สารที่มีฤทธิ์ฆ่าหอยเชอรี่จากผลบวบขม

นางสาวเสาวนิตย์ ดาวรัตนชัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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MOLLUSCICIDAL CONSTITUENTS FROM *TRICHOSANTHES CUCUMERINA* FRUITS ON *POMACEA CANALICULATA*

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Thesis title	MOLLUSCICIDAL CONSTITUENTS FROM *TRICHOSANTHES CUCUMERINA* FRUITS ON *POMACEA CANALICULATA*
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เสาวนิตย์ ดาวรัตนชัย : สารที่มีฤทธิ์ฆ่าหอยเชอรี่จากผลบวบขม (MOLLUSCICIDAL CONSTITUENTS FROM *TRICHOSANTHES CUCUMERINA* FRUITS ON *POMACEA CANALICULATA*). อ.ที่ปรึกษา รศ.ดร. ชัยโย ชัยชาญทิพยุทธ, 106 หน้า.

การศึกษาองค์ประกอบทางเคมีและฤทธิ์ทางชีวภาพของผลบวบขม (*Trichosanthes cucumerina* L.) สามารถแยกองค์ประกอบทางเคมีโดยใช้เทคนิคทางโครมาโทกราฟีจากสิ่งสกัดในชั้นเมทานอลได้ 1 ชนิด การพิสูจน์โครงสร้างทางเคมีของสารบริสุทธิ์ที่แยกได้ด้วยการวิเคราะห์เชิงสเปกตรัมของ UV, IR, MS และ NMR เปรียบเทียบกับสารที่เคยมีรายงานมาก่อน คือ คิวเคอร์บิตาซิน บี (25-acetoxy-2β,16α,20β-trihydroxy-9β-methyl-19-nor-10α-lanosta-5,23-diene-3,11,12-trione ($C_{32}H_{46}O_8$)) และพบว่าคิวเคอร์บิตาซิน บี มีฤทธิ์ฆ่าหอยเชอรี่ (*Pomacea canaliculata* Lamarck) ในขนาดเส้นผ่านศูนย์กลาง 2.0-2.5 เซ็นติเมตร โดยแสดงความเป็นพิษต่อหอยเชอรี่ที่ระดับความเข้มข้น LC_{50} ที่ 9.03 ส่วนในล้านส่วน และระดับความเข้มข้น LC_{90} ที่ 17.67 ส่วนในล้านส่วน หลังจาก 24 ชั่วโมง ที่ความเชื่อมั่นที่ 95% การทดสอบความเป็นพิษต่อเซลล์มะเร็ง 5 ชนิด ได้แก่ ลำไส้ใหญ่ (SW620), เซลล์มะเร็งตับ (HEP-G2), เซลล์มะเร็งกระเพาะอาหาร (KATO-III), เซลล์มะเร็งเต้านม (BT474) และเซลล์มะเร็งปอด (CHAGO) พบว่าคิวเคอร์บิตาซิน บี มีความเป็นพิษเฉพาะเจาะจงกับเซลล์มะเร็งลำไส้ใหญ่ (SW620) และเซลล์มะเร็งตับ (HEP-G2) มีค่า IC_{50} 0.06 และ 0.09 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ บวบขมมีศักยภาพในการนำมาใช้ฆ่าหอยแทนสารเคมีซึ่งมีพิษต่อสิ่งแวดล้อมได้

ภาควิชา.....เภสัชเวท..... ลายมือชื่อนิสิต เสาวนิตย์ ดาวรัตนชัย

สาขาวิชา.....เภสัชเวท..... ลายมือชื่ออาจารย์ที่ปรึกษา ชัยโย ชัยชาญทิพยุทธ

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KEY WORDS : MOLLUSCICIDAL/ *Trichosanthes cucumerina*/ *Pomacea canaliculata*

SAOWANIT DAORATTANACHAI: MOLLUSCICIDAL CONSTITUENTS FROM *TRICHOSANTHES CUCUMERINA* FRUITS ON *POMACEA CANALICULATA*. THESIS ADVISOR: ASSOC. PROF. CHAIYO CHICHANTIPYUTH, Ph.D., 106 pp.

The chemical study of the fruits of *Trichosanthes cucumerina* L. (Buap khom) led to the isolation of a triterpenoid saponin. The structure determination of this compound was accomplished by spectroscopic analyses (UV, IR, MS and NMR) and by comparison with previously reported data of the known compound cucurbitacin B, $C_{32}H_{46}O_8$, 25-acetoxy-2β,16α,20β-trihydroxy-9β-methyl-19-nor-10α-lanosta-5,23-diene-3,11,12-trione. Cucurbitacin B showed molluscicidal activity against *Pomacea canaliculata* Lamarck (2.0-2.5 cm, in diameter) with LC_{50} value of 9.02 ppm and LC_{90} value of 17.67 ppm after 24 h at 95% confidence interval. Assays by MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] colorimetric method with 5 cancer cell lines including human colon adenocarcinoma (SW620), human liver hepatoblastoma (HEP-G2), human gastric carcinoma (KATO-III), human breast ductal carcinoma (BT474) and human undifferentiated lung carcinoma (CHAGO) revealed that cucurbitacin B has strong cytotoxic activity against human colon adenocarcinoma (SW620) and human liver carcinoma (HEP-G2) with IC_{50} values of 0.06 and 0.09 μg/mL, respectively. It appears that *T. cucumerina* L. (Buap khom) has potential to be used as a molluscicide substitute for toxic chemicals.

Department of.....Pharmacognosy..... Student's signature.Saowanit Daorattanachai

Field of study...Pharmacognosy..... Advisor's signature.Chaiyo Chichantipyuth

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LIST OF ABBREVIATIONS AND SYMBOLS

$[\alpha]_D^{20}$	=	Specific rotation at 20°C and sodium D line (589 nm)
δ	=	Chemical shift
ε	=	Molar absorptivity
μg	=	Microgram
μL	=	Micro-liter
μm	=	Micrometer
μM	=	Micromolar
λ_{max}	=	Wavelength at maximal absorption
ν_{max}	=	Wave number at maximum absorption
Ac	=	Acetyl
BT474	=	human breast ductal carcinoma
br	=	Broad (for NMR spectral data)
°C	=	Degree Celsius
C	=	Concentration
ca.	=	Calculate
CHAGO	=	human undifferentiated lung carcinoma
$CDCl_3$	=	Deuterated chloroform
CH_3	=	Methyl
CH_2Cl_2	=	Dichloromethane
CH_3OH	=	Methanol
^{13}C-NMR	=	Carbon-13 Nuclear Magnetic Resonance
cm	=	Centimeter
cm^{-1}	=	Reciprocal centimeter (unit of wave number)
CU1	=	CU-one (Tea seed cake contained 12% saponins)
d	=	doublet (for NMR spectral data)
dd	=	doublet of doublets (for NMR spectral data)
ddd	=	doublet of doublets of doublets (for NMR spectral data)
DDI	=	Double de-ionized water

EI-MS	=	Electron Impact Mass Spectrum
EtOAc	=	Ethyl acetate
g	=	Gram (s)
^{1}H-NMR	=	Proton Nuclear Magnetic Resonance
h	=	Hour (s)
HEP-G2	=	human liver carcinoma
H_2SO_4	=	Sulfuric acid
Hz	=	Hertz
IR	=	Infrared
KATO-3	=	human gastric carcinoma
KBr	=	Potassium bromide
Kg	=	Kilogram
L	=	Liter
LC_0	=	0% Lethality concentration
LC_{50}	=	50% Lethality concentration
LC_{90}	=	90% Lethality concentration
LC_{100}	=	100% Lethality concentration
LD_{50}	=	50% Lethality dose
m	=	Multiplet (for NMR spectral data)
M^+	=	Molecular ion
m/z	=	Mass to charge ratio
MeOH	=	Methanol
mg	=	Milligram (s)
MHz	=	Mega-hertz
mL	=	Milliliter
mm	=	Millimeter
MS	=	Mass Spectrometry
MTT	=	3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide
No.	=	Number
nm	=	Nanometer

NMR	=	Nuclear Magnetic Resonance Spectrometer
ppm	=	Parts per million
R_f	=	Retardation factor
RSD	=	Relative standard deviation
q	=	Quartet (for NMR spectral data)
s	=	Singlet (for NMR spectral data)
SW620	=	human colon denocarcinoma
$t_{1/2}$	=	Time half life
t	=	Triplet (for NMR spectral data)
TLC	=	Thin Layer Chromatography
TOF	=	Time of Flight
UV	=	Ultraviolet
UV-VIS	=	Ultraviolet and visible spectrophotometry
w/w	=	Weight by weight
W.H.O.	=	World Health Organization

CHAPTER I

INTRODUCTION

1. State of problem

Besides rats, birds and crabs, Thai farmers have a new problem with golden apple snails, *Pomacea canaliculata* Lamarck, which were commonly known as cherry snail or Japanese apple snail (Chanyapate and Archwacom, 1997). The snails were first found in Thailand in 1982 coming from Japan and Philippines as a new economic farmed animal in cement tanks and ponds as promising export (Doungsawat, 1987). Unfortunately, the snails were not demanded in Thai market. Hence, the market value soon plummeted and the snails were thrown aways.

The snails can eat almost everything available in their environment, and that causes devastating damage to rice and some aquatic plants, such as grass, lotus, water morning glory, water lettuce, sulvinia. According to the recent reports, the snails quickly spread through waterways and irrigated paddy fields. In 1995, 403,896 Rai of paddy fields in 27 provinces were devastated by the snails and it increased to be 459,229 Rai of paddy fields in 30 provinces of Thailand in 1996 (Sitti, 1988). The paddy fields in Thailand devastated by golden apple snails.

The suitable method to control numbers of the snails should be identified. Although, there are many control methods, the easiest method is the use of chemicals and synthetic substances, such as niclosamide, cartap and chlorpyrifos (Chanyapate and Archawacom, 1999).

However, those chemicals are also toxic to human and other species. The effects on human include nails falling out, skin problems, blurring vision and blindness are found. There are also significant and long-lasting downstream effects on natural marine ecosystem. The types of chemicals generally used are persistent and accumulated in sediment (Chanyapate and Archwacom 1990; Isarangul na ayuthaya, 1991). Many of these chemicals have been banned in Japan, Taiwan and the

Philippines. Currently there are no chemicals, that are safe, effective and cheap enough for Thai farmers.

Integrated snail management including handpicking snails, herding ducks to eat smaller snails, improving better water control, applying spot pesticide treatment and more seeding, is being advocated. However, the results do not reveal how effective each component is. Handpicking is the most effective but also the most labor intensive. Ducks are effective but it could not hunt all existed snail and improving water control has proven very difficult.

Biological controls include raising common carp and *Nile tilapia* to eat snails, genetically manipulating snails to breed less rapidly and botanical pesticides which are biodegradable. They are believed to be less harmful to environment than synthetic pesticides (Naylor, 2000).

Among various golden apple snails controls, biological control is widely studied because of the low toxicity to environment. Therefore, this research will be conducted to study the toxicity of *T. cucumerina* L. fruits to golden apple snail, *P. canaliculata* Lamarck.

2. *Trichosanthes* spp.

Trichosanthes, a genus of climbing herbs in the family of Cucurbitaceae comprises over 100 species (most found in Southeast Asia and Australia) and 17 species are found in Thailand (Duyfjes and Pruesapan, 2004). Some *Trichosanthes* fruits posses different properties, such as edible laxative and very poisonous. *Trichosanthes* spp. is known as medicinal in China. The seeds and the pericarp of *T. kirilowii* Maxim. and *T. multiloba* Miq. are used as a tonic and as an ingredient in an astringent remedy (Yueh and Cheng, 1974).

Trichosanthes species are climbers with a stoutish habit, mostly branched tendrils, distinctly fringed petals, and often brightly colored fruits having the size of a hen's egg which can be used in the determination of the species (Phupattanapong, 1987).

2.1 *Trichosanthes* spp. in Thailand

According to Tem Smitinand (2001), the species of the genus *Trichosanthes* found in Thailand are as follows.

Trichosanthes anguina L.	นมพิจิตร Nom pichit (Nakhon Ratchasima, Trat); น้อยข้อง Noi khong; มะนอย Ma noi (Northern); **บวบงู** Buap ngu (Central); หมากนอย Mak noi (Saraburi); Snake cucumber; Snake gourd.
Trichosanthes cordata Roxb.	**ขี้กาขาว** Khi ka khao (Central).
Trichosanthes cucumerina L.	นมพิจิตร Nompichit; **บวบขม** Buap khom; มะนอยจ๋า Ma noi cha (Northern).
Trichosanthes kirilowii Maxim.	**ล่อฮังก๊วย** Lo-hang-kuai (Chinese).
Trichosanthes quinquangulata A.Gray	**บวบเหลี่ยม** Buap liam (General).
Trichosanthes tricuspidata Lour.	กระดึงช้าง Kra dueng chang; **กระดึงช้างเผือก** Kra dueng chang phueak (prachuap Khiri Khan); ขี้กาขม Khi ka khom (Phangnga); ขี้กาแดง Khi ka daeng (Ratchaburi); ขี้กาใหญ่ Khi ka yai (Nakhon Ratchasima, Surat Thani); ขี้กาลาย Khi ka lai; มะตูมกา Ma tum ka (Nakhon Ratchasima).

2.2 *Trichosanthes cucumerina* L.

Local name: Nom pichit, Buap Khom (Central), Ma noi cha (Northern).

Description: An annual, climbers; tendril trifid. Leaves deeply 5 lobed, toothed. Flowers irregularly divided into narrow, short lobed, small, white, dioecious; male flower in recemes; female flowers solitary. Berries up to 3 inch long, spindle sharp, smooth, orange or scarlet colored when ripe, green and striped when immature (Phupattanapong, 1987).

Distribution: Throughout Thailand.

Uses: The plant is a cardiotonic, laxative, alternative, and antifebrile; its decoction is given in bilious fever as a febrifuge and laxative; its efficacy is said to increase if it is given with honey; in obstinate case of fever a combined infusion of this plant and of coriander is efficacious; 180 grains of each are used as anthelmintic.

The leaves are a febrifuge, laxative and emetic; a decoction of the leaves made with the addition of coriander is given in bilious fever; the leaf juice is used as an emetic.

The leaf juice is locally applied as a liniment if the liver is congested; in remittent fever it is applied over the whole body; the juice is rubbed over the head for the cure of alopecia.

The root is a purgative and tonic; it is a strong purgative; but it has a strong irritant action on the gastro-intestinal system.

An infusion of the dried fruit is an aperient; it is also acids digestion.

The seeds are antifebrile and anthelmintic (Phupattanapong, 1987).

In 1992, cucurbitacin B and dihydrocucurbitacin B were isolated from the juice of *Trichosanthes cucumerina* L. fruits. (Jiratchariyakul and Frahm, 1992). MTT colorimetric assays for cytotoxic activity of cucurbitacin B and dihydrocucurbitacin B showed that theirs IC_{50} value against breast cancer cell line (SK-BR-3) were 0.05 and 0.40 μg/ml, respectively (Jiratchariyakul *et al*., 1999).

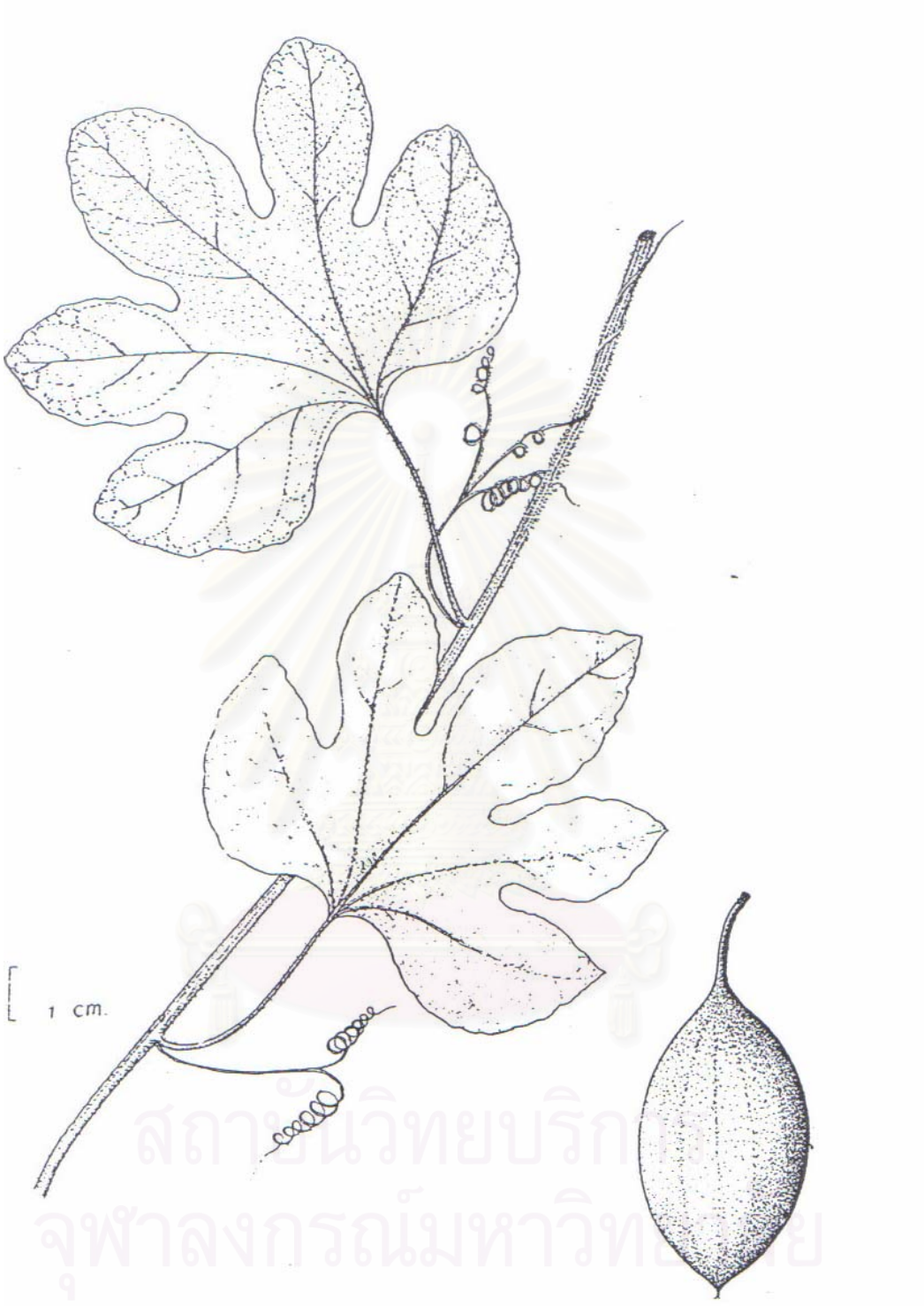


Figure 1.1 *Trichosanthes cucumerina* L.

3. The objectives of this research

3.1 To study the chemical constituents from fruit of *T. cucumerina* L.

3.2 To study the molluscicidal activity on golden apple snails (*P. canaliculata* Lamarck).

4. Scope of study

This research is laboratory scale to study *T. cucumerina* L. fruits extract that have potential molluscicidal activity on *P. canaliculata* Lamarck.

5. Limitation of study

The study has focused on sizes of *P. canaliculata* Lamarck are 2.0-2.5 cm diameters. And in case of snail collecting, it can be identified by only physiological characteristic. The snails were reared in aquaria containing dechlorinated tap water (laboratory scale) which the farmers can apply by themselves.

CHAPTER II

HISTORICAL

Trichosanthes cucumerina L. is an indigenous plant in Thailand known as **บวบขม** Buap khom (Central), นมพิจิตร Nom pichit, มะนอยจ๋า Ma noi cha (Northern) (Smitinand, 2001).

1. Review of the Genus *Trichosanthes*

The genus *Trichosanthes* is in the family of Cucurbitaceae. The *Trichosanthes* comprises 100 species of climbing herbs and can be found in subtropical and tropical eastern Asia: from India, China and Japan, through Malaysia, east to tropical Australia and Fiji. Sexual condition in most species are dioecious, and only some are monoecious, e.g. *T. cucumerina* L. Stem thickness is rather typical for the species and measured in the leafy twigs. Tendrils are variance with other scandent-climbing plant families. Leaves palmately compound (with petiolulate leaflets) or simple, with the blade either entire or variously (deeply) lobed. The lobbing may be variable within a species. Probracts are nearly always present in *Trichosanthes*, one at each node, and their shape, consistency and presence or absence of glands provides good characters. Inflorescences in the dioecious species of *Trichosanthes*, male flowers are either solitary, or usually arranged into a peduncled bracteate raceme, often with a single male flower coaxillary. Female flowers develop singly at the node. In monoecious plants, female flowers can be found singly at the nodes or singly (and developing previously) beside the male raceme; female flowers usually develop later than male flowers. Flowers, in most Cucurbitaceae family, are unisexual. In most species of *Trichosanthes*, the flowers are (partly) nocturnal, they open in the late afternoon or at night and close before sunrise when the corollas can be found fallen on the ground, but some species e.g. *T. cucumerina* L. are largely diurnal with flowers open at daytime. The flowers are mostly white, showy and large, possibly pollinated by moths. The perianths of male and female flowers are generally similar. Male flowers have three stamens, inserted in the

receptacle tube towards the throat, or in some species at the base. The filaments are free, (very) short, glabrous or hairy. The anthers have S-shaped anther cells (thecae), two anthers are 2-thecous, one 1-thecous. In Thai species, they are connate (or rarely connivent, *T. phonsenae*) into a cylindrical body (synandrium), usually with stiff hairy appendages at the apex and between the thecae. Female flowers are long, slender, with three stigmas and glabrous or hairy. The inferior ovary is wholly hairy, or glabrescent or glabrous, globose or ellipsoid of shape, three celled with three parietal placentas and numerous horizontal ovules. Fruits are indehiscent, various in size and shape, globose, ovoid or ellipsoid, rarely cylindrical (3-15 cm long; snake-like, to ca. 100 cm long in *T. cucumerina* var. *anguina*). Seeds are often decisive for the identification of *Trichosanthes* to species. The seeds are numerous, horizontal, and often densely packed (Duyfjes and Pruesapan, 2004).

2. *Trichosanthes cucumerina* L.

2.1 Traditional uses and biological activities of *Trichosanthes* spp.

Trichosanthes plants have been used in traditional medicine in many countries with several purposes. In Nepal, the fruits of *T. anguina* are used for edible, emetic, tonic and purgative (Suwal, 1970), and *T. anguina* leaves extract and larvicidal activity against *Culex quinquefasciatus* (Prabakar and Jebanesan, 2004).

