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NAME: Miss Chonchawan Jankam

THIS THESIS HAS BEEN ACCEPTED BY

THESIS ADVISOR

(Associate Professor Srunya Vajrodaya, Dr.rer.nat)

THESIS CO-ADVISOR

(Miss Nutha Sanevas, Ph.D.)

DEPARTMENT HEAD

(Assistant Professor Chatchai Ngernsaengsaruay, Ph.D.)

APPROVED BY THE GRADUATE SCHOOL ON _____

DEAN

(Associate Professor Gunjana Theeragool, D.Agr.)

THESIS

PHYTOCHEMICAL ANALYSES OF *Acmella ciliata* Cass. (FAMILY ASTERACEAE) AND ITS ACTIVE CONSTITUENTS AGAINST MALARIA

CHONCHAWAN JANKAM

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Master of Science (Botany)
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Chonchawan Jankam 2014: Phytochemical Analyses of *Acmella ciliata* Cass. (Family Asteraceae) and Its Active Constituents Against Malaria. Master of Science (Botany), Major Field: Botany, Department of Botany. Thesis Advisor: Associate Professor Srunya Vajrodaya, Dr.rer.nat. 79 pages.

Phytochemical study of the lipophilic extracts from three plant parts of *Acmella ciliata* Cass. (Pak Kard Hua Wan); leaves, inflorescence and underground part had been done during june 2009 – july 2012. Thin Layer Chromatography (TLC) screening test were shown that lipophilic extract of leaves, inflorescence and underground part contained terpenoids, steroids and triterpenoids while coumarins were only detected in underground part extract. However, the pattern of TLC profiles were similar in three plant parts (leaves, inflorescence and underground part), but they were rather different when analysed by HPLC and specific test. By mean of chromatographic technique, the pure compounds were isolated from underground part extract and purified by recrystallization. The crystals were colorless needle which were identified as mixture of stigmasterol and β-sitosterol by comparing spectroscopic data (UV, FT-IR, MS, ^{1}H and ^{13}C NMR) with the former documents.

Both extracts from inflorescence and underground part exhibited the most active antimalarial activity against *Plasmodium falciparum* to chloroquine sensitive strain 3D7 and resistant strain K1. However, the underground part showed more active antimalarial activity against both chloroquine sensitive and resistant strain of *Plasmodium falciparum* than the inflorescence extract.

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Student's signature Thesis Advisor's signature

ACKNOWLEDGEMENTS

Firstly, I would like to thanks my dearest parents, Mr. and Mrs. Jankam, my brother, I wish to sincerely thank all for giving me support in pursuing my degree and for always listening and encouraging me since the beginning until the end of my thesis.

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I would like to extend my thanks to Ms. Somnuek Promdangat the laboratory of Phytochemistry Research and Analysis Unit, Central Laboratory and Greenhouse Complex, Research and Development Institute at Kamphaeng Saen for her help and assistance in laboratory analysis to support my work.

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Chonchawan Jankam

June 2014

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LIST OF ABBREVIATIONS

α	=	alpha
β	=	beta
cm	=	centimeter
cm^2	=	square centimeter
CC	=	column chromatography
°C	=	degree Celsius
δ	=	delta
Fr	=	fraction
g	=	gram
HPLC	=	high performance liquid chromatography
IR	=	infrared
IC_{50}	=	the half maximal inhibitory concentration
MΩ	=	mega ohm
MS	=	mass spectroscopy
MHz	=	mega hertz
µg	=	microgram
µg/ml	=	microgram per milliter
µl	=	microliter
µm	=	micrometer
mg	=	milligram
mg/kg	=	milligram per kilogram
mg/ml	=	milligram per milliter
ml	=	milliter
ml/min	=	milliter per minitue
mm	=	millimeter
min	=	miniute
nm	=	nanometer
No	=	number
NMR	=	nuclear magnetic resonance

LIST OF ABBREVIATIONS (Continued)

ppm	=	parts per million
%	=	percentage
R_f	=	retardation factor
Rt	=	retention time
syn	=	synonym
TLC	=	thin layer chromatography
UV	=	ultraviolet

PHYTOCHEMICAL ANALYSES OF *Acmella ciliata* Cass. (FAMILY ASTERACEAE) AND ITS ACTIVE CONSTITUENTS AGAINST MALARIA

INTRODUCTION

In the present, Malaria remains prevalent in many tropical and subtropical regions of the world. The world Malaria report 2009 by The World Health Organization (WHO) Indicated that implementation of antimalarial intervention in Thailand has scaled up leading to case reduction up to 50% from 2000 to 2008. A considerable number of against malaria researches have also been reported (World Health Organization, 2009).

