.17</td>
</tr>
</tbody>
</table>

\[ y = 10974x \]

\[ R^2 = 0.9997 \]

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.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Concentration (ppm)</th>
<th>Mean</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rep 1</td>
<td>Rep 2</td>
<td>Rep3</td>
</tr>
<tr>
<td>Saponins crude extract of tea seed cake</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CU1-MeOH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CU1-H2O</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FP-MeOH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FP-H2O</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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-H2O, FP-MeOH and FP-H2O 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- H2O (0.25%w/w) and FP-H2O (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-H2O, 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 H₂O and CHCl₃, and then between H₂O 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 LC₅₀ 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 LC₅₀ 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- H2O 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 molluscidal activity of H2O-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.


japonica: Revised structures of camellenodiol and camelledionol. *Heterocycles*, 55(9), 1653.


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จุฬาลงกรณ์มหาวิทยาลัย
สถาบันวิจัยบัณฑิต
จุฬาลงกรณ์มหาวิทยาลัย
Fig. A3  HPLC chromatograms of 10 ppm compound D4 using condition in Table 4.5. Triplcate analyses of homogenized sample. 
A: 1st analysis,  B: 2nd Analysis,  C: 3rd 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: 3rd 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: 3rd 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 : 3rd 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: 3rd Analysis
Fig. A8  HPLC chromatograms of $5 \times 10^3$ 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 : 3rd Analysis
Fig. A9 HPLC chromatograms of $8 \times 10^3$ ppm CU1-H$_2$O extract condition in Table 4.5. Triplicate analyses of homogenized sample. A: 1st analysis, B: 2nd Analysis, C: 3rd Analysis
**Fig. A10** HPLC chromatograms of $8 \times 10^3$ ppm CU1-MeOH extract using condition in Table 4.5. Triplicate analyses of homogenized sample. A: 1st analysis, B: 2nd Analysis, C: 3rd 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: 3rd Analysis
Fig. A12 HPLC chromatograms of FP-H₂O extract using condition in Table 4.5. Triplicate analyses of homogenized sample. A: 1st analysis, B: 2nd Analysis, C: 3rd 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

<table>
<thead>
<tr>
<th>Concentration (ppm)</th>
<th>% mortality at 24 h</th>
<th>% mortality at 48 h</th>
<th>% mortality at 72 h</th>
</tr>
</thead>
<tbody>
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<tr>
<td>0 (control)</td>
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<td>0.00</td>
<td>0.00</td>
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</tbody>
</table>

95% confidence Limits of LC₅₀ and LC₉₀
Mean±S.E., calculated from Triplicate (each per 10 snails)

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

<table>
<thead>
<tr>
<th>Concentration (ppm)</th>
<th>Total snails</th>
<th>Death snails</th>
<th>% mean of mortality</th>
<th>LC₅₀ (ppm)</th>
<th>LC₉₀ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
</tr>
<tr>
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<td>13.33±3.33</td>
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<tr>
<td>5.00</td>
<td>30</td>
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<td>9.21 (7.96-10.68)</td>
<td>36.19 (29.18-48-31)</td>
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<tr>
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<td>100.00±0.00</td>
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<td>0 (control)</td>
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<td>0.00</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>Concentration (ppm)</th>
<th>% mortality at 24 h</th>
<th>% mortality at 48 h</th>
<th>% mortality at 72 h</th>
</tr>
</thead>
<tbody>
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<td>0.00</td>
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<td>26.67</td>
</tr>
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<td>0 (control)</td>
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</table>

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

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

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<tr>
<th>Concentration (ppm)</th>
<th>Total snails</th>
<th>Death snails</th>
<th>% mean of mortality</th>
<th>LC$_{50}$ (ppm)</th>
<th>LC$_{90}$ (ppm)</th>
</tr>
</thead>
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<td>0.00</td>
<td>0±0.00</td>
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<tr>
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</tr>
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<td>(0.78-1.92)</td>
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<td>25.00</td>
<td>73.33±3.33</td>
<td></td>
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</tr>
<tr>
<td>3.00</td>
<td>30</td>
<td>30.00</td>
<td>100.00±0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 (control)</td>
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<td>0.00</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Table C5** The results of % mortality at 24, 48 and 72 h against *P. canaliculata* Lamarck

<table>
<thead>
<tr>
<th>Concentration (ppm)</th>
<th>% mortality at 24 h</th>
<th>% mortality at 48 h</th>
<th>% mortality at 72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
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<td>0</td>
<td>0</td>
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<tr>
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<td>26.67</td>
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<td>66.67</td>
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<tr>
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<td>83.33</td>
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<tr>
<td>3.00</td>
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<td>100.00</td>
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<tr>
<td>0 (control)</td>
<td>0.00</td>
<td>0.00</td>
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</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Concentration (ppm)</th>
<th>Total snails</th>
<th>Death snails</th>
<th>% mean of mortality</th>
<th>LC$_{50}$ (ppm)</th>
<th>LC$_{90}$ (ppm)</th>
</tr>
</thead>
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<tr>
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<td>16.00</td>
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</table>

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

<table>
<thead>
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<th>Response</th>
<th>Sum of Squares</th>
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<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
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<td>Total</td>
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<td>17</td>
<td>3.500</td>
<td></td>
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</table>
**Multiple Comparisons**

Dependent Variable: Response

LSD

<table>
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<tr>
<th>(I) Tea</th>
<th>(J) Tea</th>
<th>Mean Difference (I-J)</th>
<th>Std. Error</th>
<th>Sig.</th>
<th>95% Confidence Interval</th>
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*. The mean difference is significant at the .05 level.
APPENDIX D

PROBIT ANALYSIS

The LC50 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 LC50 (24h) of tea seed cake in small snails (3.5-5.0 cm.)

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

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.

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

Parameter estimates converged after 15 iterations. Optimal solution found.

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

<table>
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<tr>
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<th>Regression Coeff.</th>
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<th>Coeff./S.E.</th>
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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

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สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย
### PROBIT ANALYSIS

Confidence Limits for Effective Tea

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VITA

Ms. Supunska kijprayoon was born on Wednesday 26th November, 1980, in Nakornpathom, Thailand. In 2003, she graduated with a Bachelor’s degree of Science in Chemistry, from Chulalongkorn University. After that, she has been studied for a Master’s degree of Science in Organic Chemistry, the Department of Chemistry, Faculty of Science, Chulalongkorn University, and completed the program in 2006.