In Thailand, the stem and leaves of *T. cucumerina* L. are used for emetic, antipyretic and laxative properties and the fruits are used for digestive and laxative effects (Phupattanapong, 1987), *T. cucumerina* L. fruits have cytotoxic activity due to cucurbitacin B and dihydrocucurbitacin B which showed IC_{50} value against breast cancer cell line (SK-BR-3) at 0.05 and 0.40 μg/ml, respectively (Jiratchariyakul *et al*., 1999). *T. cucumerina* L. seed showed hypoglycemic activity in alloxan-diabetic rats (Kar *et al*., 2003). In China, the decoctions of fruits of *T. kirilowii* are used to cure lung problems: takes moisture, relieves coughs and clears mucus and the roots are used to induce abortion (Shaw *et al*., 1994). The crude extract of *T. kirilowii* seeds showed antibacterial activity against *Streptococcus mutans* Serotype C (MT 5091) (MIC = 93.8 mg/mL) and Serotype D (OMZ 176) (MIC = 62.5 mg/mL) (Chen *et al*., 1989). Moreover,

T. kirilowii are reported to have trichosanthin, a protein possesses immunomodulatory, anti-tumor and anti-human immunodeficiency virus (HIV) properties (Shaw *et al*., 1994) and the seeds of *T. kirilowii* have 7-oxodihydrokarounidiol-3-benzoate which possesses anti-inflammatory effect (Akihisa *et al*., 1997).

In Australia, the leaves of *T. palmata* are smoked as a remedy for asthma (Schramm, 1956).

Lectin have been purified from seed extracts of *T. kirilowii , T. rosthornii, T. truncate, T. laceribractea, T. hupehensis, T. schizostroma, T, dunniana, T. pedata, T. cucumeroides, T. trichocarpa* and *T. ovigera.* Then compound have hemagglutinating activity (Dong *et al*., 1993).

Trichosanic acid, a major component of *T. japonica* has effects on platelet aggregation and arachidonic acid metabolism in human platelets (Takenaga *et al*., 1988).

2.2 Chemical constituents of *Trichosanthes* spp.

Various secondary plant products in Genus *Trichosanthes* have been extensively investigated. The following substances have been reported for this genus.

2.2.1 Amino acids

In the cucurbitaceous seeds examined, very large differences in the concentration of citrullin (Silapa-archa, 1980) existed between different species. Other amino acids such as *m*-carboxyphenylalanine occurred in the Cucurbitaceae. Citrullin and *m*-carboxyphenylalanine are found in *Trichosanthes* spp. (Patricia and Fowden, 1965).

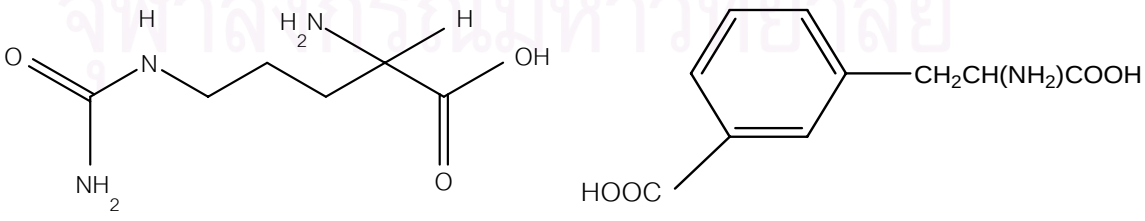


Figure 2.1 Structure of citrullin and *m*-carboxyphenylalanine

2.2.2 Proteins

Trichosanthin, a protein of the root of tuber the Chinese medicinal herb. *T. kirilowii,* was found to be effective in inducing abortion in several animal models and in humans (Kuo-Fen, 1982). It has been used in China for the abortion and the therapy of choriocarcinoma. The isoelectric point of trichosanthin is 9.4. It is composed of 233 or 234 amino acid residues (M.W. 25,700), and contained no cysteine or carbohydrate. Trichosanthin was found to be a potent inhibitor of protein synthesis in a cell frees translation system (Maraganore, Joseph and Bailey. 1987a; Zhang and Wang, 1986), and also found to inhibit selective, *in vitro* the human immunodeficiency virus (HIV) (Buderi, 1989).

Beside trichosanthin, several abortificients ribosome-inactivating proteins with the range from 27,000-32,000, were isolated from several *Trichosanthes* spp. They were β-trichosanthin (from roots of *T. cucumeroides*) (Yeung, 1987), trichosanthin ZG (from the roots of *T. kirilowii*) (Maragonore and Kindsuogel, 1987b), trichokilin (from the seeds of *T. kirilowii*) (Barbieri, Casellas and Stirpe, 1986; Casellas *et al*., 1988), karasurin (from the root of *T. kirilowii* Maxim var. japonica) (Shunsuke, Tadahiro and Yukio, 1991).

However, other types of proteins were also found in the plants such as serine protease (Kanaeda *et al*., 1985; Kanaeda *et al*., 1986; Uchikoba *et al*., 1987) and trypsin inhibitor (Qian, Tan and Qi, 1990).

2.2.3 Fatty acids

Seed oils of the Cucurbitaceous fell into two distinct groups, one having a considerable proportion of conjugated triene fatty acids in the glycerides. The first group included the common cultivated cucurbits. The second or conjugated oil groups were found in *Cucurbita, Momordica, Telfairia* and *Trichosanthes* (Chisclm and Hopkins, 1964).

There was evidently some loss of the conjugated triene ester on the GLC column during the analysis. Accordingly, the content of conjugated triene was determined by

ultraviolet absorption analysis and the content of each non-conjugated acid was calculated as a percentage of the remainder (Chisclm and Hopkins, 1964).

Punicic acid (Lakshminarayana *et al*., 1988) was the major component of the oil of *Trichosanthes*. Other fatty acids were also found, such as trichosanic acid (Kitajima and Tanaka, 1989a), palmitic acid (Silapa-archa, 1980) and bryonolic acid (Kitajima and Tanaka, 1989b).

2.2.4 Polysaccharide

A number of glycans were isolated from the roots of *T. kirilowii*, namely trichosan A, B, C, D and E, which possessed hypoglycemic activity in normal mice. The main glycan, trichosan A, also exhibited the activity in alloxan-induced hyperglycemic mice (Hikino *et al*., 1989).

The pure lecithin from *T. kirilowii* was used as an antigen immunizes rabbits (Wang and Zhang, 1990). It showed antitumor in mice bearing sarcoma-180 (Shiio and Che, 1986) and stimulated the corporation of D-(3-H) glucose into lipids in isolated rat epididymal adipocytes. The lecithin, however, did not inhibit lipolysis induced by either epinephrine or corticotrophin in these adipocytes. It did not suppress corticotrophin-induced lipolysis in isolated haster adipocytes. At a dose that did not stimulate lipolysis on its own, the lecithin slightly potentiated the lipolytic effect of corticotropin and epinephrine (Ng *et al.,* 1985).

The polysaccharide fraction from the rhizome of *T. kirilowii* showed antitumor and cytotoxic activity with the immunopotentiating activity (Chung and Lee, 1990).

2.2.5 Flavonoids

Eleven flavonoids were isolated and identified from the leaves of the genus *Trichosanthes*, they are luteolin-7-O-β-D-glucopyranoside (a), 4'-O-β-D-glucopyranoside (b), 3'-O-β-D-glucopyranoside (c), apigenin-7-O-β-D-glucopyranoside (d), apigenin-6,8-di-O-β-D-glucopyranoside (e), kaempferol-3,7-di-O-α-L-rhamnopyranoside (f), kaempferol-3-O-β-D-glucopyranoside-7-O-α-L-rhamnopyranoside (g), kaempferol-3-O-

β-D-galactopyranoside (h), kaempferol-3-O-β-sophopyranoside (i), quercetin-3-O-β-rutinoside (j) and kaempferol-3-O-β-rutinoside (k) in Table 2.1.

Table 2.1 Flavonoids in *Trichosanthes* spp.

Plants	flavones					flavonols					
	a	b	c	d	e	f	g	h	i	j	k
T. kirilowii Maxim.	+	+	+	+							
T. kirilowii Maxim. var.*japonica* Kitam.	+	+	+	+	+						
T. cucumeroides Maxim.						+	+				
T. anguina Linne								+	+		
T. multiloba Miquel										+	
T. rostrata Kitam.										+	
T. bracteata Voiqt.					+						+

Trichosanthes flavonoids could be divided into two groups on the basis of their aglycones, i.e. the flavones group and flavonols group (Yoshizaki *et al*., 1987).

2.2.6 Triterpenes and steroids

Terpenoids are dimers and polymers of five-carbon precursors called isoprene units (Figure 2.2). The smallest terpenoids, the monoterpenes, have two isoprene units. Monoterpenes such as geraniol and menthol, from the leaves of mints and eucalyptus, are volatile and usually have strong odors. Pines and other resinous plants also synthesize terpenoids made from four isoprene units (diterpenes) or from six isoprene units (triterpenes).

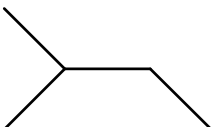


Figure 2.2 Isoprene unit

One subclass of triterpenes is the sterols, which are chemically similar to the steroidal hormones of animals. Sterols may be combined with nitrogen to form alkaloids or with sugar in steroidal glycosides. Terpenoids having eight isoprene units form a class of yellow to red pigments called carotenoids. The largest terpenoids, which can have more than 6,000 isoprene units in a single molecule, as known as rubber. Rubber is made by about 2,000 plant species, but most of them make this substance in amounts too small for commercial use.

Although terpenoids are considered secondary metabolites, they include some compounds that have clear roles in plants. The most prominent terpenoids are abscisic acid, which are diterpenes, and the carotenoid β-carotene. Abscisic acid is important plant hormone that regulates plant growth and development. β-carotene is a universally occurring accessary pigment in phytosynthesis. Abscisic acid and β-carotene are examples of basic metabolites that are derived from secondary metabolic pathways (Moore *et al*., 1995).

Triterpenoids can be divided into at least four groups of compounds. They are true triterpenes, steroids, saponins and cardiac glycosides (Harborne, 1973).

Sterols are triterpenes, which are based on the cyclopentano-perhydro phenanthrene ring system (Figure 2.3). Sterols were mainly considered to be animal substances but such compounds have been detected in plants tissues, so called “phytosterol” (Harborne, 1973).

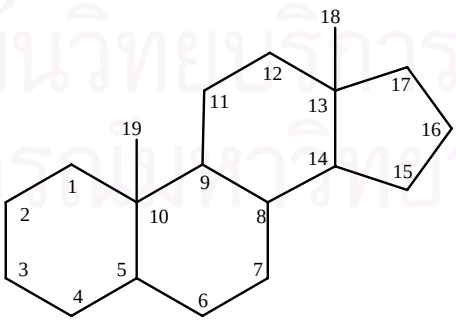


Figure 2.3 Structure of cyclopantano-perhydro phenanthrene ring system

A widely used test was the Libermann-Burchard reaction (acetic anhydride, conc. H_2SO_4), which produced a blue green color with most triterpenes and steroids. (Harborne, 1973).

The investigations of sterols of *Trichosanthes* plants have been reported. The sterol compositions in various *Trichosanthes* species were found to be similar but quantitatively different.

The triterpenes in Cucurbitaceous plants were tetracyclic triterpenes, e.g. cucurbitacin (which was designated as a chemotaxonomic character) (Silapa-Archa *et al.*, 1981) and pentacyclic triterpenes, e.g. α-amyrin, β-amyrin and bryonic acid (Silapa-Archa *et al.*, 1981).

Many types of tetracyclic and pentacyclic triterpenes in *Trichosanthes* plants have been investigated and reported in many papers (Silapa-Archa *et al.*, 1981). The pentacyclic triterpenes in the fruits of *T. anguina* was α-amyrin which was found in the *Trichosanthes* plants (Chandra and Sastry, 1990).

2.2.7 Bitter principles

The major bitter principles of the Cucurbitaceae were cucurbitacins which possessed the skeleton of tetracyclic triterpenes, 19(10 ⟶9β)-abeo-5α-lanost-5-ene (cucurbitane) (Dinan *et al.*, 2001). Most of the naturally occurring cucurbitacins contained a double bond between C-5 and C-6, but some representatives of this compound class were known in which formal cyclization between C-16 and C-24 provided an extra ring (e.g. cucurbitacin S); certain cucurbitacin glycosides were also discovered that possessed the 5β-cucurbitane skeleton (e.g.momordicoside G (Che *et al.*, 1985). Cucurbita-5,24-dienol was considered to be an important intermediate in the biogenesis of cucurbitacins found in the Cucurbitaceae (Akihisa *et al.*, 1986).

Biologically, the cucurbitacins exhibited a wide range of activities, for example, increasing the capillary permeability in rats, antifertility effects in female mice, and presentative action on experimental hepatitis and cirrhosis induced in rats. These compounds exerted a gibberellins-antagonistic activity in rice seedling, feeding

stimulant and antifeedant effects in insects. The cucurbitacins have been investigated for their cytotoxic and anticancer effects (Akihisa *et al*., 1986).

However, the cucurbitacins also possessed the acute mammalian toxicity (Sasamori *et al*., 1983).

The known tumor inhibitors are classified to 2 types; one which involve electrophilic addition to an SH group and the others which feature displacement reaction by the SH group (Fujita and Nagao, 1977). When tested against cell cultures derived from human carcinoma of the nasopharynx (Eagle's KB strain), cucurbitacin B, E, O, P and Q showed cytotoxic activity (Lavie and Glotter, 1971).

Cucurbitacin B and D were found in *T. kirilowii* Maxim. var. *japonica* root (Kitajima and Tanaka, 1989a), and cucurbitacin B and dihydrocucurbitacin B in *T. cucumerina* L. fruits (Tiangda *et al*., 1986).

Table 2.2 Chemical constituents of the genus *Trichosanthes*.

Species	Category	Reference
Trichosanthes anguina **L.**		
Entire Plant		
Chondrillasterol **[1]**	Steroid	Desai *et al.,* 1966.
Fruit		
α - Amyrin **[2]**	Triterpene	Chandra *et al*., 1990.
Daucosterol **[3]**	Steroid	Chandra *et al*., 1990.
Leaf		
β - Sitosterol **[4]**	Steroid	Chandra *et al*., 1990.
Taraxerone **[5]**	Triterpene	Chandra *et al*., 1990.
α - Tocopherol **[6]**	Flavonoid	Mannan & Ahmad, 1966.
Trifolin **[7]**	Flavonoid	Yoshizaki *et al*., 1987.
Seed		
Cucurbitacin B **[8]**	Triterpene	Guha *et al.,* 1975.
Cucurbitacin K **[9]**	Triterpene	Guha *et al.,* 1975.
Isolectin	Protein	Anuradha & Bhide, 1999.
Kaempferol **[10]**	Flavonoid	Yadav *et al.,* 1994a & Yadav *et al.,* 1994b.
Lectin	Protein	Gowrikumar *et al*., 1981;
Punicic acid	Lipid	Lakshminarayana *et al.,* 1982.
Quercetin **[11]**	Flavonoid	Yadav & Sveda.1994a & Yadav & Sveda.1994b.
Trichosanthes bracteata **Voigt**		
Root		
Cucurbitacin B **[8]**	Triterpenoid	Kitajima *et al*., 1989c.

Species	Category	Reference
Seed		
Punicic acid	Lipid	Lakshminarayana *et al.,* 1988.
***Trichosanthes cucumerina* L.**		
Fruit		
Cucurbitacin B **[8]**	Triterpenoid	Jiratchariyakul *et al*., 1992.
Dihydrocucurbitacin B **[12]**	Triterpenoid	Jiratchariyakul *et al*., 1992.
Leaf		
Daucosterol **[3]**	Alkane	Mallavarapu & Ramadhandra. 1979.
β - Sitosterol **[4]**	Steroid	Mallavarapu & Ramadhandra. 1979.
Seed		
Lectin	Protein	Kenoth & Swamy, 2003.
Trichosanic acid	Lipid	Kenoth & Swamy, 2003.
Trichosanthes cucumeroides		
Fruit		
Arachidonic acid **[13]**	Lipid	Kato, 1961.
β - Carotene **[14]**	Carotenoid	Matsuno *et al*., 1971.
α - Carotene **[15]**	Carotenoid	Matsuno *et al*., 1971.
Lycopene **[16]**	Carotenoid	Matsuno *et al*., 1971.
α - Spinasterol **[17]**	Steroid	Matsuno *et al*., 1971.
Leaf		
Kaempferitrin **[18]**	Flavonoid	Nakaoki *et al*., 1957.

Species	Category	Reference
Root		
Erythrodiol **[19]**	Triterpene	Akihisa *et al*.,1997.
β-Trichosanthin	Protein	Ng *et al*., 1992.
Trichobitacin	Protein	Zheng *et al*., 2000.
Vanillic acid **[20]**	Benzenoid	Kitajima *et al*., 1989b.
Seed		
Betulin **[21]**	Triterpene	Akihisa *et al*., 1997.
Bryonolol **[22]**	Triterpene	Akihisa *et al*., 1997.
Isoleucine	Protein	Akihisa *et al*., 1997.
Karounidiol-3-benzoate **[23]**	Triterpene	Akihisa *et al*., 1997.
7-Oxodihydrokarounidiol **[24]**	Triterpene	Akihisa *et al*., 1997.
Pipecolic acid	Protein	Akihisa *et al*., 1997.
Trichosanic acid	Lipid	Toyama *et al*., 1935.
Trichosanthes dioica		
Fruit		
Cucurbitacin B **[8]**	Triterpene	Guha *et al*., 1975.
Root		
n-Hentriacontane **[25]**	Alkane	North *et al*., 1945.
Seed		
Betulin **[21]**	Triterpene	Akihisa *et al*., 1997.
Cucurbitacin B **[8]**	Triterpene	Guha *et al*., 1975.
Karounidiol **[26]**	Triterpene	Akihisa *et al*., 1997.
Karounidiol-3-benzoate **[23]**	Triterpene	Akihisa *et al*., 1997.
Lectin	Triterpene	Dharkar *et al*., 2006.
7-Oxodihydrokarounidiol **[24]**	Triterpene	Akihisa *et al*., 1997.
7-Oxodihydrokarounidiol-3-benzoate	Triterpene	Akihisa *et al*., 2001.

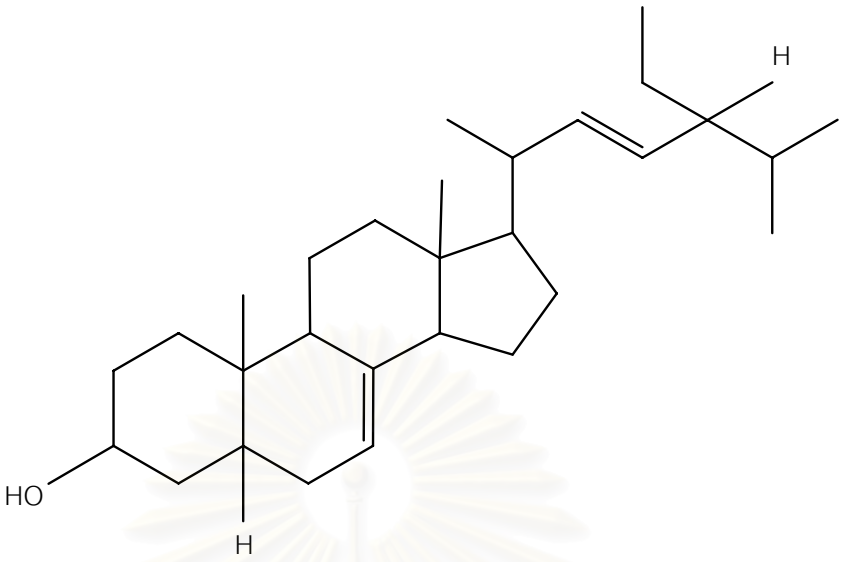
Species	Category	Reference
Trichosanthes hupehensis		
Root		
Cucurbitacin B [8]	Triterpene	Huang *et al*., 1993.
Trichosanthes integrifolia Kurz.		
Fruit		
Cucurbitacin B [8]	Triterpene	Silapa-archa *et al*. 1981.
Root		
Chondrillasterol [1]	Steroid	Itoh *et al*., 1982.
22-Dihydrospinasterol [27]	Steroid	Itoh *et al*., 1982.
Seed		
Trichosanic acid	Lipid	Takenaga *et al*., 1988.
Trichosanthes kirilowii Maxim.		
Entire Plant		
α - Trichosanthin	Protein	Zhang *et al*., 1982.
Leaf		
Apigenin 6-8-di-C-β-glucoside	Flavonoid	Yoshizaki *et al*., 1987.
Luteolin 3'-O-β-D-Glucoside	Flavonoid	Yoshizaki *et al*., 1987.
Luteolin 4'- O-β-D-Glucoside	Flavonoid	Yoshizaki *et al*., 1987.
α - Trichosanthin	Protein	Chow *et al*., 1990.
Root		
Bryonolic acid [28]	Triterpene	Ogiwara *et al*., 1995.
		Takeda *et al*., 1978.
Chondrillasterol [1]	Steroid	Kitajima *et al*., 1989a.
Cucurbitacin B [8]	Triterpene	Kitajima *et al*., 1989a.
Cucurbitacin D [29]	Triterpene	Oh *et al*., 2002.

Species	Category	Reference
23,24-Dihydrocucurbitacin B [12]	Triterpene	Kitajima *et al*., 1989a.
Ethyl α-L-arabinofranoside	Carbohydrate	Ogiwara *et al*., 1995.
Karasurin A	Protein	Kondo *et al*., 1996.
Karasurin B	Protein	Kondo *et al*., 1996.
Karasurin C	Protein	Kondo *et al*., 1996.
Lectin	Protein	Yeung *et al*., 1980.
α - Spinasterol [17]	Steroid	Kitajima *et al*., 1989a.
Tianhuafen	Protein	Sun *et al*., 1992.
Trichobitacin	Protein	Jin *et al*., 1997.
Trichosan A	Carbohydrate	Hikino *et al*., 1989.
Trichosan B	Carbohydrate	Hikino *et al*., 1989.
Trichosan C	Carbohydrate	Hikino *et al*., 1989.
Trichosan D	Carbohydrate	Hikino *et al*., 1989.
Trichosan E	Carbohydrate	Hikino *et al*., 1989.
Trichosanthin	Carbohydrate	Shaw, *et al*., 2005.
Vomifoliol [30]	Sesquiterpene	Kitajima *et al*., 1989a.
Seed		
Bryonolol [22]	Triterpene	Akihisa *et al*., 1994b.
Campesterol [31]	Steroid	Homberg & Seher, 1977.
10α-cucurbitadienol [32]	Triterpene	Akihisa *et al,* 1994a.
10α-cucurbitadienol acetate [33]	Triterpene	Akihisa *et al*., 1994a.
Cyclokirilodiol	Triterpene	Akihisa *et al,* 1992a.
5-Dehydrokarounidiol	Triterpene	Akihisa *et al*, 1992a.
3-Epibryonolol [34]	Triterpene	Akihisa *et al*., 2001.
3-Epikarounidiol [35]	Triterpene	Akihisa *et al,* 2001.
Karounidiol [26]	Protein	Akihisa *et al,* 2001.
Karounidiol-3-benzoate [23]	Triterpene	Akihisa *et al*, 2001.
α - Kirilowin	Protein	Wong *et al*., 1996.
β - Kirilowin	Protein	Kimura *et al*., 1995.
7-Oxo-10α-cucurbitadienol [36]	Triterpene	Akihisa *et al*, 1992.
7-Oxodihydrokarounidiol [24]	Triterpene	Akihisa *et al*., 1994b.