Malaria is one of the most frequent causes of illness in many tropical and subtropical regions of the world. There have been reported that malaria mainly caused from four species of protozoa, that are *Plasmodium falciparum, P. vivax, P. ovale* and *P. malariae*. In tropical regions, malaria is mostly maintained by the evolution of therapy-resistant strains of *P. falciparum*, as well as the by insufficient resistance in the inhabitants (Hausmann and Hülsmann, 1996). Malaria has been developed and evolved continuously although there are many effective medicines. Therefore, the ethnopharmacological research is willing to discover new plant extract against drug resistant strain of *P. falciparum.* For example, crude extract of native herbs in Africa has been shown to inhibit *P. falciparum* (Kaou *et al*., 2008). The study of antimalarial activity from ethnomedicine in Kenya was also reported (Muregi *et al*., 2007). In Thailand, the researches involving medicinal plants which are able to express activation against malaria have been carried out continuously. However, the evolutionary drug of resistance strains of *P. falciparum* has been still developed continuously. The phytochemical study is still playing as significant role in providing information and knowledge for antimalarial drug investigation.

OBJECTIVES

1. To investigate the chemical constitutents from *Acmella ciliata*

2. To investigate pure compounds from *Acmella ciliata* for inhibition of protozoa causing malaria.

LITERATURE REVIEW

Para cress *(Acmella* spp.)

Thais have used traditional herbs as food and medicine for more than hundred years ago. One of traditional herb which used for relieve toothache is “Para cress” or “toothache plant”. The para cress belongs to genus *Acmella* in Asteraceae. *Acmella*, is closely allied to *Spilanthes* in the subtribe Verbesininae, consists of annual and perennial herbs with matted to erect growth habits. The genus *Acmella* consists of thirty species which are mainly distubuted in the tropical and subtropical regions around the world (Jansen, 1985).

Morphology

The broad range of variation in duration and habit, duration include annuals and perennials.

Stem : usually decumbent and ascending, 30-40 cm high; stem cylindric, succulent, rooting at nodes, green to purple (Figure 1) (Jansen, 1985).

Figure 1 Stem of *Acmella* sp.

leaves : simple leaf, opposite, leaf blades 23-75 mm long, 10-59 mm wide, ovate to broadly ovate, base usually truncate or cordate, apex acute, glabrous to sparsely pilose above and below, margin denticulate to coarsely dentate (Jansen, 1985).

Inflorescence : radiate and discoid heads are occured in *Acmella*. Phyllaries are uni-, bi- and triseriate, shorter or longer than ray florets (Figure 2) (Jansen, 1985).

-Ray florets: In Thailand, ray florets were found in *A. brachyglossa*, *A. ciliata* and *A. uliginosa*. For *A. ciliata*, ray florets are slightly shorter to only slightly exceeding the phyllaries, often inconspicuous. Corollas are 2.5 - 6.5 mm long, yellow to yellow-orange, with evident cork-like margin, lacking shoulders. Pappus usually absent or sometimes of 2 short subequal bristles, 0.2 - 0.4 mm long. *A. brachyglossa* and *A. uliginosa* have mature achenes lacking evident, stramineous, cork-like margins (Jansen, 1985).

-Disc florets: aspects of the disc corollas and anthers which are taxonomically valuable, are the number of parts and their color. Disc florets have yellow to orange-yellow. Anthers are brown, brown-black and black. In Thailand, there are three species of *Acmella* which have heads discoid (without ray florets); *A. calva*, *A. paniculata* and *A. oleracea* (Jansen, 1985).

Achene: the shape and margin of both ray and disc achenes are very useful taxonomically within *Acmella*. The shape varies from ellipsoid to broadly ovoid. Some taxa have achenes with apical shoulders extended beyond the attachment of the corolla in contrast to the more typical condition in which these extensions are lacking. The mature achene have an evident, stramineous, cork-like margin, which has been useful in seperating taxa.