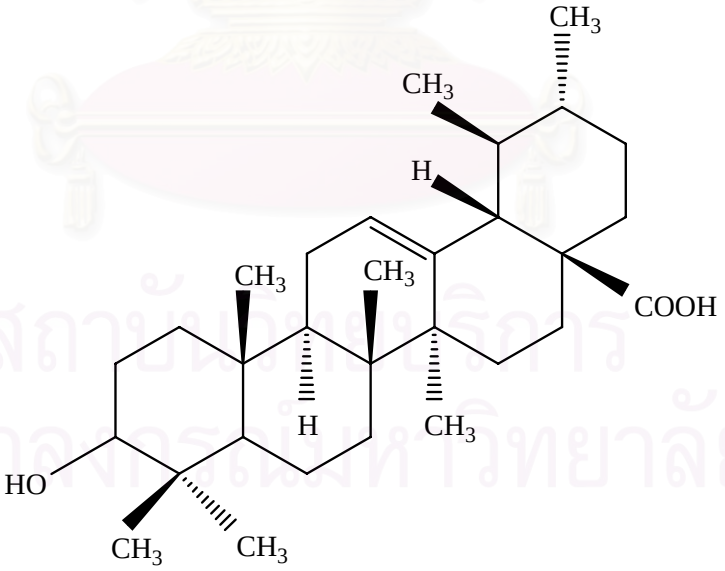
Species	Category	Reference
Sitosterin [37]	Steroid	
β - Sitosterol [4]	Steroid	Kimura *et al*., 1995.
Spinosterol [38]	Steroid	Kimura *et al*., 1995.
Stigmasterol [39]	Steroid	Homberg & Seher, 1977.
		Kimura *et al.,* 1995.
Trichosanthes lepiniate		
Root		
Trichomaglin	Protein	Gan *et al.,* 2004.
Trichosanthes miyagii		
Root		
Cucurbitacin B [8]	Triterpene	Kitajima *et al*., 1989d.
Cucurbitacin D [29]	Triterpene	Kitajima *et al*., 1989d.
23,24-dihydrocucurbitacin B [12]	Triterpene	Kitajima *et al*., 1989d.
Trichosanthes palmata		
Leaf		
Cycloeucalenol [40]	Triterpene	Kocor *et al*., 1973.
Cyclotrichosantol	Triterpene	Kocor *et al*., 1973.
α - Spinasterol [17]	Steroid	Kocor *et al*., 1973.
Stigmastanol [41]	Steroid	Kocor *et al*., 1973.
Trichosanthes pedata		
Seed		
Capric acid [42]	Lipid	Cheng *et al*., 1981.
Linoleic acid [43]	Lipid	Cheng *et al*., 1981.
Oleic acid [44]	Lipid	Cheng *et al*., 1981.
Punicic acid	Lipid	Cheng *et al*., 1981.

Species	Category	Reference
Trichosanthes rosthornii		
Fruit		
Trichosanatine	Alkaloid	Chao *et al*., 1995.
Trichosanthes rostrata		
Leaf		
Rutin [45]	Flavonoid	Yoshizaki, *et al*., 1987.
Trichosanthes tricuspidata Lour.		
Aerial Part		
Cycloeucalenol [40]	Triterpene	Bhandari & Rastogi, 1983.
Daucosterol [3]	Steroid	Bhandari & Rastogi, 1983.
Hexacosanoic acid [46]	Lipid	Bhandari & Rastogi, 1983.
Spinosterol [38]	Steroid	Bhandari & Rastogi, 1983.
β - Sitosterol [4]	Steroid	Bhandari & Rastogi, 1983.
Fruit		
Cucurbitacin B [8]	Trirerpene	Lotulung *et al*., 1998.
23,24-Dihydrocucurbitacin B [12]	Trirerpene	Lotulung *et al*., 1998.
Leaf		
Apigenin 6-8-di-C-β-glucoside	Flavonoid	Yoshizaki *et al*., 1987.
Cyclotricuspidoside A [47]	Trirerpene	Kasai *et al.,* 1999.
Cyclotricuspidoside B [48]	Trirerpene	Kasai *et al.,* 1999.
Cyclotricuspidoside C [49]	Trirerpene	Kasai *et al.,* 1999.
Kaempferol-3-O-β -rutinoside	Flavonoid	Yoshizaki, *et al*., 1987.
Seed		
m-Carboxyphenylalanine [50]	Protein	Dunnill *et al*., 1965.
Citrullin [51]	Protein	Dunnill *et al*., 1965.

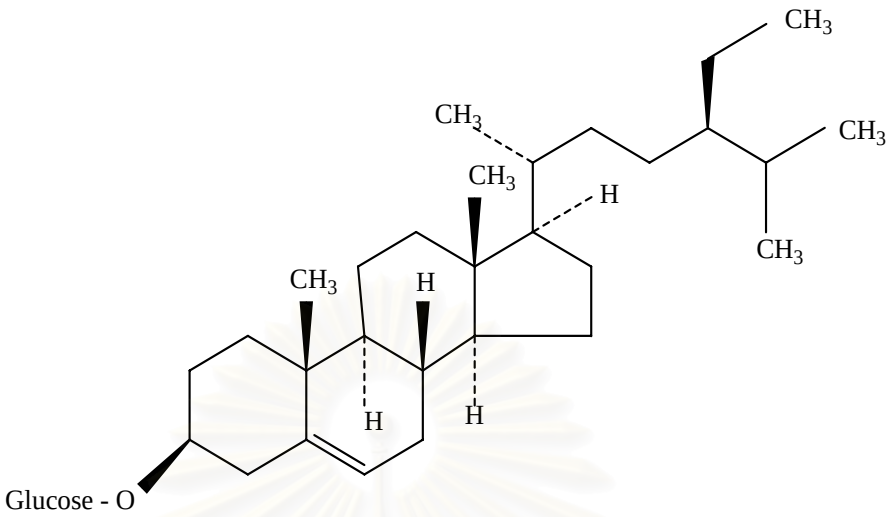
Species	Category	Reference
Stem		
Cyclotricuspidoside A **[47]**	Trirerpene	Kasai *et al.,* 1999.
Cyclotricuspidoside B **[48]**	Trirerpene	Kasai *et al.,* 1999.
Cyclotricuspidoside C **[49]**	Trirerpene	Kasai *et al.,* 1999.
Trichosanthes uniflora		
Seed		
Tricin **[52]**	Protein	Arisawa *et al*., 1985.



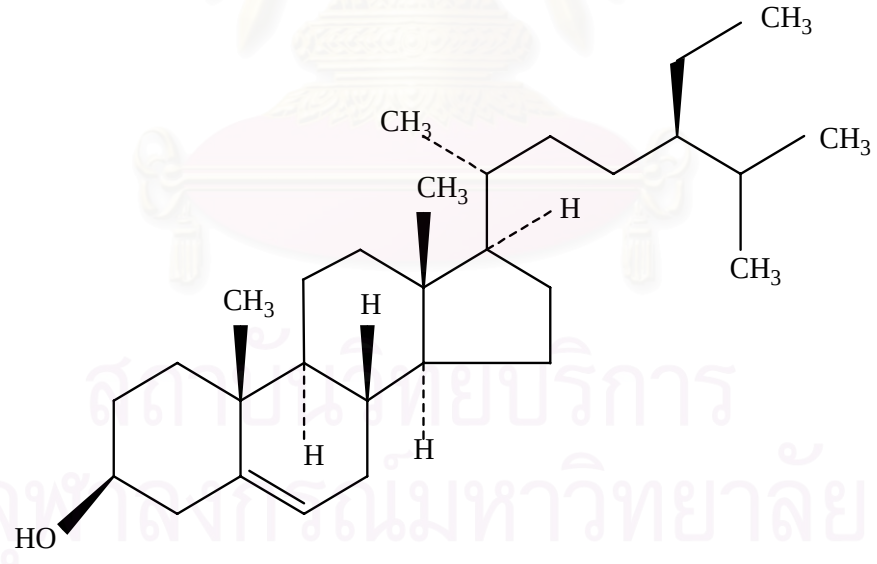
[1] Chondrillasterol



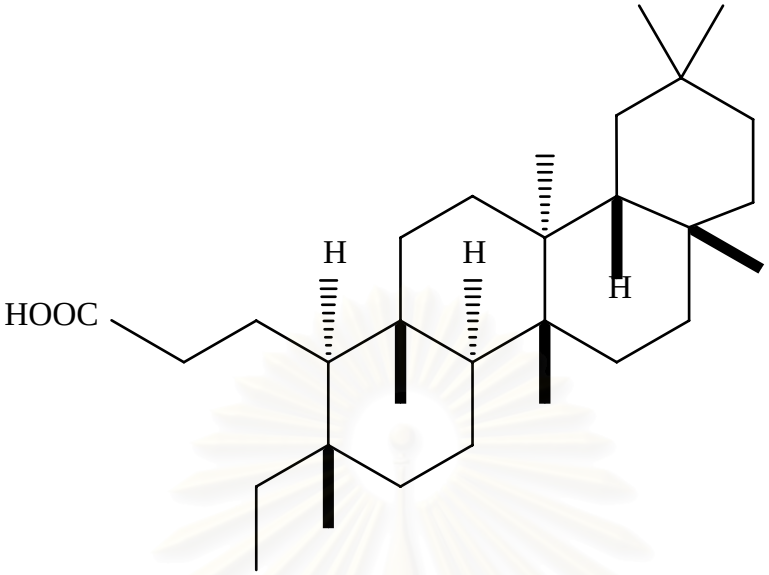
[2] α-Amyrin



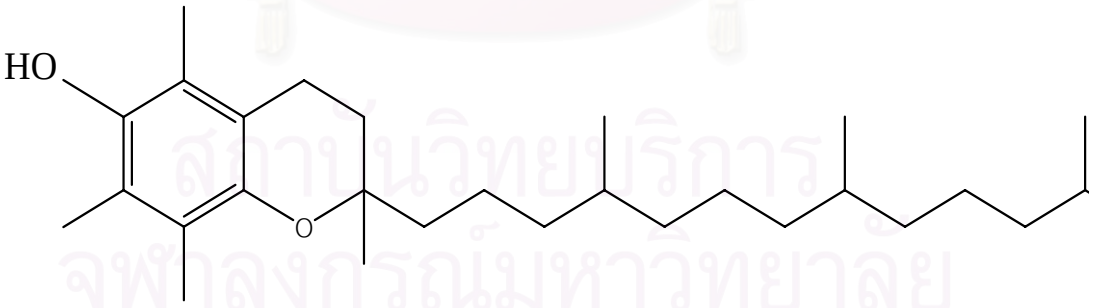
[3] Daucosterol



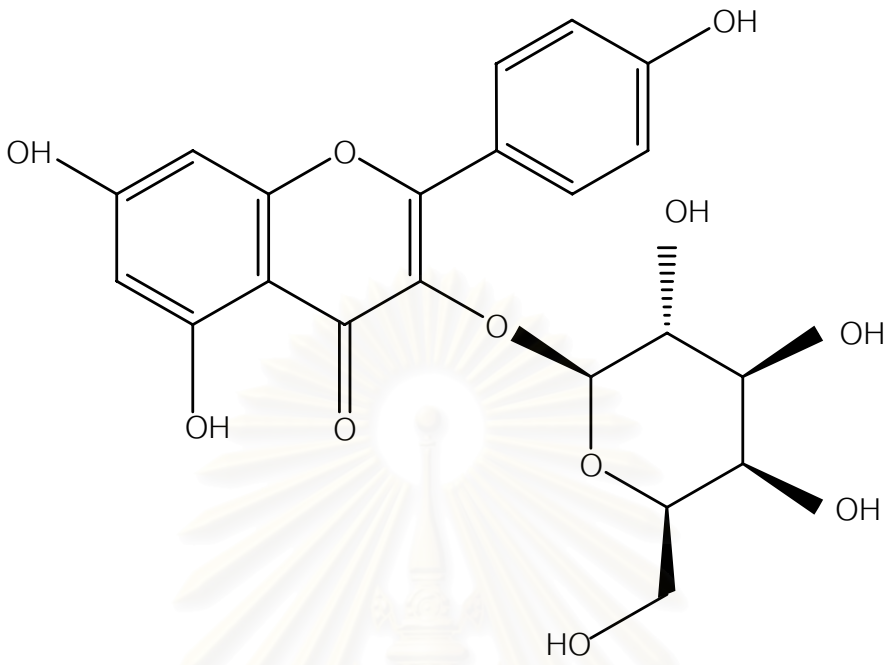
[4] β-Sitosterol



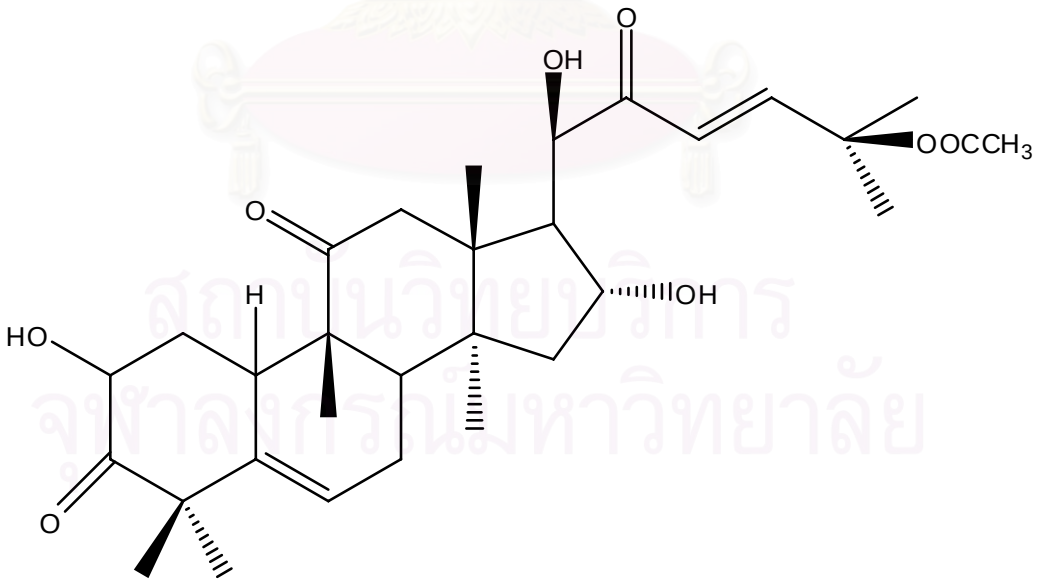
[5] Taraxerone



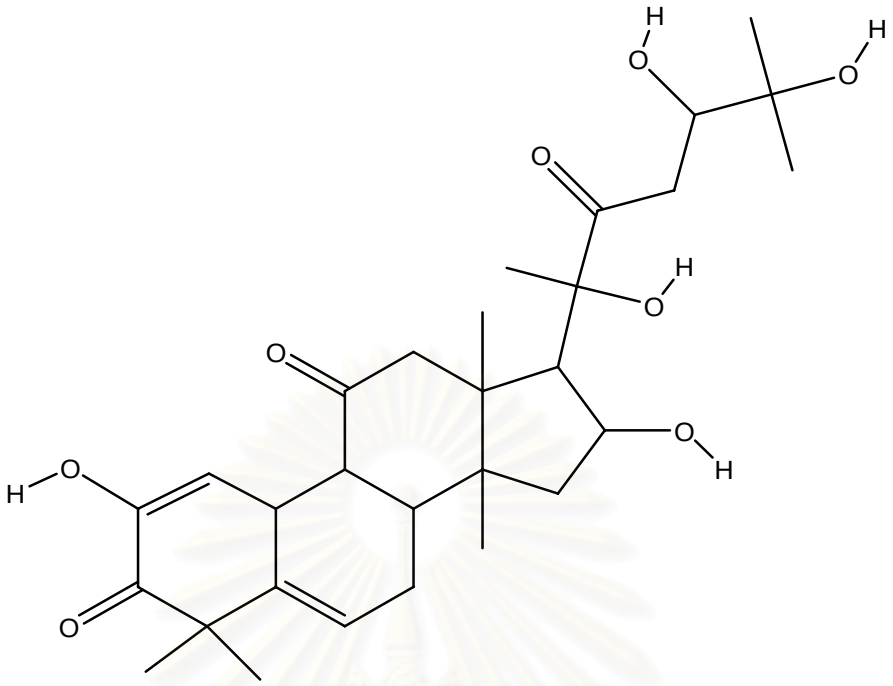
[6] α-Tocopherol



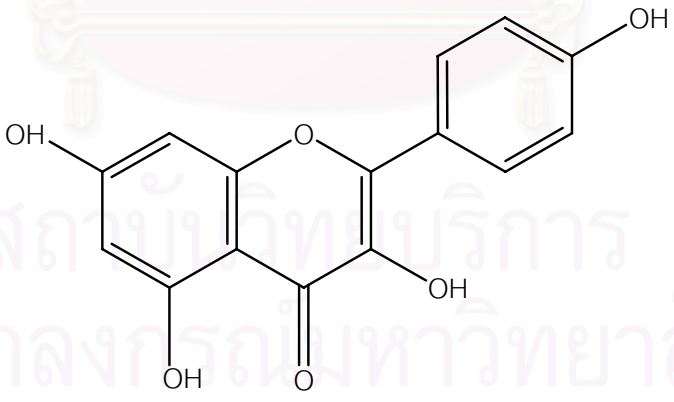
[7] Trifolin



[8] Cucurbitacin B



[9] Cucurbitacin K



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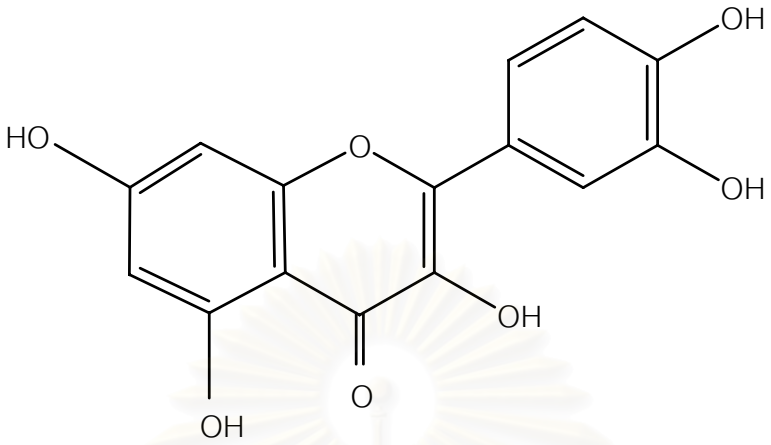
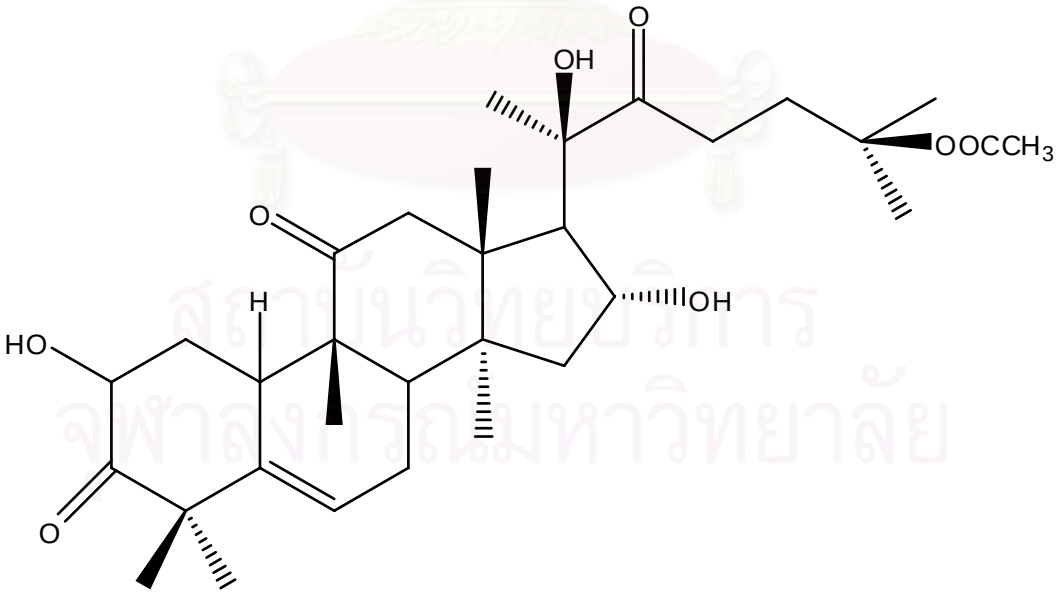
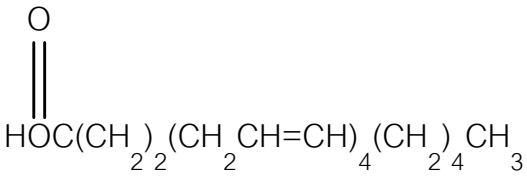


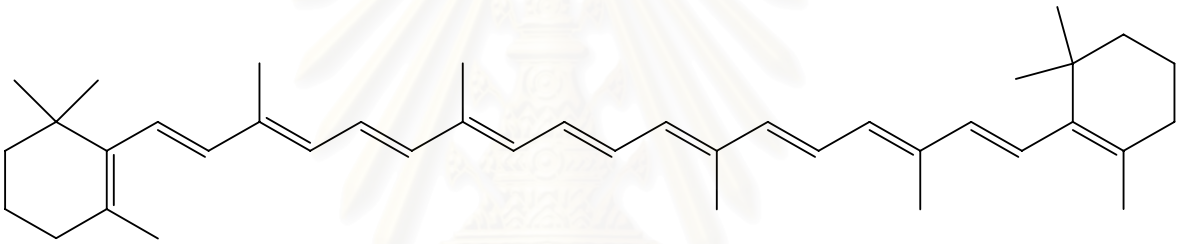
[11] Quercetin



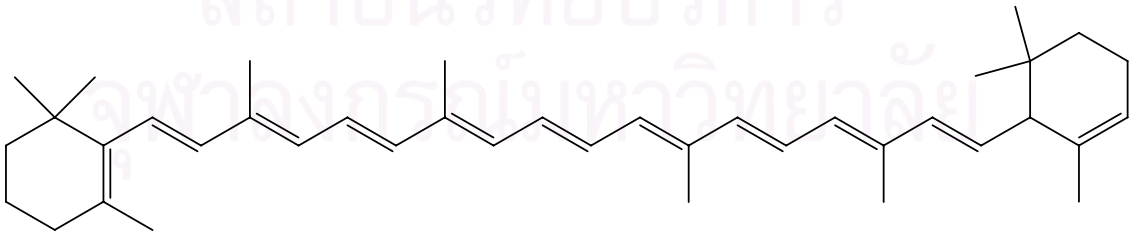
[12] Dihydrocucurbiacin B



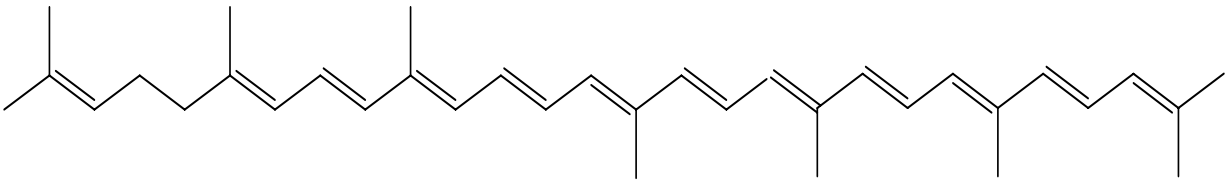
[13] Arachidonic acid



[14] β-Carotene



[15] α-Carotene



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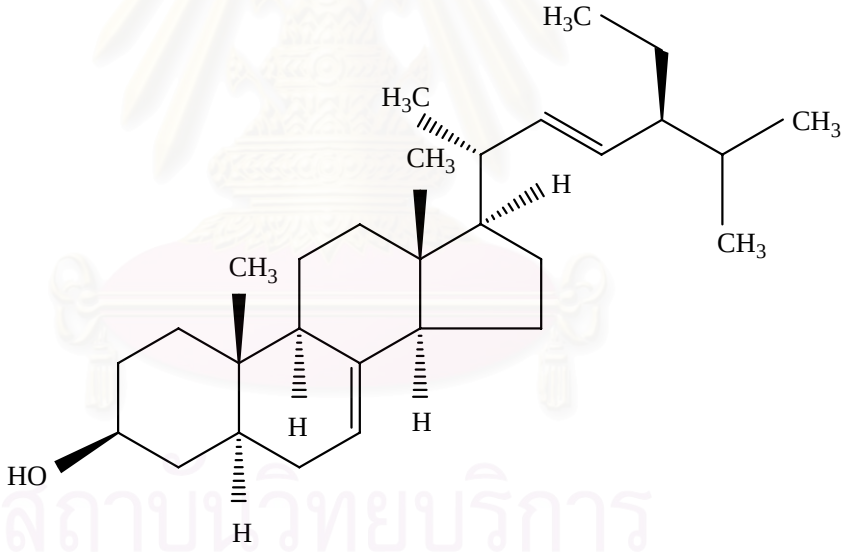
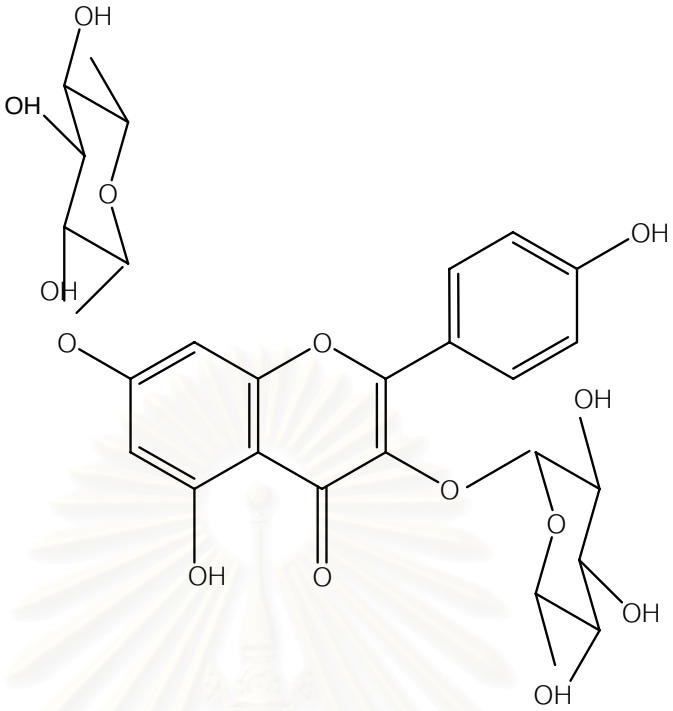
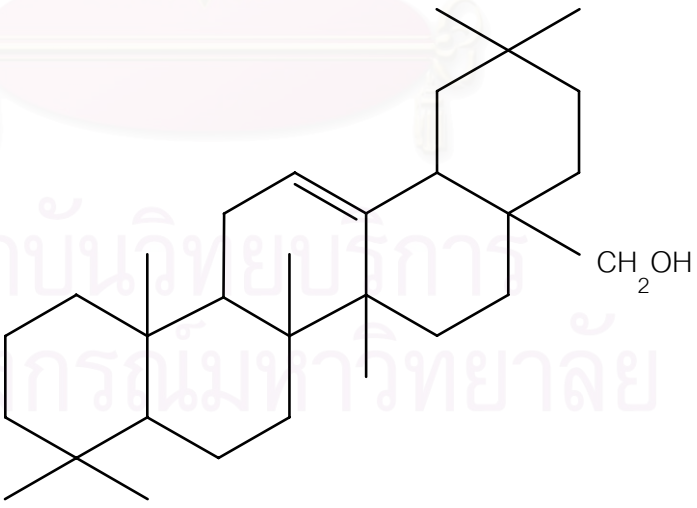


[17] α-spinasterol



[18] Kaempferitrin



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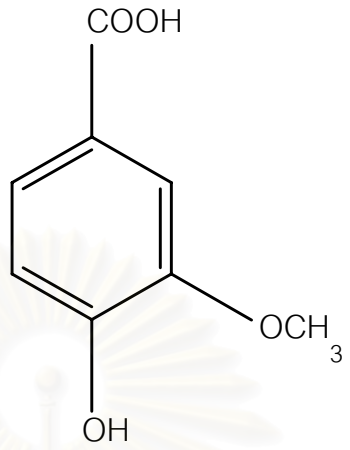
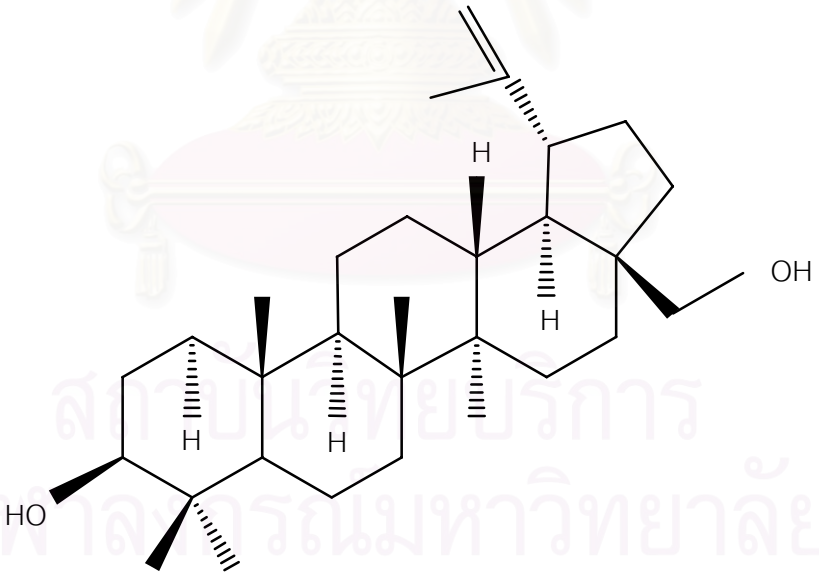


[20] Vanillic acid



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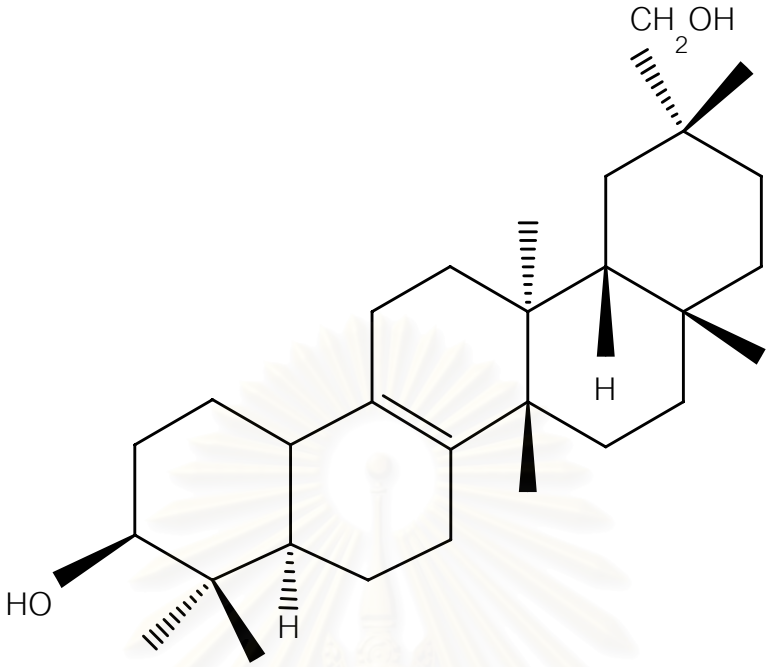
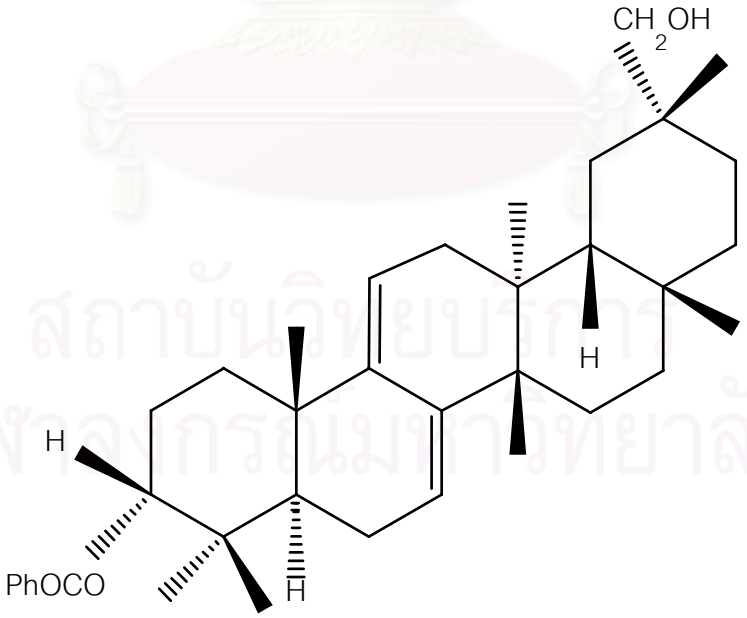
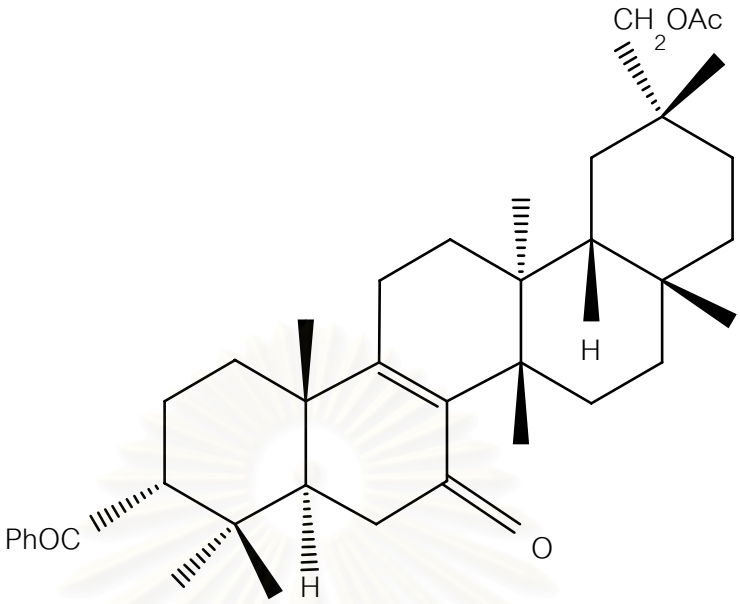