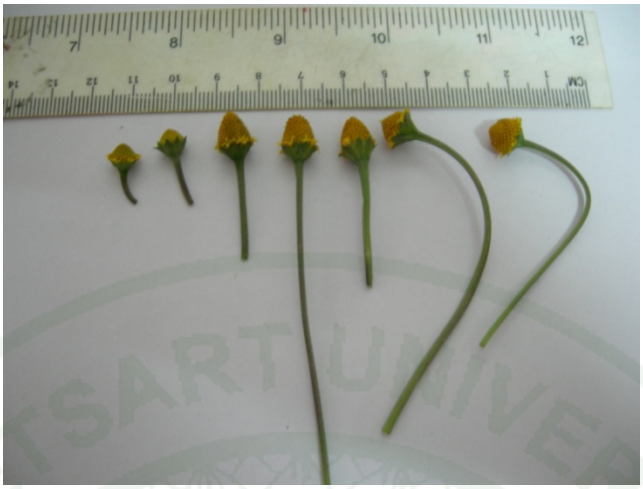


Figure 2 Inflorescence of *A. ciliata* with ray florets.

Distribution

The distribution is in moist weedy habitats especially along roadsides, cultivated fields, and stream banks. Some species are known only from cultivation and found in weedy habitats. It is grown primary in botanical and herbs garden. In Thailand, there are two species of genus *Acmella* which has been reported they are *Acmella oleracea* (L.) R. K. Jansen and *Acmella paniculata* (Wall. ex DC.) R.K. Jansen. However, The systematics of *Acmella* (Asteraceae-Heliantheae) shows that there are 6 species distribute in Thailand (Figure3)(Jansen, 1985).

A. brachyglossa Cass.

A. calva (DC) R.K. Jansen

A. ciliata Cass.

A. oleracea (L.) R.K. Jansen

A. paniculata (Wall ex DC) R.K. Jansen

A. uliginosa (Swartz) Cass.

Some species of this genus such as *A. ciliata* is mostly widely distributed in South America (Figure 4) and introduced to Thailand which now are naturalized in almost every parts (especially in the northeastern part).

Figure 3 Distribution of *A. brachyglossa*, *A. ciliata* and *A. uliginosa*in Thailand.

Source: Jansen (1985).

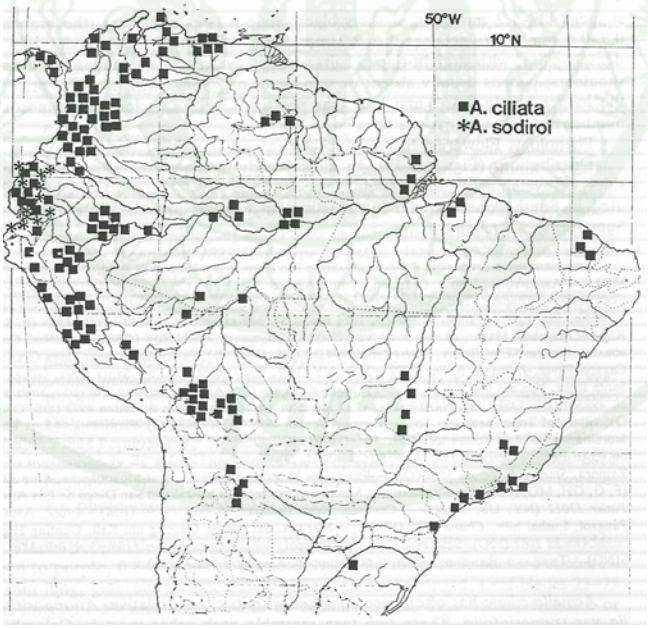


Figure 4 Distribution of *A. ciliata* in South America.

Source: Jansen (1985).

The ethnobotanical usage of *Acmella* spp.(Table 1)

Thais have used *Acmella* as toothache plant and healing the fever for long time ago.

In western Uganda, leaves and flowers of *A. caulorrhiza* Del. are squezzed by hand and was used as traditional medicine in childbirth (Mugisha and Origa, 2007).

The whole plant of *A. paniculata* are used for dysentery, scabies, psoriasis and diuretic. The flowers are chewed for relief of throat afflictions, toothache and paralysis of tongue. In Papua New Guinea, people chew the root to relieve the toothache. The flowers of *A. oleracea* are mainly used for healing in the oral, paralysis of tongue, throat and gum infection. It is also used to cure children's stammering, a sialogogue and stimulant (Jain and Defilipps, 1991). In Indonesia, the flowers of *A. paniculata* are also used to promote the secretion of saliva. The leaves are applied to the head in case of migraine (Wiart, 2002).