[22] Bryonolol

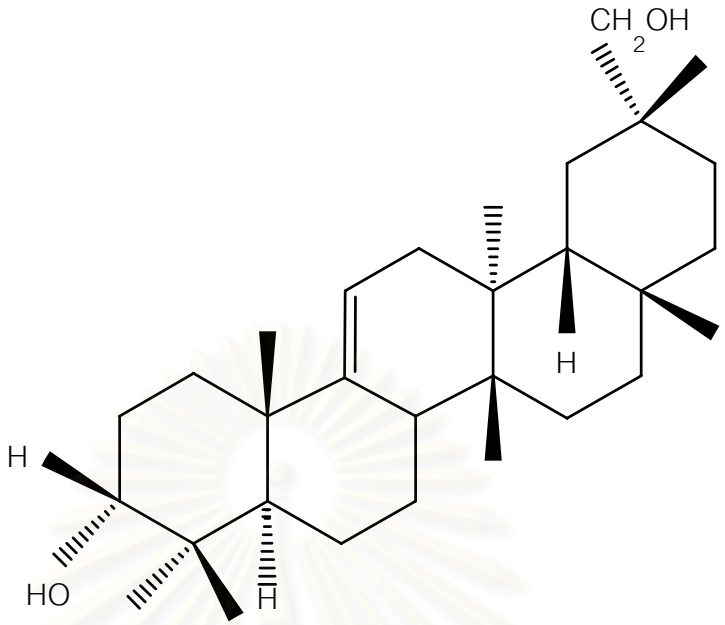


[23] Karounidiol-3-benzoate

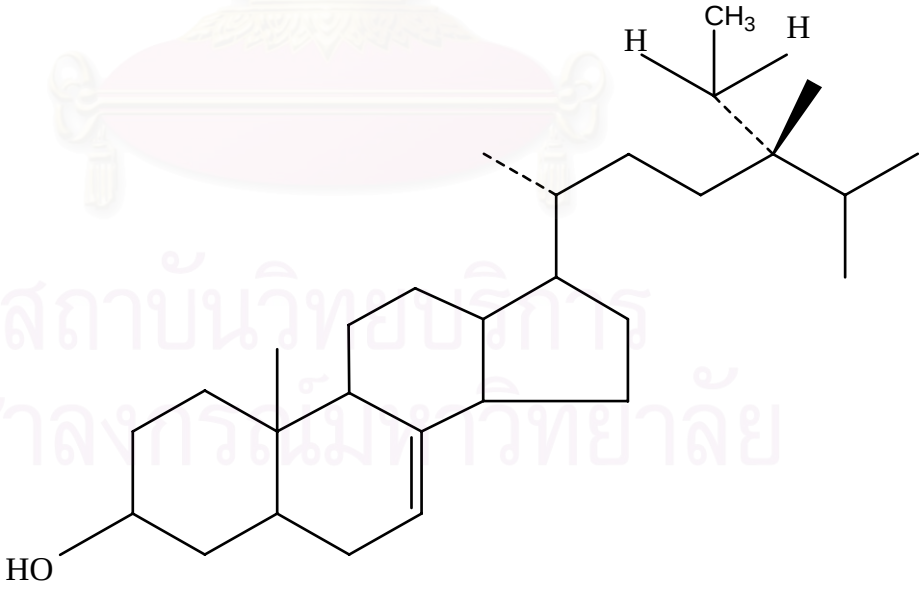


[24] 7-Oxodihydrokarounidiol

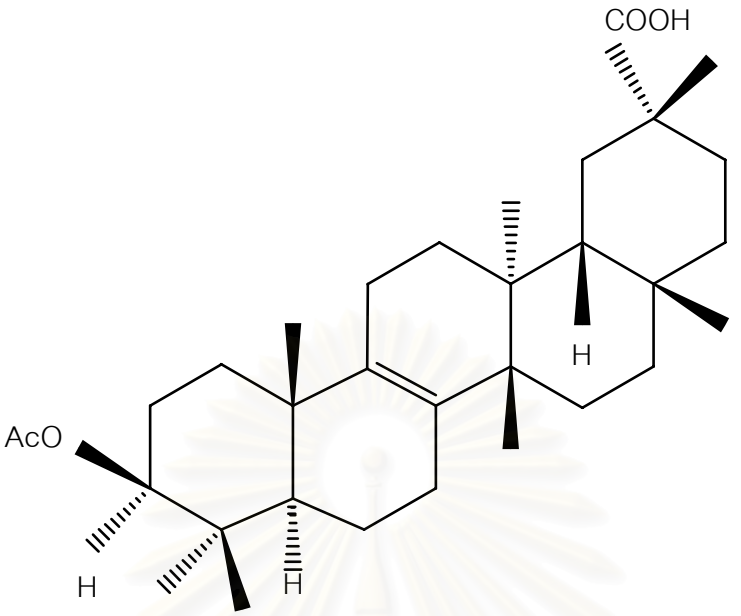
[25] n-Hentriacontane



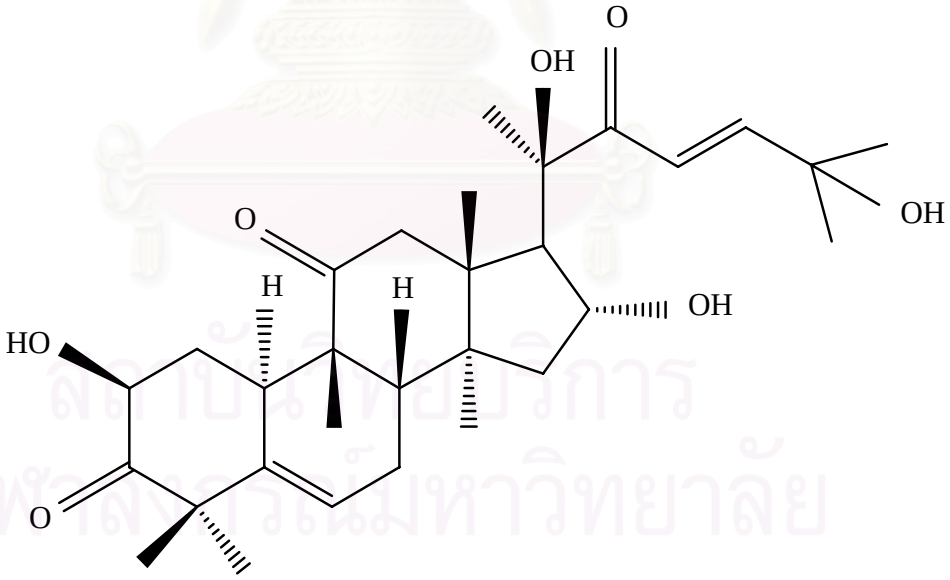
[26] Karounidiol



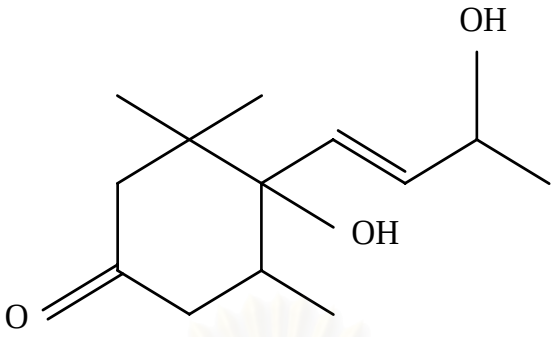
[27] 22-Dihydrospinasterol



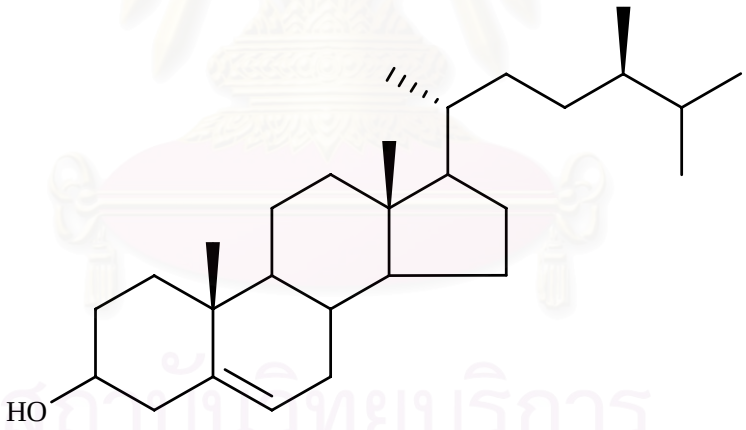
[28] Bryonolic acid



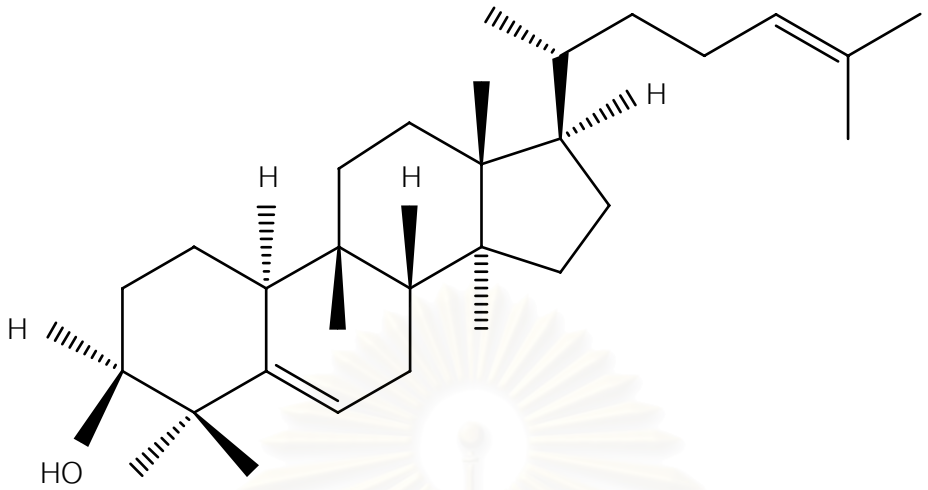
[29] Cucurbitacin D



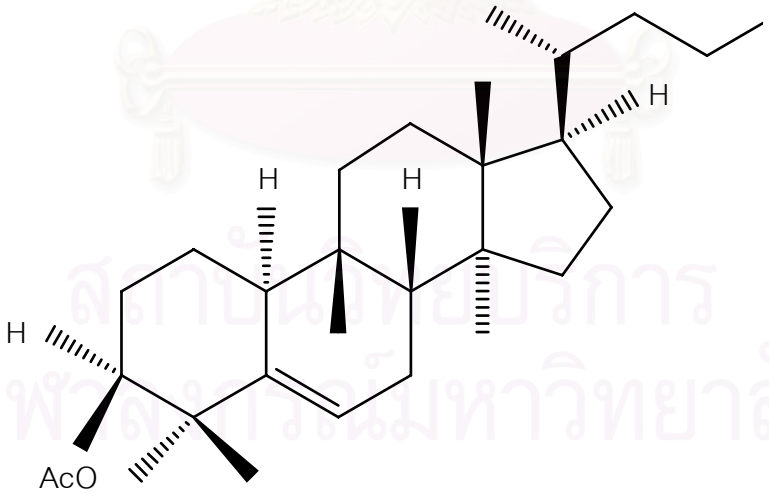
[30] Vomifoliol



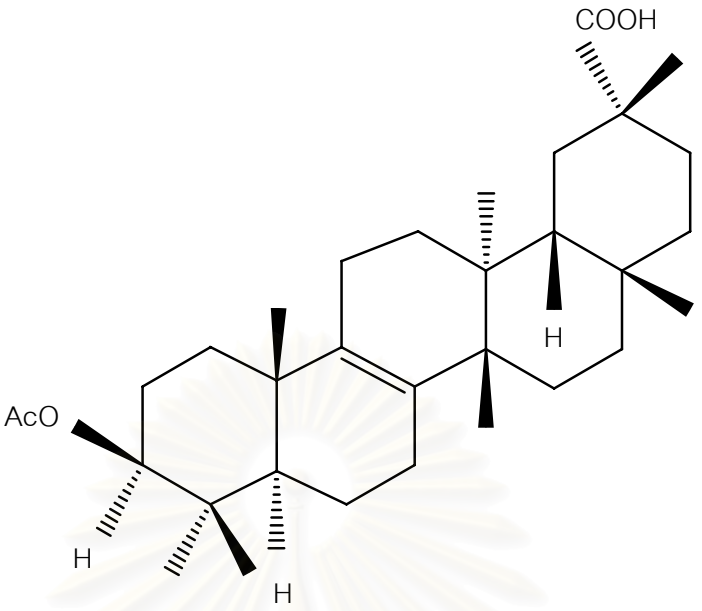
[31] Campesterol



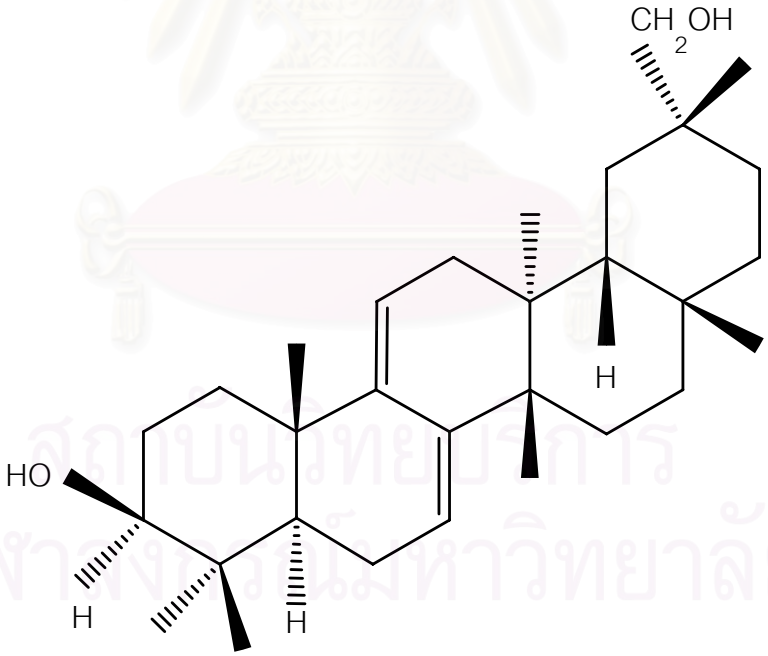
[32] 10α-Cucurbitadienol



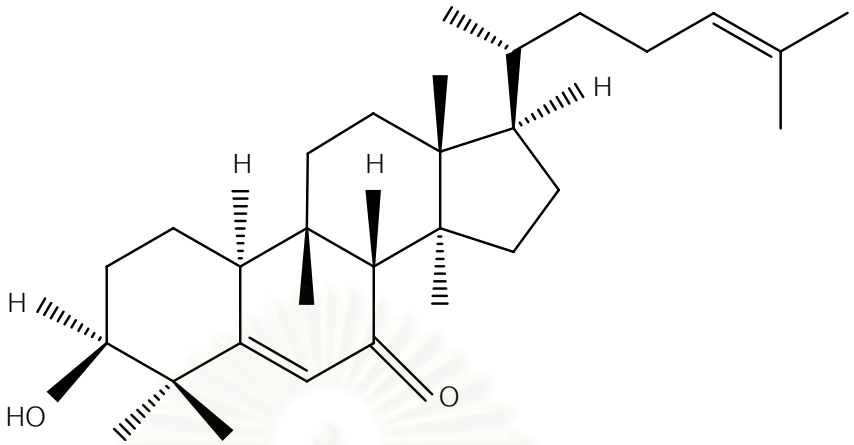
[33] 10α-Cucurbitadienol acetate



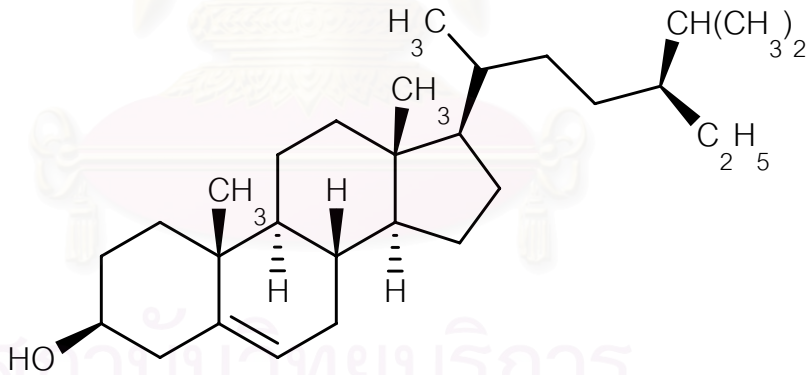
[34] 3-Epibrynolol



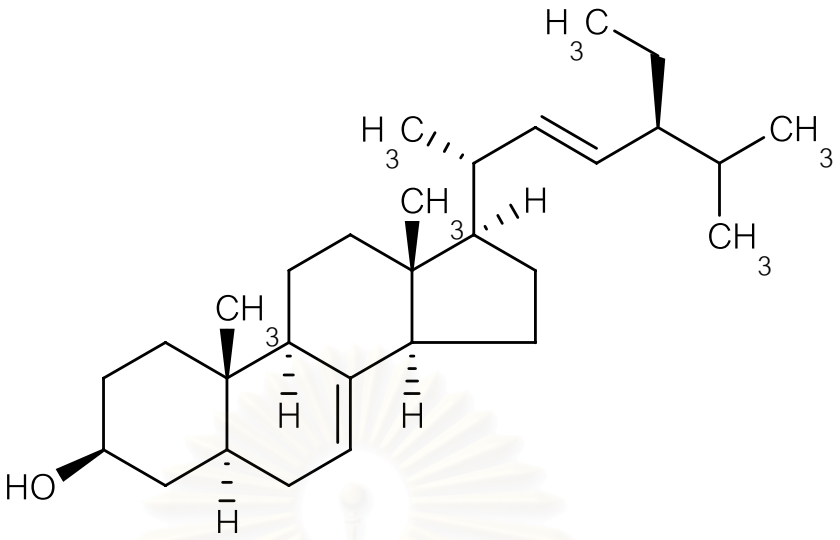
[35] 3-Epikarounidiol



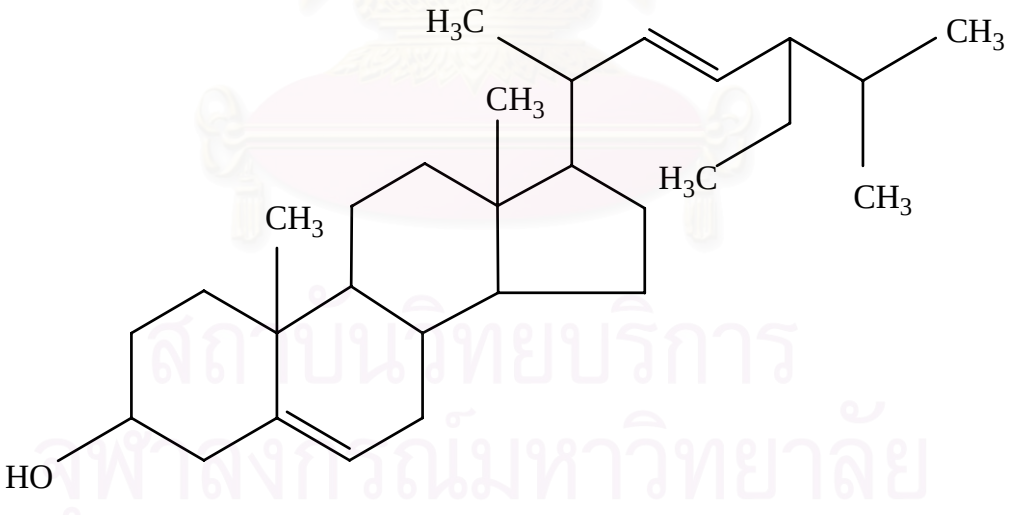
[36] 7-Oxo-10α-cucurbitadienol



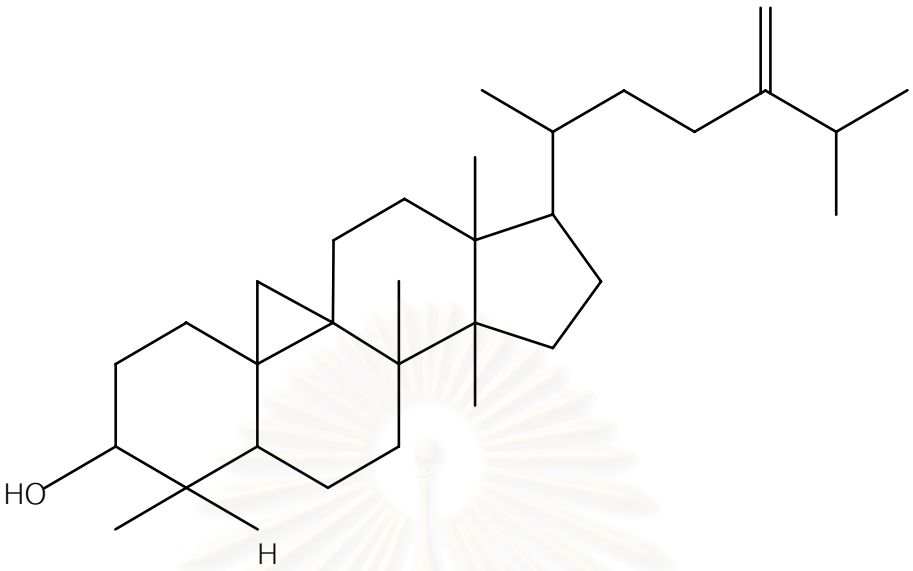
[37] Sitosterin



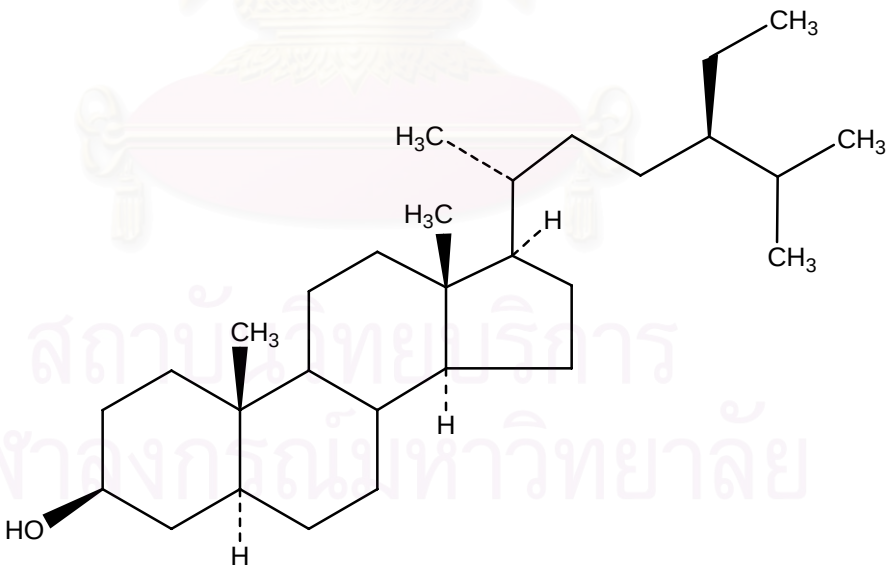
[38] Spinosterol



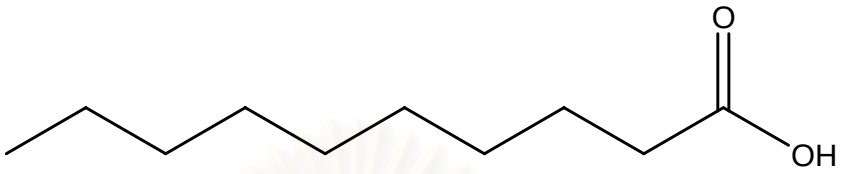
[39] Stigmasterol



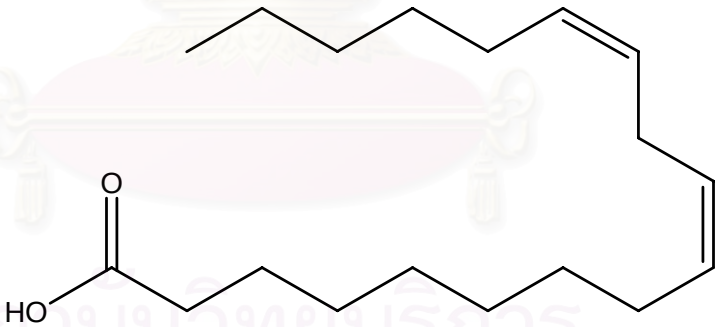
[40] Cycloeucalenol



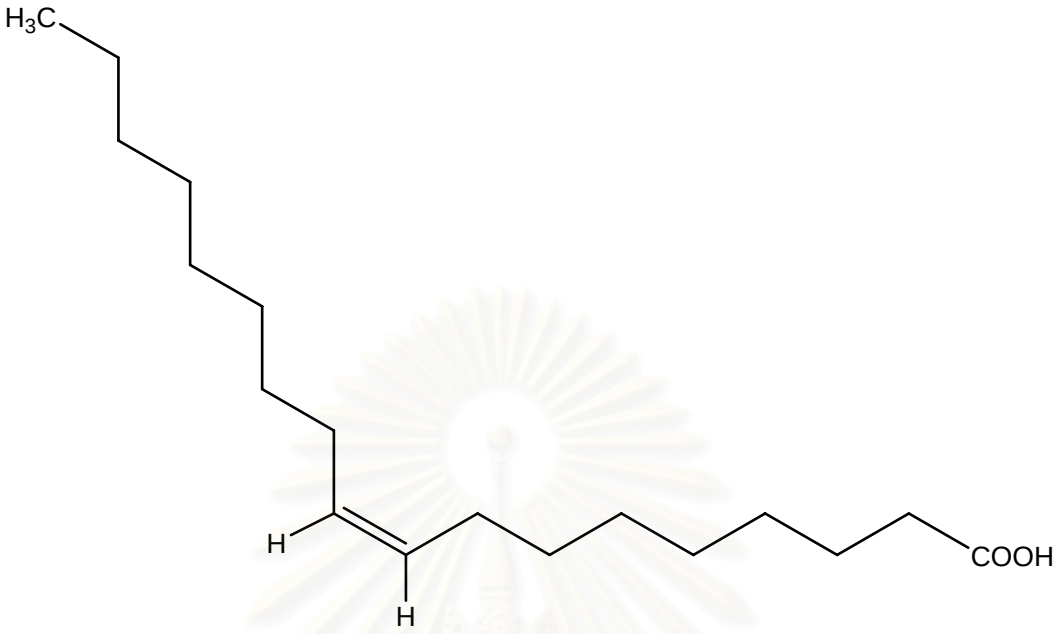
[41] Stigmastanol



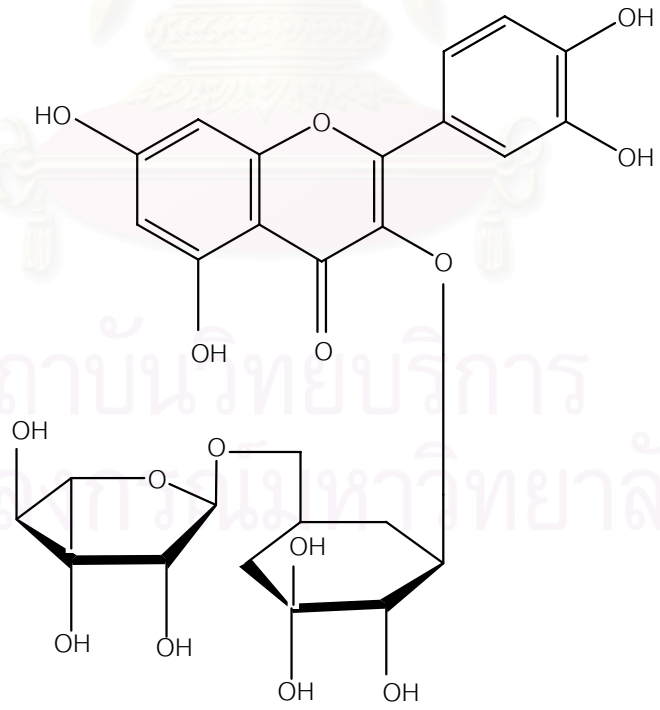
[42] Capric acid



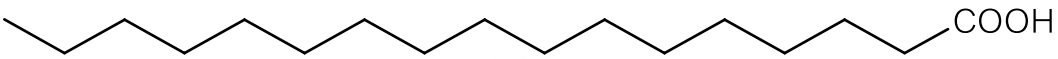
[43] Linoleic acid



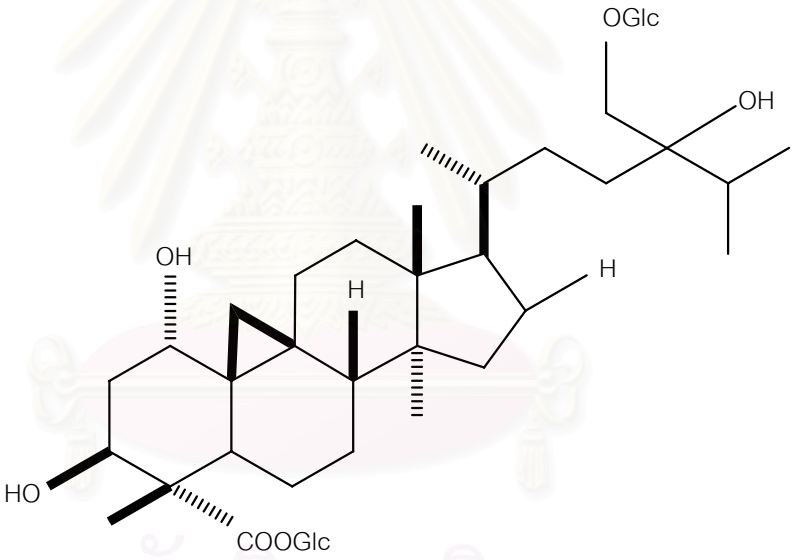
[44] Oleic acid



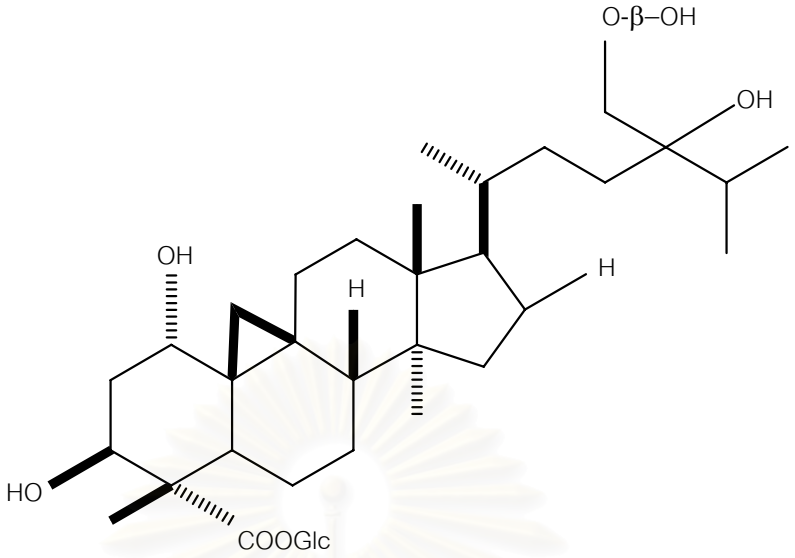
[45] Rutin



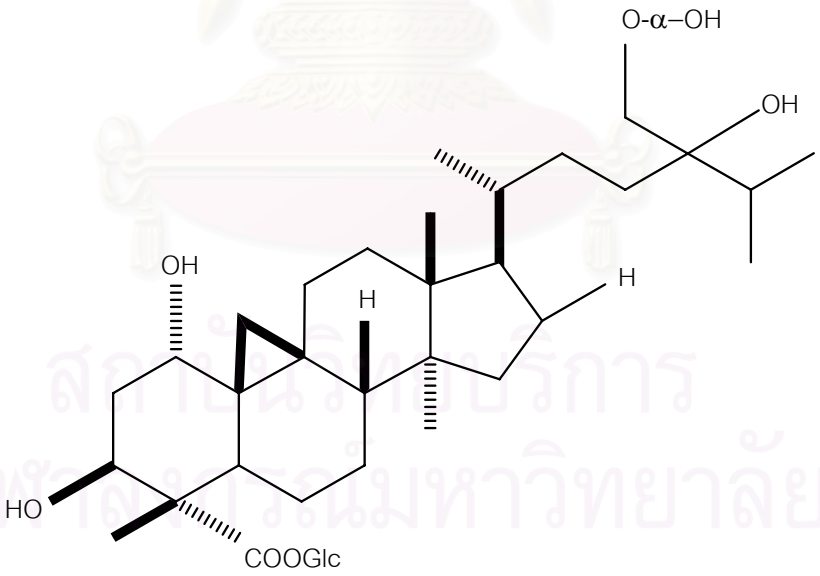
[46] Hexacosanoic Acid



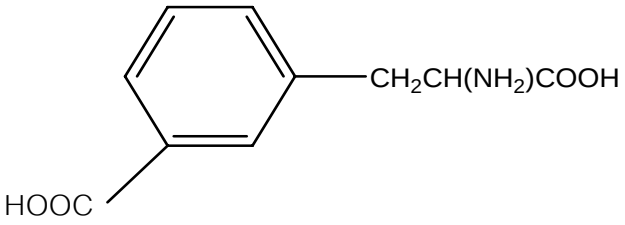
[47] Cyclotricuspidoside A



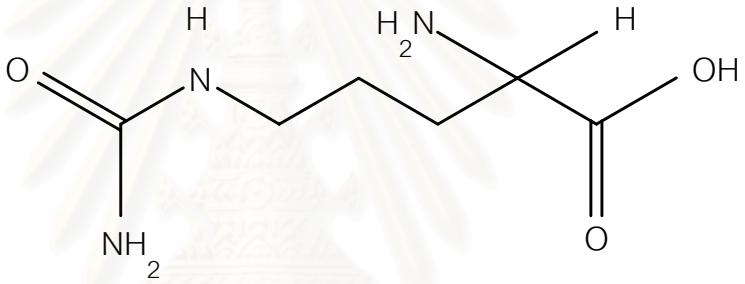
[48] Cyclotricuspidoside B



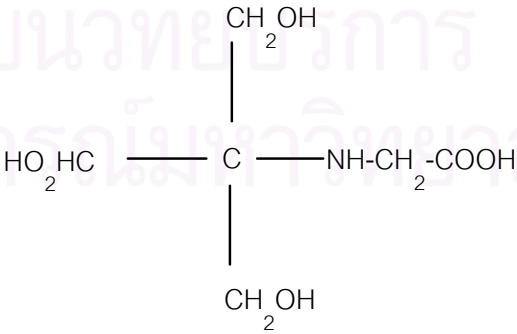
[49] Cyclotricuspidoside C



[50] *m*-carboxyphenyl alanine



[51] Citrullin



[52] Tricin

3. *Pomacea canaliculata* Lamarck

3.1 Taxonomy of the golden apple snails

Keawjam (Kaewjam, 1986) reported that South African apple snail, *Pomacea canaliculata* Lamarck has an appearance like an apple. They are commonly known as golden apple snail because of their large size and globosely shape. The classification of the golden apple snails as:

Phyllum : Mollusca
Class : Gastropoda
Subclass : Prosobranchia
Order : Mesogastropoda
Family : Ampullariidae
Subfamily : Viviparoidea
Genus : *Pomacea*

There are three species of *Pomacea* that devastated the rice fields in Thailand as follow, *P. canaliculata* Lamarck, *P. insularus* and *Pomacea* sp. (Kaewjam, 1990;Chanyapate and Archwacom, 1997).

3.2 General characteristics of the golden apple snails

These snails have a dextral shell (shell opening at the right) in Figure 2.4. The shell color varies from yellow-brown, brown to greenish brown, with or without dark spiral bands. The surface of the shell can be smooth or rough, depending on the environment. When snails grow, the shell has to be enlarging to fit the snail's body. To accomplish that, a snail gradually extents its shell by adding new parts at the shell opening. Shell opening is large, oval to round and have operculum (shell door), corneous plate and moderately thick that is able to close off its shell to survive and as protection against predators (Chanyapate and Archwacom, 1997; ศักดา, 2543).

The muscular foot is used for locomotion and consisted of several muscular layers. There are two types of walking in apple snails: with creeping locomotion and

with small wave locomotion. The creeping locomotion is consisted of elongation-shortening movement of foot (Ghesquiere, 2000) in Figure 2.5.

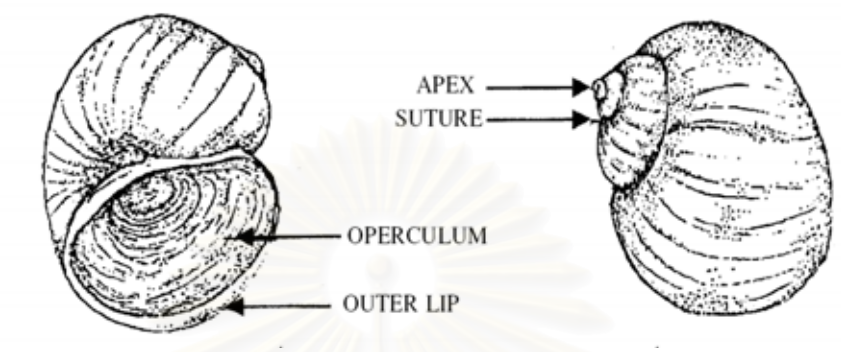


Figure 2.4 The dextral shell of golden apple snail. [Online] Available from: http://applesnail.net

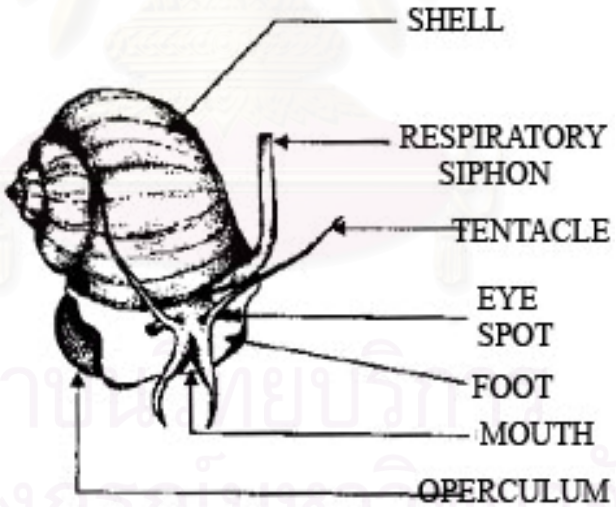


Figure 2.5 General characteristics of the golden apple snail. [Online] Available from: http://applesnail.net

3.3 Life cycle

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

3.3.1 Egg 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 eggs masses gradually lighten and hatching commences. Hatch ability was highly variable, ranking from 7 to 90%.

3.3.2 Pre-adult stage

The newly hatched neonate snail drops into the water and soon starts moving about, feeding on algae and detritus aggregates. The abundance of juvenile snail is negatively corrected with the density of adult snails. When grown to shell heights of about 1.5 cm juveniles start consuming plant material.

3.3.3 Adult stage

The golden apple snail has separate sexes, which can be morphologically distinguished by curve of the operculum. Under favorable conditions, females reach maturity 60-85 days after hatching and may spawn at weekly intervals throughout the year. [Online] Available from: http://www.applesnail.net

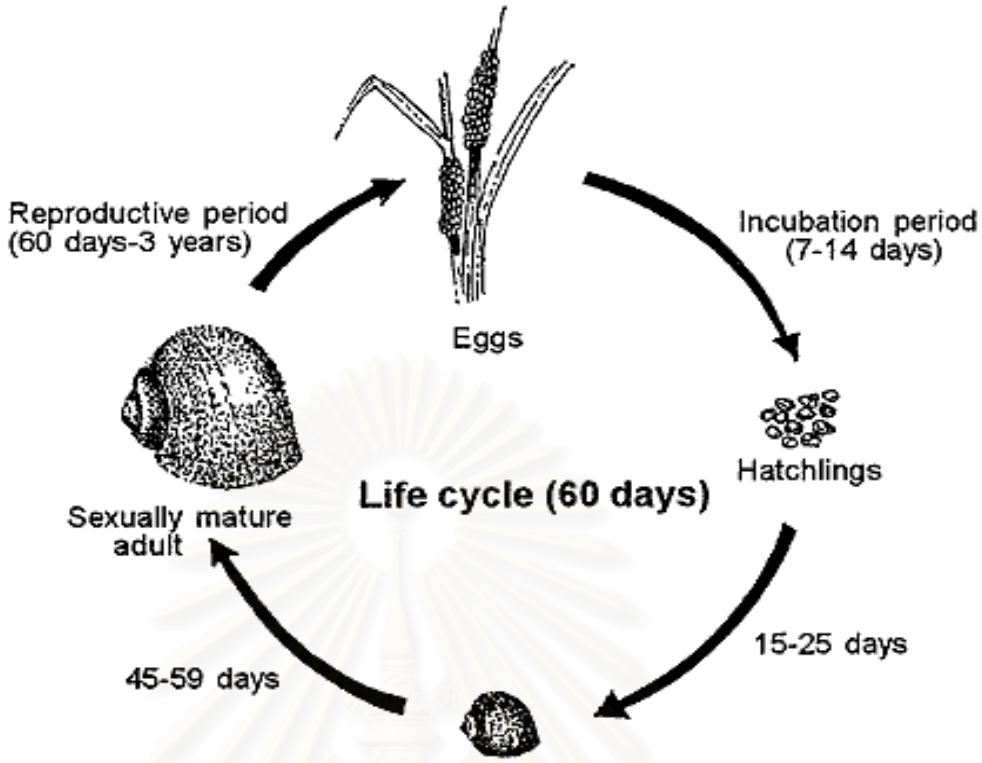


Figure 2.6 Life cycle of apple golden snail. [Online] available from: http://applesnail.net

3.4 Cytotoxicity

3.4.1 Definition

The natural Cancer Institute of the United States of America classified the compounds into three types. These are following

Cytotoxic agents as toxic agents to tumor cells in culture *(in vitro)*.

Antitumor or antineoplastic agents as agent that have *in vivo* activity in experimental systems.

Anticancer agents as agents reported in clinical trials.

In order to avoid false reports and the false hopes of cancer patients who heard of “anticancer” agents which were only in early experimental stages of development (Suffnes and Douros, 1982).

3.4.2 Cytotoxicity test

The method of investigation of cytotoxic agents from natural substances, which were the investigation *in vitro*. The method can be classified by determination of the cell killing effect into five methods, as follows

3.4.2.1 Viable cell count (Trypan-blue exclusion method)

A cell population in variably contains some dead or degenerated cells. For experimental work, living or viable cells are to be used; therefore a viable cell count is necessary. In living cells the cells membrane is active and it permits the selective intake of substances inside the cell. Use is made of this property to differentiate between viable and non-viable cells by subjecting these to contain dyes, e.g. trypan blue. This stain diffuses freely in a dead cell, as opposed to a living cell which does not permit the entry of this dye.

Consequently, living cells are unstained and non-viable cells take up the stain. Viable cells can therefore be count under the microscope (W.H.O., 1973).

3.4.2.2 Protein determination

The measurement of the cell growth by the determination of cell protein concentration is a rapid and reliable method which can be employed in large-scale testing. This method is a modification of the method for measuring protein using a phenol reagent. The amount of cell growth can be determined with cultures grown in suspension and also with culture adhering to a surface. The case of determination of the initial protein concentration and of the subsequent concentration during the course of the experiment makes this method very attractive. With appropriate conversion factors the values may be changed to dry weight, protein, nitrogen or cell count. This method has found great utility for monitoring the dynamics of cell population during the logarithmic phase of growth (Holden, Litchter and Sigel, 1973).

3.4.2.3 Release ^{51}Cr as a measure of cell death

This method utilized the release of ^{51}Cr to measure cell death. After the target cells are labeled, the isotope is slowly released (spontaneously) over a rapid of 24 to 48

h; however, if the cells are injured or killed, the isotope is released very quickly. Quantitation of the amount of isotope release by the culture gives a measurement of the percentage of cells that were killed or died during a prescribed time interval. Excellent correlation has been shown with other techniques (e.g. dye exclusion) which measure cell death due to virus infection, to detect cytotoxic lymphocytes, and to detect and quantitate cytotoxic antibodies (Holden, Litchter and Sigel, 1973).

3.4.2.4 Human tumor stem cell (clonogenic) assays

In this procedure, single-cell suspension is exposed to drugs for 1 h prior to seeding the cells in an agar overlay. The use of 1 h exposure period allows the selection of test levels that approximate the pharmacokinetic condition *in vivo*, where most drugs have short half-lives and are rapidly cleared from the plasma. However, for most experimental agents pharmacokinetic information are not available and alternative continuous exposure method using standard test concentrations would be more convenient. In vitro sensitivity to each concentration of test compound is calculated as average percentage of survival of drug-exposed colonies (x colonies treated) divided by the average number of colonies in the control (x colonies control). An arbitrary end point defining an acceptable level of activity may be established from log dose-survival curves as the concentration of drug effecting 50% survival. The arbitrary selection of a minimum-sized colony for inclusion in the surviving colony count may not exclude small abortive colonies of cells whose continued growth may have been interrupted by slow-acting cytotoxic compounds. The incorporation of an appropriate drug standard at concentrations spanning the entire range of activity would be useful in identifying artifactual plateaus in the dose-response curves, and defining the limits of assay sensitivity in terms related to minimum colony size (Adorjan, 1986).

3.4.2.5 Agar diffusion disk assay

The inhibition of dye-reducing activity of mammalian cells suspended in agar has been applied to the preliminary screening of cytotoxic substances by a number of investigators. All these methods rely on the inhibition of dehydrogenase activity of cells

visually detected by the lack of reduction of a redox indicator. The assay method that follows was developed for use with suspension-growth culture of P388 and L1210 cells and differed from other diffusion assays in one or more aspects, such as cell lines, nutrient media, incubation conditions, or reagent. The results produced more accurate and better defined zones of activity. Although this method has not been described in detail previously, its use as a prescreen for the detection of antitumor in fermentation products has been reported. This method has been proven to be particularly useful in the isolation and purification of cytotoxicity substances from the mixture of unidentified antimicrobial products. Both agar disks saturated with chemical extracts and thin-layer chromatograms (TLC) may be utilized in this assay system. Although inhibition assays are more sensitive, dehydrogenase inhibition in agar plate has distinct advantage, namely, ease of operation, rapidity, small amount of the test substances required, and the suitability for bioautography (Adorjan, 1986).

4. Literature review

4.1 Literature review of molluscicidal activity

Adewunmi and Sofowora (Adewunmi and Sofowora, 1980) reported that 181 plant extracts employed in Nigerian herbal medicine were screened for molluscicidal activity to *Bulinus globosus*. Only 23 (12.7%) of these plant extracts showed 100% mortality to the snails at a concentration of 100 ppm of extract. Futhermore, Adewunmi *et al* (Adewunmi, Oguntimein and Furu, 1990) studied the major constituents of *Zingiber officinale* responsible for its molluscicidal activity and the effect of the active component on different stages of *Schistosoma mansoni*. Gingerol and shogaol exhibited potent molluscicidal activity on *Biomphalaria glabrata.* Gingerol (5 ppm) completely abolished the infectively of *S. mansoni* miracidia and cercariae in *B. glabrata* in mice. This report also indicated that the gingerol is capable of interrupting *Schsitosoma* transmission at a concentration lower than its molluscicidal concentrations.

Maini and Morallo (Maini and Morallo, 1992) reported seventeen volatile oils were evaluated for their biotoxicity to golden apple snails at 1-100 ppm. Oils of sassafras, star anise and oregano showed 100% snail mortality at 10 and 20 ppm

against young snails with operculum diameter of 6 mm and mixed populations with 6-18 mm operculum diameter, respectively. In addition, Maini and Morallo (Maini and Morallo, 1993) reported, the aqueous extract of *Derris elliptica* (Lotin) root was toxic to golden apple snail at 2,000 ppm. While the stem gave only 30% mortality at 1,000 ppm. Small-scale field experiments showed Derris root to be effective in snail control at the rate at 90 kg/Hectares either by spraying or broadcasting.

Bergeron *et al* (Bergeron *et al*, 1996) studied the molluscicidal activity of nineteen plant species from fourteen families in the traditional medicine of North America Indians. And it was found that the eleven plant species had molluscicidal activity to *Biomphalaria glabrata.* Clark and Appleton (Clark and Appleton, 1997) studied the activity of crude aqueous suspensions prepared of leaf of six South African plants. The results suggested that three plants namely *Gardenia thunbergia, Apodytes dimidiate* and *Warburgia salutaris* had high potential in *Bulinus africanus* killing activity and receive priority for further studies should be investigated for their toxicology and stability.

Somkasettrin (Somkasettrin, 1996) studied efficacy of neem extracts on golden apple snails. Efficacy test of neem extracts on golden apple snail was studied under laboratory conditions. The study showed that the concentration of 3 ppm caused 100% mortality to small size (20-30 mm operculum diameter) at 48 hours while the medium size snails (30-40 mm operculum diameter) and large size snails (50-60 mm operculum diameter) reached 100% mortality at 72 hours. Field test result showed that 6 ppm concentration has highest efficacy with average mortality of 79.33% at 72 hours. The behavior of the snail after received the neem extract was produced copious and was unable to close the operculum. Many plant extracts were used as pesticide.

Lauhachinda *et al* (Lauhachinda *et al*., 1999) reported, neem seed extract to control the snail directly revealed that the concentration at 2-3 ppm caused the tested animals to die 73-100% at 72 hours after application. Field test of neem seed extract on various sizes of snails demonstrated that concentration at 6 ppm can cause 70-80% of all sized snails to die at 72 hours after application.

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.

Molgarrd (Molgarrd *et al*., 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 hours). The saponins were completely consumed within 10 days which indicates their abilities to degrade in aquatic environments under aerobic conditions.

Aspers (Aspers *et al*., 2001) tested 10 saponins isolated from 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 is responsible for a large part of the activity of the mixture. This saponin had been LC_{50} values of 0.05 mg/mL in its isolated form.

Santos and Sant Ana (Santos and Sant Ana, 2001) studied the ethanolic extracts from different parts of six species namely *Annona glabra, A. muricata, A. pisonis, A. crassiflora, A. salzmani* and *A. squamosa* of the Annonaceae family against adult forms and egg masses of *Biomphalaria glabrata*. Results from accurate experiments indicate that the majority of analyzed extracts possess properties lethal to *B. glabrata* some of them with significant LD_{50} values (<20 ppm), as showed for *A. crassiflora* [pulp and seed (LD_{50} = 13.1 ppm), stem (LD_{50} = 2.34 ppm), root bark (LD_{50} = 3.79 ppm)].

Derris root and tea seed cake were extracted by distilled water at 12 h of golden apple snail maceration for 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 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).

Moungnoi (Moungnoi, 2002) studied the toxicity of indigenous plant extracts to golden apple snail. The result showed that strong activity was observed in aqueous extracts of *Bougainvillea spectabilis, Calotropis gigantea* and *Croton tiglium* and many types of plant in ethanolic extract. When the toxicity of aqueous and ethanolic extract of each plants was compared, it was found that the ethanolic extract of some plants (for examples; *Alpinia galanga* and *Calotropis gigantea*) were significantly higher than aqueous extract while the aqueous extract of *Mimusops elengi* had more toxicity than the ethanolic extract.

Kitprayoon (Kitprayoon, 2005) studied the toxicity of tea seed extract from *Camellia oleifera* Abel. against golden apple snail (*P. canaliculata* Lamarck) LC_{50} showed values at 6.79 ppm after 24 h.

Triterpenoid saponins isolated from *Sapindus mukorossi* Garetn. (Sapindaceae) had molluscicidal effects against the golden apple snail, *P. canaliculata* Lamarck, 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

1. Source of Plant Material

The fruits of *Trichosanthes cucumerina* L. were collected from Pichit, Thailand in November 2005. The plant material was authenticated by comparison with the voucher specimen no. BKF 70279, at the Royal Forest Department, Bangkok, Thailand.

2. Tested animals

Golden apple snails (*Pomacea canaliculata* Lamarck) with 2.0-2.5 cm operculum diameters (3.5-5.0 cm shell length) and average weight of about 12-15 grams were used for molluscicidal activity test. The snails were collected from Amphoe Wang Noi, Ayuthaya Province, Thailand, in 2005-2006.

Figure 3.1 The golden apple snails (*Pomacea canaliculata* Lamarck) with 2.0-2.5 cm operculum diameter were used for molluscicidal activity test.

3. General techniques

3.1 Chromatography

3.1.1 Analytical Thin-layer Chromatography (TLC)

Technique : One dimension, ascending

Adsorbent : Silica gel 60 GF_{254} precoated plate (E. Marck)

Layer thickness : 0.25 mm

Developing distance : 5 cm

Temperature : Laboratory room temperature (30-35°C)

Detection : 1. Ultraviolet light at wavelengths of 254 and 365 nm

2. Anisaldehyde-H_2SO_4 and heated at 100-105 °C for a few minutes

3.1.2 Column Chromatography

3.1.2.1 Conventional Column Chromatography

Adsorbent : Silica gel 60 (No.7734) (E. Merck)
particle size 0.063-0.200 nm (70-230 mesh ASTM)

Packing method : Wet packing

Sample loading : The sample was dissolved in a small amount of eluant, then applied gently on top of the column.

Examination of eluate : Fractions were examined using TLC observing under UV light at wavelength of 254 and 365 nm. The TLC plate was then detected by exposing to anisaldehyde-H_2SO_4 reagent and heating respectively. Fractions of similar chromatographic pattern were combined.

3.1.2.2 Quick Column Chromatography

Adsorbent : Diaion HP-20 (Mitsubishi Chemical Co., Tokyo, Japan)

Packing method : Wet packing

Sample loading : The sample was dissolved in a small amount of eluant, then applied gently on top of the column.

Examination of eluate : Fractions were examined using TLC observing under UV light at wavelength of 254 and 365 nm. The TLC plate was then detected by exposing to anisaldehyde-H_2SO_4 reagent and heating. Fractions of similar chromatographic pattern were combined.

3.2 Spectroscopy

3.2.1 Ultraviolet (UV) Absorption Spectra

UV (in MeOH) Spectra were obtained on a Shimadzu UV-160A UV/vis spectrophotometer (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

3.2.2 Infrared (IR) Absorption Spectra

FT-IR Spectra were recorded on a Nicolet Impact 410 Spectrometer. Spectra of solid samples were recorded as KBr pellets at The Scientific and Technological research Equipment Center, Chulalongkorn University.

3.2.3 Proton and Carbon Nuclear Magnetic Resonance (^{1}H and ^{13}C NMR) Spectra

^{1}H- NMR (300 MHz) and ^{13}C-NMR (75 MHz) spectra were obtained with a Bruker Advance DPX-300 FT-NMR spectrometer (Faculty of Pharmaceutical Sciences, Chulalongkorn University).

3.2.4 Mass Spectra (MS)

High Resolution mass spectra were recorded on the Time-of-Flight (TOF) mode. The TOF-MS technique was used to study the molecular weight of this compound. Mass spectra were obtained with a Bruker Daltonics MicrOTOF time-of-flight mass (National Science and Technology Development Agency).

4. Physical Properties

4.1 Melting point

Melting points were obtained on a Buchi Melting point B-545 Apparatus (Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

4.2 Optical rotation

Optical rotation was measured on a Perkin-Elmer Polarimeter model 341 (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

5. Chemicals

5.1 Solvent for column chromatography

All solvents used for column chromatography such as methanol, hexane, ethyl acetate, dichloromethane were commercial grade and redistilled prior to use.

5.2 Chemical test

p-Anisaldehyde reagent consists of 15 ml methanol, 2 ml acetic acid, 10 ml sulfuric acid and 0.2 ml *p*-anisaldehyde. The reagent was sprayed to the spots on TLC plate and the plate was heated for detection of the spots of steroids or triterpenoid compounds. If the spots change to dark blue or greenish blue spots appear, the sample may contain steroid or triterpenoid compounds.