Table 1 The ethnobotanical usage of genus *Acmella*

Sources	Places	Plant part/ Usage	Reference
A. caulorrhiza	western Uganda	1) leaves and inflorescence/ traditional medicine for heal in oral of childbirth	Mugisha and Origa, 2007
A. paniculata	Indonesia	1) inflorescence/ promote the secretion of saliva. 2) leaves/ relief of migraine	Wiart, 2002
	Papua New Guinea	1) inflorescence/ relief of throat afflictions, toothache and paralysis of tongue. 2) root/ relieve the toothache.	Jain and Defilipps, 1991
A. oleracea	-	1) healing in the oral, paralysis of tongue, throat and gum infection. 2) cure children's stammering, a sialogogue and stimulant	Jain and Defilipps, 1991

Bioassay of *Acmella*spp. (Table 2)

Thus, there are many case studies of this genus such as the study of ethnopharmacological, methanolic extract of *S. mauritiana* (syn. *A. paniculata*) from flowers and roots showed the minimum inhibitor concentration value ≥ 8 mg/ml which against bacteria 6 genera that are *Staphylococcus, Enterococcus, Pseudomonas, Escherichia, Klebsiella* and *Salmonella* (Fabry *et al*., 1997). The study of hexane extract which obtain from flowers of three species that are *S. acmella* var *oleracea* L. (syn. *A. oleracea), S. calva* L. (syn. *A. calva*) and *S. paniculata* (syn. *A. paniculata*). It shows variable mortality against the late third and early fourth instar larvae of *Anopheles stephensi* Liston, *A. culicifcies* species C. and filaria vector (Pandey *et al*., 2007). The root extract of *S. acmella* (syn. *A. paniculata*) which has the most toxic against to larvae of *Anopheles stephensi* and *Culex quinquefasciatus* Say. It has a hundred percent mortility that was obtained at 6.25 ppm (*A. stephens*) and 3.125 ppm (*Cx. quinquefasciatus*)(Pandey and Agrawal, 2008).

The micropropagated flower head of *S. acmella* (syn. *A. paniculata*) are extracted by hexane then isolate into separated fractions by silica gel column chromatography. The fractions are collected then combined by base on TLC analysis into 7 fractions. The fractional groups C, D, E and F that show larvicidal activity against *Anopheles stephensi*larvae, except fractional group A, B and G (Pandey *et al*., 2010).

The ethanolic extract of *S. acmella* (syn. *A. paniculata*) which obtain from flower buds. The extract show significantly reduced the *in vitro* activity of the human pancreatic lipase (Ekanem *et al*., 2007).

The study of antifungal activity from Thai medicinal plants show that the chloroform extract of *S. acmella* (syn. *A. paniculata*) is able to inhibit *Cryptococcus neoformans.* The minimum inhibitory concentration (MIC) is 128 µg/ml that has the inhibition zone of fungal strains around 7.5 mm (Phongpaichit *et al*., 2005).

The study on antinociceptive activity of methanolic extract of *A. uligonosa* (Sw.) Cass show the extract can produced significant antinociceptive response in hot plate test and all the chemical induced nociception models (acetic acid –induced abdominal constriction and formalin-, capsaicin-, glutamate-induced paw licking test) (Ong *et al*., 2011).

The flowers of *S. acmella* (syn. *A. paniculata*) are extracted by cold water for diuretic activity study in rats. The different concentration of cold water extract (500,1000 and 1500 mg/kg) are compared with furosemide at concentration value 13 mg/kg. The experiment shows the highest dose of cold water extract has the peak effect in the first and second hour however the furosemide increase the urine output only in the first hour (Ratnasooriya *et al*., 2004).

The hexane extract from aerial parts of *A. oleracea* exhibited the highest activity, causing 100% mortality in *Tuta absoluta* at a concentration of 10 µg of extract per mg of insect. The ethanol extract of *A. oleracea* also showed high activity (88.3 % mortality) against *T. absoluta*. (Moreno *et al*., 2011)

Table 2 Biological activities of genus *Acmella*

Plants	Extract	Biological activities	Reference
S.mauritiana (syn.*A. paniculata*)	methanolic extract	antibacterial activitiy	Fabry *et al*., 1997
S. acmella var. *oleracea* (syn. *A. oleracea)*	hexane extract	larvicidal activity	Pandey *et al*., 2007
S. calva L. (syn. *A. calva*)	hexane extract	larvicidal activity	Pandey *et al*., 2007
S. paniculata (syn. *A. paniculata*)	hexane extract	larvicidal activity	Pandey *et al*., 2007
S. acmella (syn. *A. paniculata*)	root extract	Insecticidal activity	Pandey and Agrawal, 2008
S. acmella (syn. *A. paniculata*)	Inflorescence	larvicidal activity	Pandey *et al*., 2010
S. acmella (syn. *A. paniculata*)	ethanolic extract/ Inflorescence	human pancreatic lipase activity.	Ekanem *et al*., 2007
S. acmella (syn. *A. paniculata*)	chloroform extract	antifungal activitiy	Phongpaichit *et al*., 2005
S. acmella (syn. *A. paniculata*)	cold water	diuretic activity	Ratnasooriya *et al*., 2004
S. acmella (syn. *A. paniculata*)	hexane extract	larvicidal activity	Ramsewak *et al*., 1999
A. uligonosa (Sw.) Cass	methanolic extract	antinociceptive activity	Ong *et al*., 2011
A. oleracea	hexane extract of aerial parts	Insecticidal activity	Moreno *et al*., 2011
A. oleracea	ethanol extract of aerial parts	Insecticidal activity	Moreno *et al*., 2011