5.3 Other chemicals

5.3.1 Niclosamide

Niclosamide 70% wettable powder (WP) (Bayerthai, Co.Th), Bionene (Cothtech, Co.Th), Metaldehyde 80% wettable powder (WP) (Sotus International, Co.Th) and CU1 (tea seed granule with 12% saponin) (Siriwat Product. Co.) were used as the positive control chemicals.

5.3.2 CU1 samples

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

6. Extraction

6.1 Methanol crude extract

The dried powdered whole fruits of *T. cucumerina* L. (6 kg) were extracted with methanol (20 L) by soxhlet apparatus. Methanol extract was filtered. The filtrate was evaporated under reduced pressure to give a green methanol crude extract sample.

6.2 Water crude extract

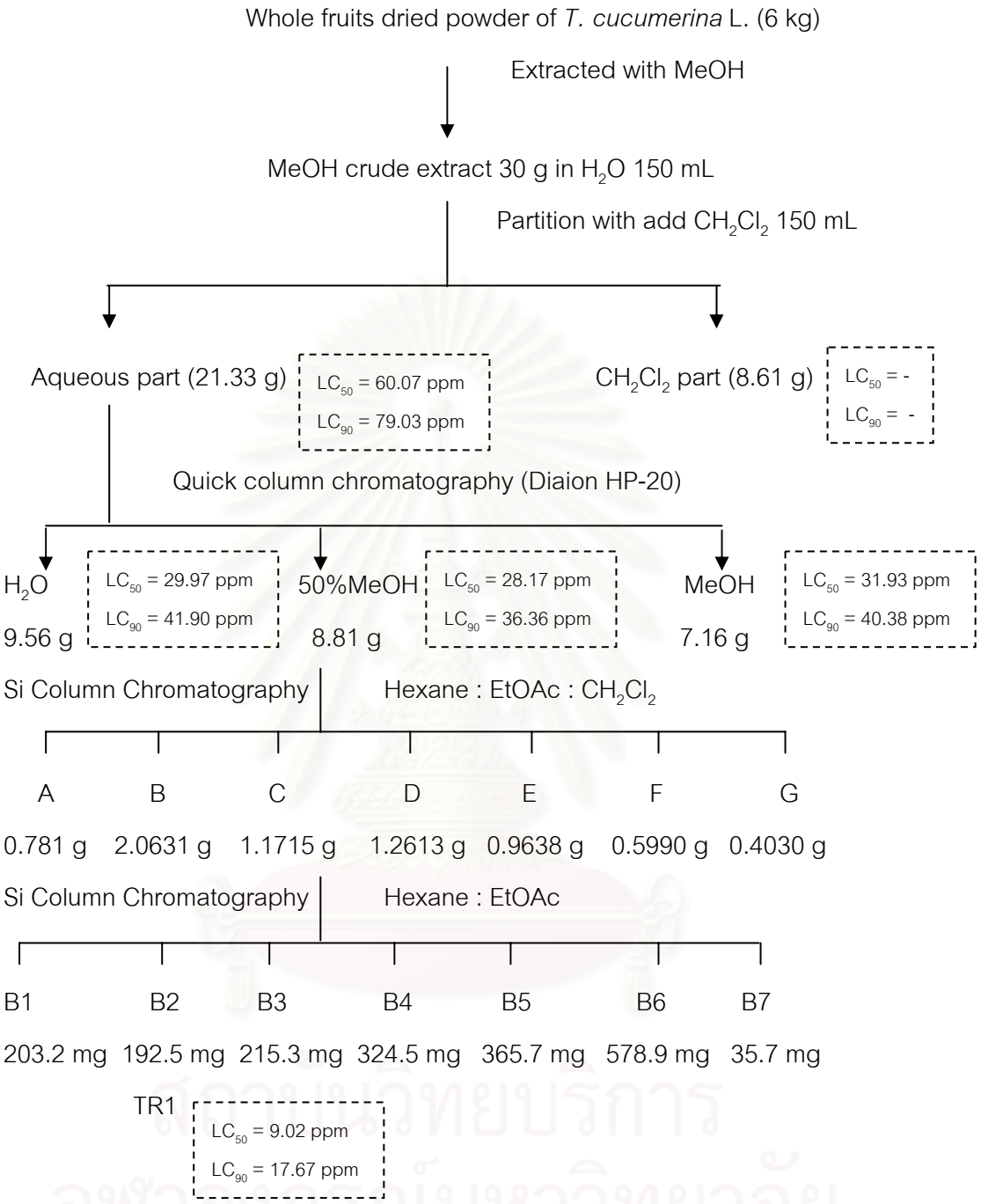
The dried powdered whole fruits of *T. cucumerina* L. (500 g) were extracted with water by decoction. The brown solution was filtered and evaporated on the water bath to give water crude extract sample.

6.3 Extraction and isolation

The methanol crude extract sample from whole fruits of *T. cucumerina* L. (30 g) was suspended in 100 ml water and partitioned with CH_2Cl_2 (3 times) at room temperature. Each layer was evaporated under reduced pressure until dryness to give the dichloromethane extract and the aqueous extract as shown in scheme 3.1

6.4 Separation of the aqueous extract obtained from methanol crude extract

The aqueous extract, which was the most active fraction for molluscicidal activity testing, was obtained as brown powder after evaporation. Preliminary purification of the aqueous layer was made by using quick column chromatography over 7 x 4 cm the Diaion HP-20 porous polymer resin column. The column was eluted with water, 50% MeOH, MeOH, successively. Each fraction was analyzed by TLC and spot was detected with *p*-anisaldehyde solutions and molluscicidal activity testing. The result of preliminary purification of aqueous layer was showed in Scheme 3.1.



Scheme 3.1 Extraction and isolation of the molluscicidal substance from the whole fruits dried powder of *T. cucumerina* L.

According to molluscicidal activity guide, each partition-layer was subjected to molluscicidal testing. The result indicated that the aqueous part had the highest molluscicidal activity. The aqueous extract was separated in 3 fractions by Diaion HP-20 column chromatography.

The 50% methanol fraction was further purified by silica gel column chromatography, eluted with hexane and increased polarity by adding ethyl acetate as 30, 50, 70, 100% ethyl acetate, and then by adding dichloromethane 30, 50, 70, 100% dichloromethane. Each fraction was analyzed by thin layer chromatography and gave 7 fractions as shown in Table 3.1.

Table 3.1 The result of preliminary purification of the 50% methanol fraction of *T. cucumerina* L. fruits.

Group of compounds	Fraction number	Appearance	%w/w of aqueous fraction
A	1-4	Colorless powder	10.00
B	5-18	Colorless powder	33.33
C	19-33	Colorless powder	15.00
D	34-46	Colorless powder	16.15
E	47-60	Colorless powder	12.34
F	61-74	Yellowish powder	7.67
G	75-82	Yellowish powder	5.16

Fraction B was further purified by silica gel column chromatography, eluted with hexane and increasing polarity by adding ethyl acetate as 30, 50, 70 and 100% to give compound TR1.

7. Identification of compound TR1

Compound TR1 was obtained as colorless crystals, soluble in dichloromethane (192.5 mg).

$[\alpha]^{20}_{D}$	:	+19º (MeOH)
Melting point	:	165.6 ^{o}C
UV	:	λ_{max} nm (log ε), in MeOH; 228.6 nm (3); Figure A6
IR	:	ν_{max} cm^{-1}; 3462.66, 1718.32, 1693.61 and 1629.67; Figure A5
TOF MS	:	$[M+Na]^{+}$ *m/z* : 581.32 (100%), 499.42 (60), 481.42 (60); Figure A3-A4
^{1}H NMR	:	δ_{H} (ppm), 300 MHz, in $CDCl_3$; Table 4.1; Figure A1
^{13}C-NMR	:	δ_{C} (ppm), 75 MHz, in $CDCl_3$; Table 4.2; Figure A2

8. Evaluation of biological activity

8.1 Molluscicidal activity test

8.1.1 Preparation of tested snails

The snails used for this study were the golden apple snails (*P. canaliculata* Lamarck). For preparation of dechlorinated water for golden apple snails, tap water was left at room temperature at least 24 h before use. The golden apple 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 leaves. Before molluscicidal testing, the snails were selected by diameter of operculum at about 2.0-2.5 cm.

8.1.2 Molluscicidal activity

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 concentrations 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 and extracting solvent. The amount

of mortal snails of range finding test was recorded and these data were then used to establish a more 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 except that 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 respectative 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 detect their response and the number of dead snails was count after 24 h (Huang, 2003).

8.1.3 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 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.

Data on accumulated mortality of snails after 24 h were analyzed by Probit Analysis (Meeposom, 2004; Finney, 1925) that was done by SPSS computer software (Version 11.5). The 95% confidence intervals of the LC_{50} were determined as a measure of the toxicity. The details of Probit Analysis are shown in appendix C.

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

8.2 Cytotoxicity test (*in vitro*)

Cytotoxicity test was carried out at the Institute of Biotechnology and Genetic of Engineering. Bioassay cytotoxic activity against human tumor cell culture in vitro was performed by the MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetry method (Carmichael *et al.*, 1987; Twentyman and Luscombe, 1987). In principle, the viable cell number/well is directly proportional to the production of formazan which, following solubilization, can be measured spectrophotometrically.

The human tumor cell line was harvested from exponential - phase maintenance culture (T-75 cm^2 flask), counted by trypan blue exclusion, and dispensed within replicate 96-well culture plates in 100 μl volumes using a repeating pipette. Following a 24 hours incubation at 37°C 5%CO_2, 100% relative humidity and 100 μl of culture medium. Culture medium containing sample was dispensed within appropriate wells. Peripheral wells of each plate were utilized for sample blank and medium/tetrazolium reagent blank “background” determinations. Culture plates were then incubated for 4 days prior to the addition of tetrazolium reagent. MTT stock solution was prepared as follows: 5 mg MTT/ml PBS was steriled and filtered with 0.45 μm filtered units. MTT working solution was prepared just prior to culture application by diluting MTT stock solution 1:5 (v/v) in prewarmed standard culture medium. MTT working solution (50 μl) was added to each culture well resulting in 50 mg MTT/250 μl total medium volume) and culture were incubated at 37°C for 4-24 h depending upon individual cell line requirements. Following incubation, cell monolayer and formazan were inspected microscopically: culture plates containing suspension lines or any detached cells were centrifuged at low speed for 5 min. All 10-20 μl of culture medium supernatant was

removed from wells by slow aspiration through a blunt 18-guage needle and replaced with 150 μl of DMSO using a pipette. Following through formazan solubilization, the absorbance of each well was measured using a micro culture plate reader at 540 nm (single wavelength, calibration factor = 1.00).

Cell line growth and growth inhibition were expressed in terms of mean (±SD) absorbance units and/or percentage of control absorbance (±SD%) following subtraction of mean "background" absorbance.

Samples were also tested for cytotoxic activity towards 5 cancer cell lines, including SW620 (human colon adenocarcinoma), HEP-G2 (human liver hepatoblastoma), KATO-III (human gastric carcinoma), BT474 (human breast ductal carcinoma) and CHAGO (human undifferentiated lung carcinoma) following the experimental method for bioassay of cytotoxic activity.

CHAPTER IV

RESULTS AND DISCUSSION

According to the preliminary investigation the methanolic extract of *T. cucumerina* L. fruits exhibited high molluscicidal activity against *P. canaliculata* Lamarck.

The extract of *T. cucumerina* L. fruits was separated by chromatographic method to give one pure compound. The structure of the isolated compound was identified by spectroscopic data (UV, IR, NMR and MS) and comparison with the literature values.

1. Structure elucidation of compound TR1

Compound TR1 as colorless crystalline and purified by recrystallization in ethyl acetate gave white crystal (192.5 mg), has a melting point at 165.6°C, the specific rotation of +19° (*c* 1, methanol) and the hR_f value 20 with the solvent system of Hexane : EtOAc (2:1).

The UV spectrum of compound TR 1 has the maximum absorption at 228.6 nm (log ε = 3.00), which indicated the $\pi \rightarrow \pi^*$ transition of α,β-unsaturated ketone.

The TOF-MS technique was used to study the molecular weight of this compound. Appendix A3-A4 showed the TOF-MS spectrum of compound TR1 showed peak at 581.3 $[M+Na]^+$ which indicates that the molecular weight of TR 1 is 588.3.

From the comparison of the TOF MS spectrum of compound TR1 with literature review of cucurbitacin B in *T. cucumerina* L. (Jiratchariyakul and Frahm. 1992), it was found that the component with m/z at 558.3 had molecular formula $C_{32}H_{46}O_8$. Thus compound TR1 was identified as cucurbitacin B as shown in Figure 4.1.

The IR spectrum showed the vibration of OH-stretch at 3462.66 cm^{-1}, carbonyl absorption at 1718.32, 1693.61 and 1629.67 cm^{-1} and the secondary alcohol deformation vibration at 1126.61 cm^{-1}.

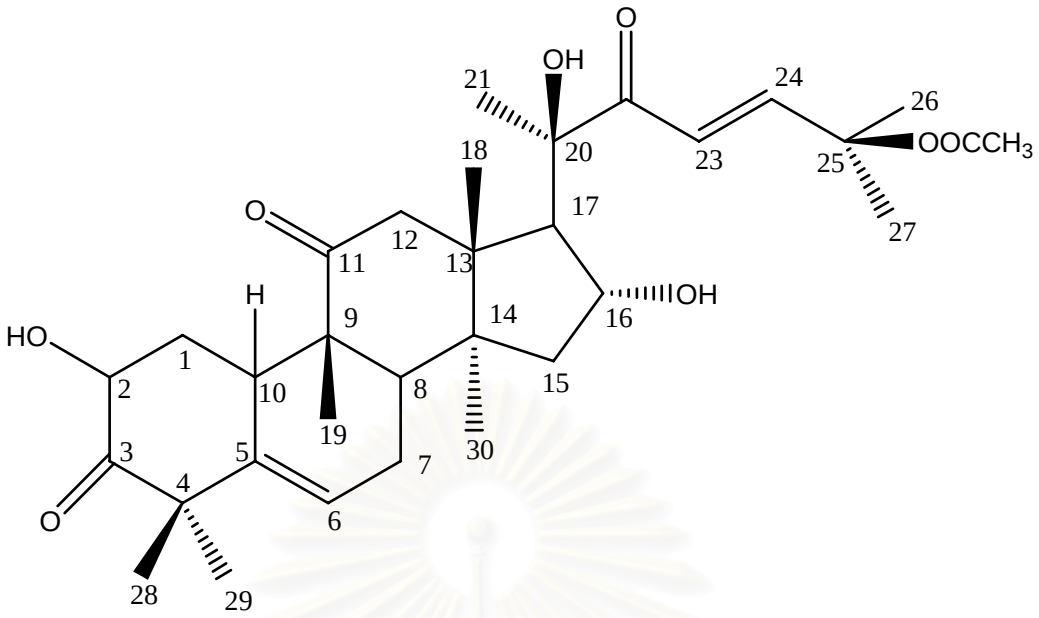


Figure 4.1 The structure of cucurbitacin B (M.W.558.3)

Table 4.1 300 MHz ^{1}H NMR data of compound TR1 in $CDCl_3$ as compared with cucurbitacin B (in $CDCl_2$)

H	δ_H (ppm)		H	δ_H (ppm)	
	TR1	Cucurbitacin B		TR1	Cucurbitacin B
18-CH_3	0.95 (s)	0.98 (s)	25-OAc	1.97 (s)	2.00 (s)
30-CH_3	1.04 (s)	1.07 (s)	1αeq	2.27 (ddd)	2.30 (ddd)
1βax	1.13 (ddd)	1.23 (ddd)	7αeq	2.38 (ddd)	2.40 (ddd)
29αax-CH_3	1.25 (s)	1.28 (s)	17α	2.47 (dddd)	2.48 (d)
28βeq-CH_3	1.28 (s)	1.34 (s)	12βax	2.65 (d)	2.65 (d)
19-CH_3	1.30 (s)	1.35 (s)	10α	2.72 (dddd)	2.75 (dddd)
15β	1.39 (dd)	1.40 (dd)	12αeq	3.10 (d)	3.19 (d)
21-CH_3	1.42 (s)	1.44 (s)	OH	3.25 (dd)	3.59 (dd)
26-CH_3	1.51 (s)	1.54 (s)	OH	4.10 (m)	4.25 (m)
27-CH_3	1.55 (s)	1.57 (s)	16	4.32 (ddd)	4.35 (ddd)
OH-	1.67 (m) OH	1.70 (m) OH	2	4.40 (ddd)	4.41 (ddd)
15α	1.81 (dd)	1.87 (dd)	6	5.72 (dddd)	5.77 (dddd)
8β	1.85 (ddd)	1.93 (ddd)	23	6.33 (d)	6.39 (d)
7βax	1.95 (dddd)	1.97 (dddd)	24	7.01 (d)	7.03 (d)

Table 4.2 75 MHz ^{13}C-NMR data of compound TR1 in $CDCl_3$ as compared with cucurbitacin B (in $CDCl_2$)

C	δ_H (ppm)		C	δ_H (ppm)	
	TR1	Cucurbitacin B		TR1	Cucurbitacin B
1	34.634 (t)	35.678 (t)	17	56.817 (d)	57.867 (d)
2	70.300 (d)	71.340 (d)	18	19.500 (q)	19.544 (q)
3	211.300 (s)	211.866 (s)	19	18.500 (q)	18.609 (q)
4	49.108 (s)	49.935 (s)	20	76.739 (s)	77.936 (s)
5	139.040 (s)	140.027 (s)	21	21.700 (q)	23.630 (q)
6	119.089 (d)	120.131 (d)	22	201.222 (s)	202.159 (s)
7	20.550 (t)	23.550 (t)	23	119.089 (d)	119.971 (d)
8	41.061 (d)	42.403 (d)	24	150.482 (t)	151.684 (t)
9	46.859 (s)	47.785 (s)	25	78.132 (s)	79.018 (s)
10	32.374 (d)	33.417 (d)	26	25.030 (q)	26.100 (q)
11	211.706 (s)	212.744 (s)	27	24.727 (q)	25.619 (q)
12	48.907 (t)	48.333 (t)	28	20.000 (q)	20.932 (q)
13	47.284 (s)	48.119 (s)	29	28.016 (q)	29.064 (q)
14	49.308 (s)	49.935 (s)	30	20.000 (q)	19.744 (q)
15	44.149 (t)	45.021 (t)	C=OAc	168.914 (s)	169.965 (s)
16	69.823 (d)	70.953 (d)	CH_3-Ac	21.700 (q)	21.640 (q)

The ^{1}H-NMR and ^{13}C- NMR spectra of compound TR1 were compared with those of curbitacin B in Tables 4.1 and 4.2.

The^{13}C- NMR spectrum of compound TR1 showed 32 carbon resonances. Cucurbitacin B have eight primary carbon atoms, four secondary carbon atoms, tertiary carbon atoms and eleven quaternary carbon atoms and four carbonyl carbons (δ_C 211.300 (s), 211.706 (s), 201.222 (s) and 169.965 (s)). The first two carbonyl signals indicated the cyclohexone, the third carbonyl signal represented the side chain carbonyl.

The molecular formula $C_{32}H_{46}O_8$ of compound TR1 defined degree of unsaturation of six; therefore, compound TR1 must consist of three rings in addition to the two double bonds and one carbonyl group.

2. Molluscicidal activity testing of *T. cucumerina* L. fruits 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 4.2 and chemical controlled samples were subjected to 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 were shown in Table 4.3.

The results of molluscicidal activity test with LC_{50} and LC_{90} were displayed in Table 4.3.

The W.H.O. quantities toxicity by means of LC_{90} values but LC_{50} values and 100% snail kill values, all in ppm were also currently used (Marston and Hostettmann, 1985).

Table 4.3 The results of molluscicidal activity testing with LC_{50} and LC_{90} at 24 h intervals.

Extract	LC_{50} (ppm) (P<0.05)	LC_{90} (ppm) (P<0.05)
MeOH extract	80.58	100.28
H_2O extract	176.99	203.10
CH_2Cl_2 fraction	-	-
H_2O fraction	60.07	79.03
H_2O Q	29.97	41.90
50%MeOH Q	28.17	36.36
MeOH Q	31.93	40.38
TR1	9.02	17.67
Chemical controlled		
CU1	60.32	99.32
CU1 No.2	60.56	108.18
Niclosamide	0.27	1.12
Metaldehyde	34.98	89.57
Bionene	0.2*	0.68*

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

3. Cytotoxic activity of TR1 and chemical control

TR1 was also tested for cytotoxic activity towards 5 cancer cell lines, including SW620 (human colon adenocarcinoma), HEP-G2 (human liver hepatoblastoma), KATO-III (human gastric carcinoma), BT474 (human breast ductal carcinoma) and CHAGO (human undifferentiated lung carcinoma) as shown in Table 4.4.

Table 4.4 Cytotoxic activity by MTT colorimetric assay

Cell line	% survival (average)				
	10 μg/mL	1 μg/mL	0.1 μg/mL	0.01 μg/mL	0.001 μg/mL
SW620	20	32	30	53	98
HEP-G2	32	36	43	93	100
KATO-3	47	44	54	76	101
BT474	38	63	55	70	110
CHAGO	85	91	93	99	101

TR1 is high strong inhibiting growth cancer cell as doxorubicine, medicine for treat cancer as shown in Table 4.5.

Table 4.5 Cytotoxic activity by MTT colorimetric assay of cucurbitacin B (compound TR1) compare positive control (doxorubicine)

Cell line	IC_{50} (μg/ml)	
	Cucurbitacin B (TR1)	Doxorubicine
SW620	0.06	0.1
HEP-G2	0.09	0.61
KATO-3	10	>10
BT474	10	0.10
CHAGO	> 10	0.55

CHAPTER V

CONCLUSION

In this study, a known triterpenoid saponin named cucurbitacin B was isolated from the methanolic extract of fruits of *Trichosanthes cucumerina* L. (Buap khom).

The cytotoxicity of cucurbitacin B was evaluated by MTT [3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] colorimetric assay. Cucurbitacin B has strong cytotoxic activity against human colon denocarcinoma (SW620) and human liver carcinoma (HEP-G2), with IC_{50} values of 0.06 and 0.09 μg/mL, respectively. This study is the first report of a tetracyclic triterpene aglycone (i.e. cucurbitacin B) with molluscicidal activity.

Cucurbitacin B exhibited strong molluscicidal activity against *P. canaliculata* Lamarck by submersion method with LC_{50} value of 9.02 ppm and LC_{90} value of 17.67 ppm after 24 h at 95% confidence interval. *T. cucumerina* L. (Buap khom) has potential to be used as molluscicide substitute for the toxic chemicals.

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APPENDICES

Appendix A

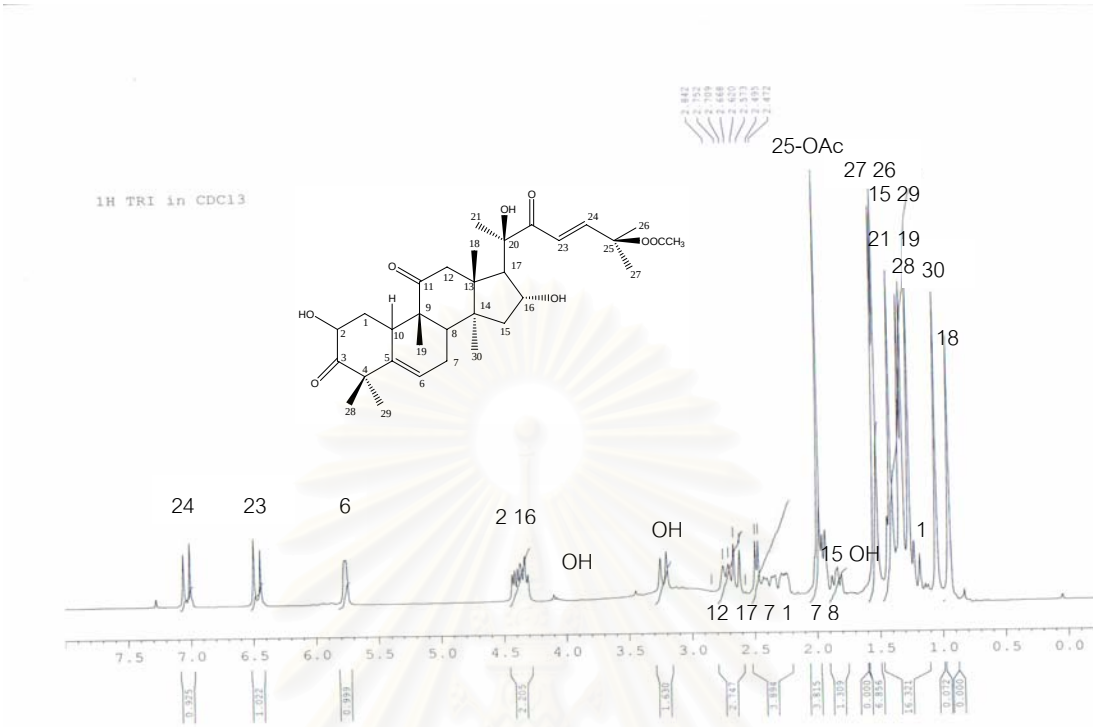


Figure A1 ^{1}H NMR (300 MHz) spectrum of compound TR1 (in CDCl2)

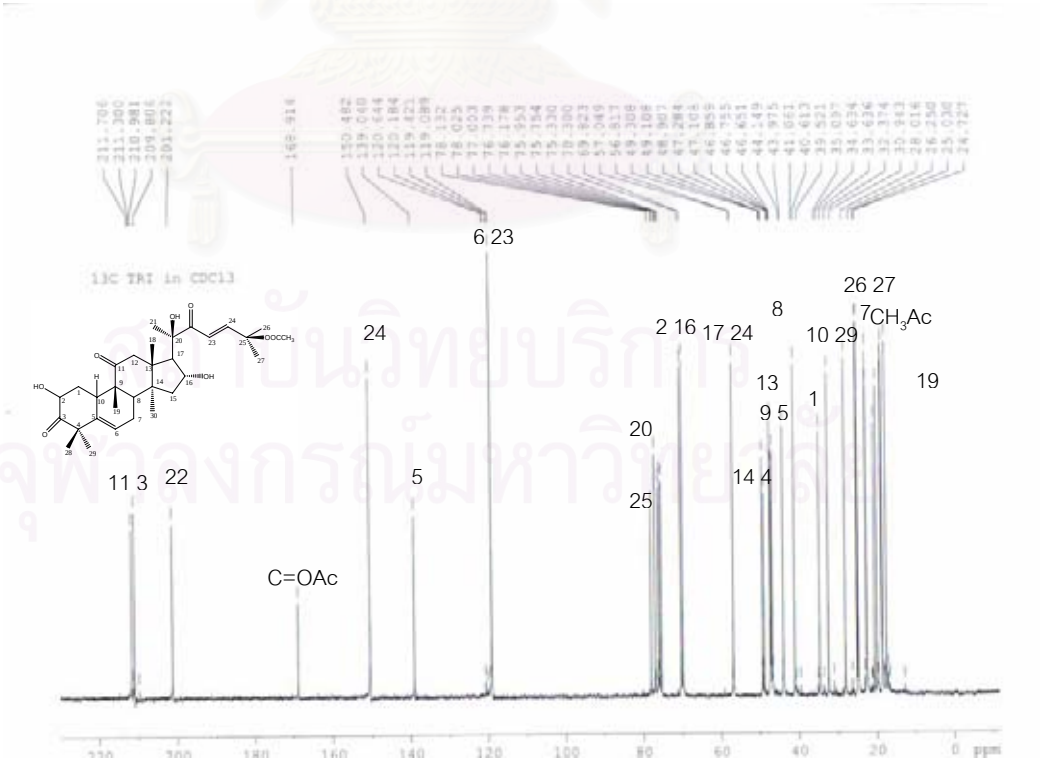


Figure A2 ^{13}C NMR (75 MHz) spectrum of compound TR1 (in CDCl2)

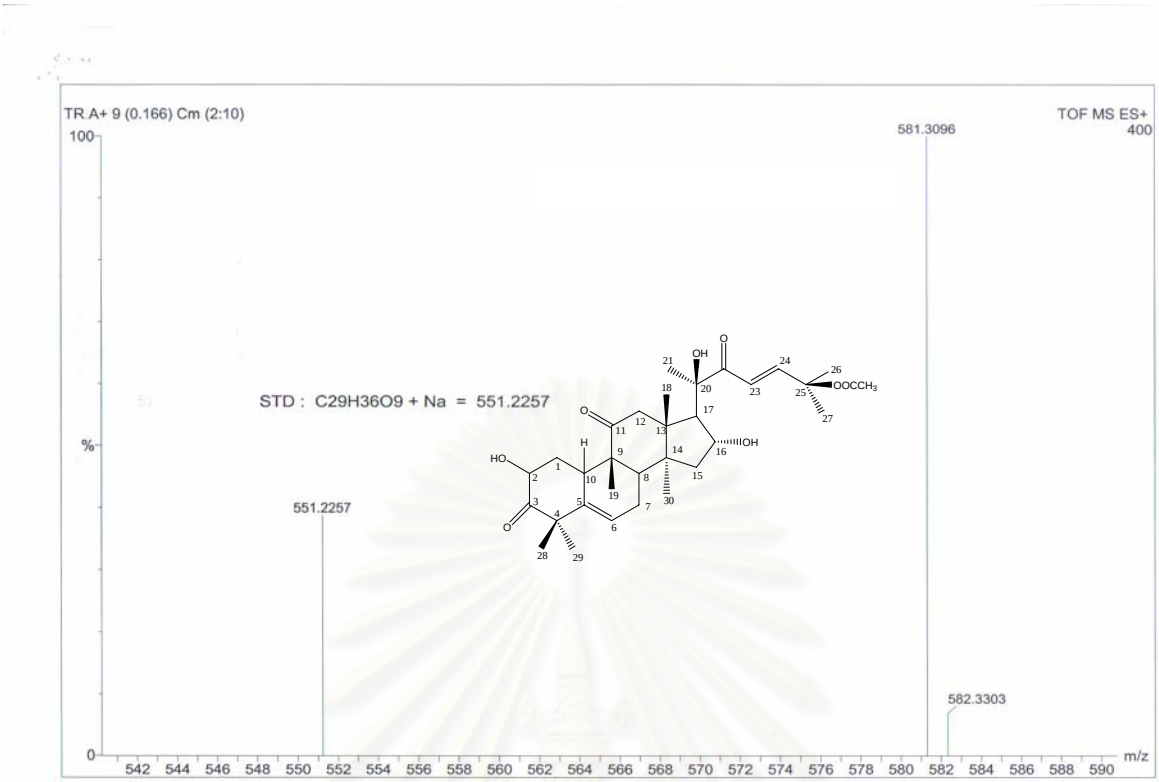


Figure A3 Mass spectrum of compound TR1

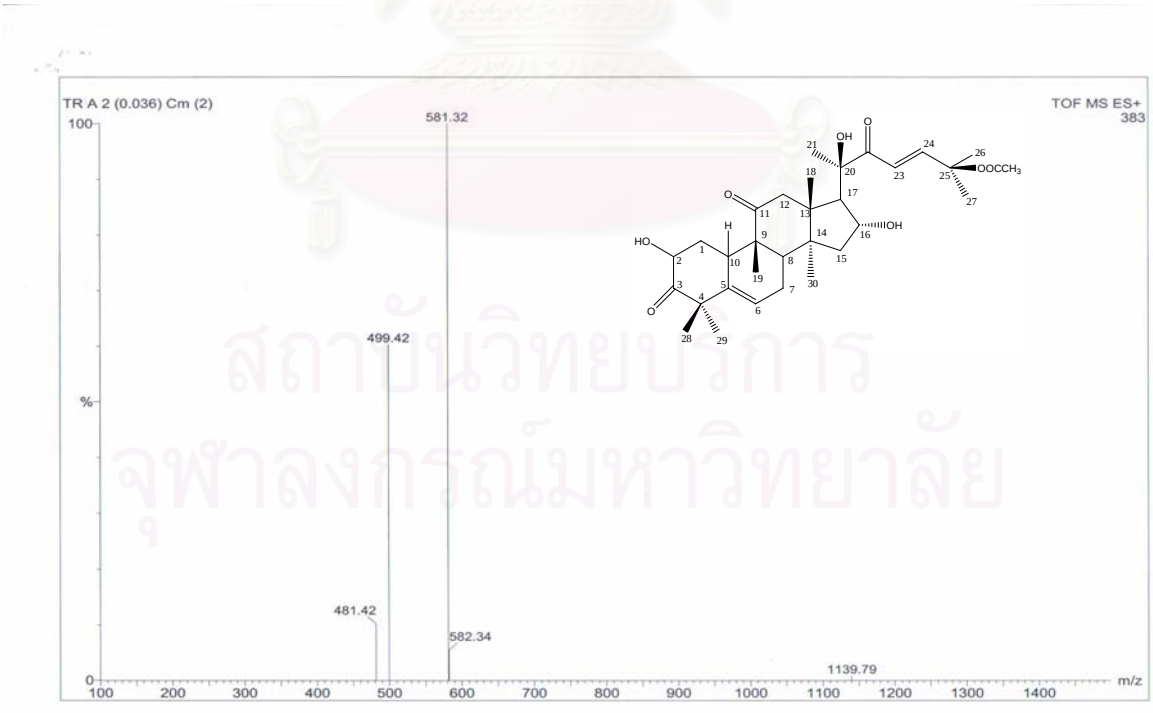


Figure A4 Mass spectrum of compound TR1

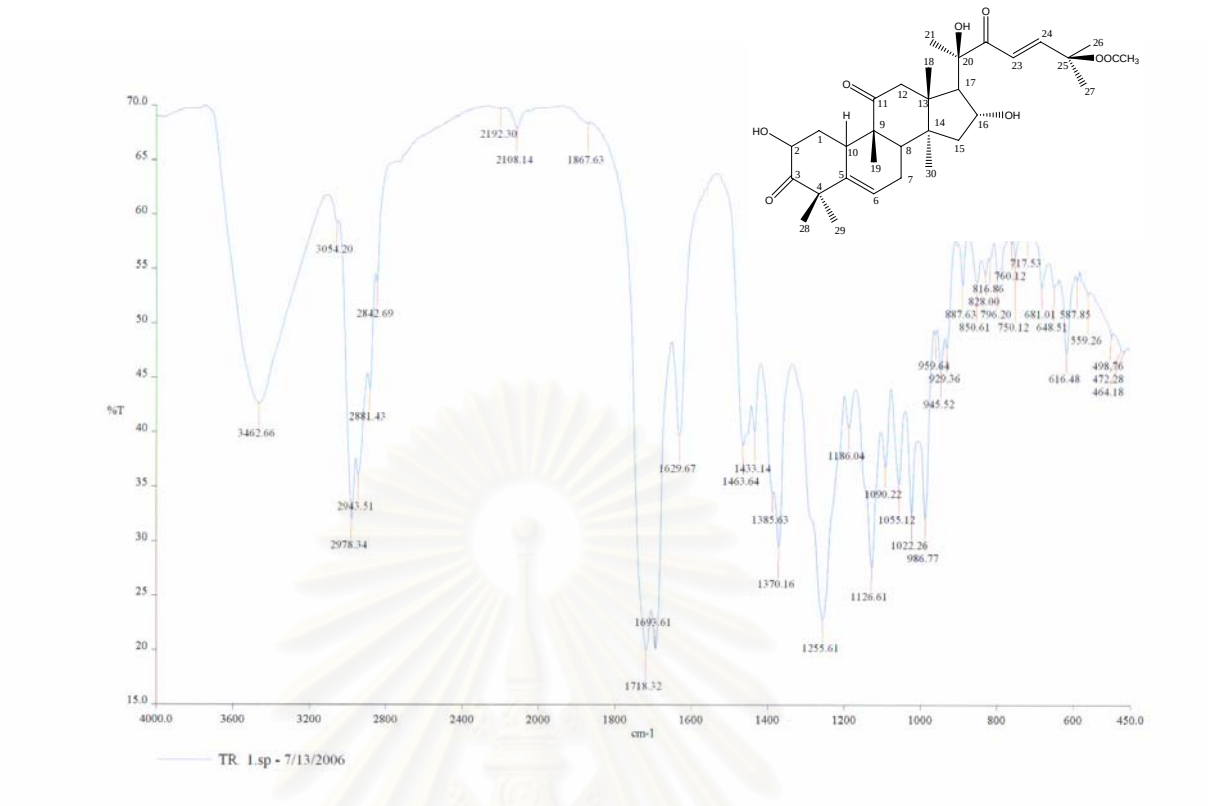
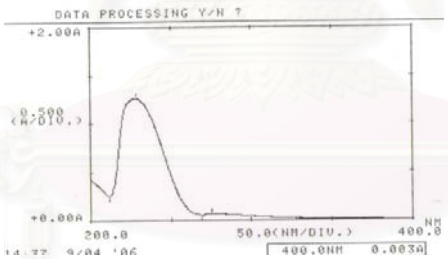


Figure A5 FT-IR of compound TR1



*** PEAK-PICK ***

-- PEAK --		-- VALLEY --	
λ	ABS	λ	ABS
275.0	0.071	269.0	0.049
228.6	1.273	211.8	0.255

Figure A6 UV spectrum of compound TR1

Appendix B

Molluscicidal activity test

The result of molluscicidal testing of methanolic exteact of *T. cucumerina* L. fruits against golden apple snails with 2.0-2.5 cm diameter.

Table B1 The result 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
20	0	0	0
40	0	0	0
60	0	6.67	20
80	36.67	46.67	56.67
100	73.33	93.33	100
120	100	100	100
140	100	100	100
160	100	100	100
180	100	100	100
200	100	100	100
0 (Control)	0.00	0.00	0.00

Table B2 The result of molluscicidal activity testing of methanolic exteact against *P. canaliculata* Lamarck at 24 h intervals.

Concentration (ppm)	Total snails	Death snails	%mean of mortality	LC_{50} (ppm)	LC_{90} (ppm)
20	30	0	0.00	80.58 (77.86-83.30)	100.28 (96.55-105.04)
40	30	0	0.00		
60	30	0	20.00±3.33		
80	30	11	36.67±3.33		
100	30	22	73.33±6.67		
120	30	30	100±0.00		
140	30	30	100±0.00		
160	30	30	100±0.00		
180	30	30	100±0.00		
200	30	30	100±0.00		
0 (Control)	0	0	0.00±0.00		

95% confidence Limits of LC_{50} and LC_{90}

Mean±S.E., calculated from triplicate (each per 10 snails)

The result of molluscicidal testing of aqueous of partition-fraction of *T. cucumerina* fruits against golden apple snails with 2.0-2.5 cm diameter

Table B3 The result 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
40	0	0	0
50	0	0	26.67
60	53.33	86.67	100
70	66.67	73.33	96.67
80	70.00	83.33	96.67
90	80.00	96.67	100
100	100	100	100
0 (Control)	0.00	0.00	0.00

Table B4 The result of molluscicidal activity testing of aqueous of partition-fraction against *P. canaliculata* Lamarck at 24 h intervals.

Concentration (ppm)	Total snails	Death snails	%mean of mortality	LC_{50} (ppm)	LC_{90} (ppm)
40	30	0	0	60.07 (54.73-64.80)	79.05 (72.49-91.05)
50	30	0	0		
60	30	16	53.33±3.33		
70	30	20	66.67±3.33		
80	30	21	70.00±3.33		
90	30	24	80.00±3.33		
100	30	30	100.00±0.00		
0 (Control)	0	0	0.00±0.00		

95% confidence Limits of LC_{50} and LC_{90}

Mean±S.E., calculated from triplicate (each per 10 snails)

The result of molluscicidal testing of 50% methanolic fraction of *T. cucumerina* L. fruits against golden apple snails with 2.0-2.5 cm diameter

Table B5 The result 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
15	0	0	0
20	0	0	6.67
25	26.67	40.00	53.33
30	36.67	53.33	70.00
35	60.00	80.00	96.67
40	100	100	100
45	100	100	100
50	100	100	100
0 (Control)	0.00	0.00	0.00

Table B6 The result of molluscicidal activity testing of methanolic fraction against *P. canaliculata* Lamarck at 24 h intervals.

Concentration (ppm)	Total snails	Death snails	%mean of mortality	LC_{50} (ppm)	LC_{90} (ppm)
15	30	0	0.00	28.17 (26.79-29.50)	36.36 (34.34-39.34)
20	30	0	0.00		
25	30	8	26.67±3.33		
30	30	11	36.67±3.33		
35	30	18	60±3.33		
40	30	100	100±0.00		
45	30	100	100±0.00		
50	30	100	100±0.00		
0 (Control)	0	0	0.00±0.00		

95% confidence Limits of LC_{50} and LC_{90}

Mean±S.E., calculated from triplicate (each per 10 snails)

The result of molluscicidal testing of compound TR1 against golden apple snails with 2.0-2.5 cm diameter

Table B7 The result 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
5	0.00	6.67	30.00
10	36.67	70.00	83.33
15	56.67	70.00	96.67
20	83.33	93.33	100.00
25	100.00	100.00	100.00
30	100.00	100.00	100.00
0 (Control)	0.00	0.00	0.00

Table B8 The result of molluscicidal activity testing of compound TR1 against *P. canaliculata* at 24 h intervals.

Concentration (ppm)	Total snails	Death snails	%mean of mortality	LC_{50} (ppm)	LC_{90} (ppm)
5	30	0	0.00±0.00	9.03 (7.18- 10.71)	17.67 (14.69-23.43)
10	30	11	36.67±3.33		
15	30	17	56.67±3.33		
20	30	25	83.33±3.33		
25	30	30	100±0.00		
30	30	30	100±0.00		
0 (Control)	0	0	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

PROBIT ANALYSIS

The LC_{50} value was determined by Probit Analysis using SPSS version 11.5 for window at 95% confidence intervals. This is the example of the result from Probit Analysis for LC_{50} (24 h.) of compound TR1 in small snails (2.0-2.5 cm).

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

DATA Information

18 unweighted cases accepted.

0 cases rejected because of missing data.

0 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 12 iterations.

Optimal solution found.

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

	Regression Coeff.	Standard Error	Coeff./S.E.
CONCE TR1	4.39394	.32965	13.32910

Intercept	Standard Error	Intercept/S.E.
-4.19916	.35930	-11.68706

Pearson Goodness-of-Fit Chi Square = 65.669 DF = 16 P = .000

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

CONCE TR1	Number of Subjects	Observed Responses	Expected Responses	Residual	Prob
.70	30.0	.0	3.890	-3.890	.12968
.70	30.0	2.0	3.890	-1.890	.12968
.70	30.0	10.0	3.890	6.110	.12968
1.00	30.0	11.0	17.317	-6.317	.57722
1.00	30.0	21.0	17.317	3.683	.57722
1.00	30.0	25.0	17.317	7.683	.57722
1.18	30.0	17.0	25.008	-8.008	.83361
1.18	30.0	21.0	25.008	-4.008	.83361
1.18	30.0	29.0	25.008	3.992	.83361
1.30	30.0	25.0	28.063	-3.063	.93543
1.30	30.0	28.0	28.063	-.063	.93543
1.30	30.0	30.0	28.063	1.937	.93543
1.40	30.0	30.0	29.220	.780	.97401
1.40	30.0	30.0	29.220	.780	.97401
1.40	30.0	30.0	29.220	.780	.97401
1.48	30.0	30.0	29.671	.329	.98902
1.48	30.0	30.0	29.671	.329	.98902
1.48	30.0	30.0	29.671	.329	.98902

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

Confidence Limits for Effective CONCE TR1

		95% Confidence Limits	
Prob	CONCE TR1	Lower	Upper
.01	2.66824	1.28657	3.93386
.02	3.07798	1.58373	4.39566
.03	3.36998	1.80635	4.71788
.04	3.60776	1.99382	4.97674
.05	3.81350	2.16026	5.19850
.06	3.99784	2.31255	5.39566
.07	4.16678	2.45464	5.57522
.08	4.32409	2.58904	5.74156
.09	4.47232	2.71744	5.89761
.10	4.61325	2.84105	6.04542
.15	5.24556	3.41266	6.70361
.20	5.80936	3.94301	7.28651
.25	6.34111	4.45798	7.83601
.30	6.85999	4.97156	8.37473
.35	7.37864	5.49310	8.91836
.40	7.90700	6.02991	9.48028
.45	8.45417	6.58848	10.07394
.50	9.02963	7.17515	10.71461
.55	9.64426	7.79677	11.42133
.60	10.31165	8.46131	12.21928
.65	11.05003	9.17908	13.14358
.70	11.88547	9.96483	14.24563
.75	12.85803	10.84195	15.60541

.80	14.03496	11.85130	17.35811
.85	15.54347	13.07270	19.76282
.90	17.67394	14.69000	23.42547
.91	18.23086	15.09634	24.42912
.92	18.85579	15.54553	25.57647
.93	19.56769	16.04927	26.90973
.94	20.39459	16.62476	28.49211
.95	21.38040	17.29874	30.42407
.96	22.59966	18.11619	32.87891
.97	24.19427	19.16179	36.19345
.98	26.48956	20.62704	41.15983
.99	30.55727	23.13001	50.48798

VITA

Miss Saowanit Daorattanachai was born on May 19th, 1977 in Ubonratchatani, Thailand. She received her Bachelor's degree of Pharmaceutical Science from the Faculty of Pharmacy, Mahidol University in 2002. After graduation, she worked as Medicinal Plant Information Center for 2 years at Faculty of Pharmacy, Mahidol University. Since 2004, she has been a graduate student at Department of Pharmacognosy, Faculty of Pharmaceutical Science, Chulalongkorn University. She finished her postgraduate study in leading of Master's degree of Pharmaceutical Science in 2006.