Chemical constituents in the genus *Acmella* (Table 3).

The chemical constituents in genus *Acmella* can be found in different parts of plants. The main amide component has been known which is spilanthol. Spilanthol is the active major constituent with a pungent taste. The spilanthol-related amides from *Acmella ciliata* has been examined and isolated that possess isobutylamine, 2-phenylamine and 2-mthylbutylamine.(Martin and Becker, 1984). Beside, fourteen highly unsaturated amides are isolated from *Acmella ciliata* (Martin and Becker, 1985). The flower head of *Spilanthes acmella* (syn. *Acmella paniculata*) are extracted by hexane that afford three N-isobutyl amides: spilanthol, undeca-2*E*,7*Z*,9*E*-trienoic acid isobutylamide and undeca-2*E*-en-8,10-diynoic acidisobutylamide (Ramsewak *et al*., 1999).

Table 3 Chemical constituents of plants in the genus *Acmella*

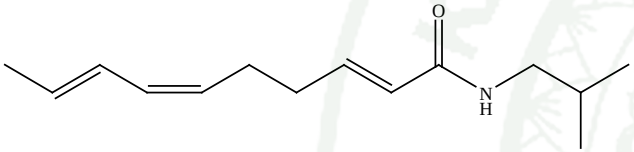
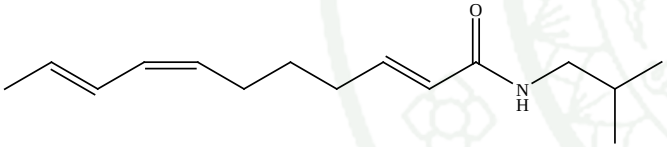
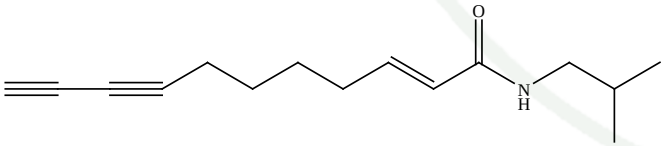
Chemical type/ Chemical compounds	Plant	Reference
Spilanthol 	*A. ciliata* *A. oleracea (*syn. *S. acmella L.* var. *oleracea)*	Martin and Becker, 1984 Nakatani and Nagashima, 1992 Moreno *et al*., 2011
Undeca-3*E*,7*Z*,9*E*- trinoic acid isobutylamide 	*A. paniculata* (syn. *S. acmella*)	Ramsewak *et al*., 1999
Undeca-2*E*-en-8,10-diynoic acid isobutylamide 	*A. paniculata* (syn. *S. acmella*)	Ramsewak *et al*., 1999

Table 3 (Continued)

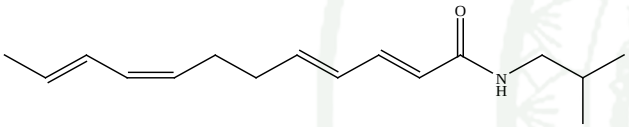
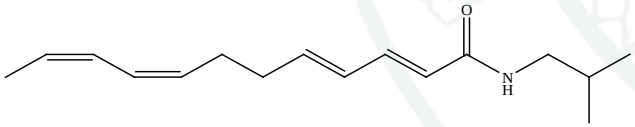
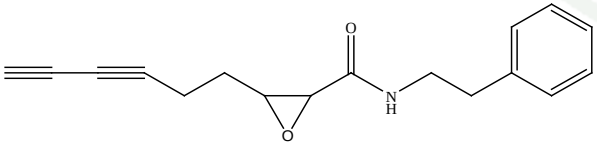
Chemical type/ Chemical compounds	Plant	Reference
Dodeca-2*E*,4*E*,8*Z*,10*E*- tetraenoic acid isobutylamide 	*A. paniculata* (syn. *S. acmella*)	Raner *et al*., 2007
Dodeca-2*E*,4*E*,8*Z*,10*Z*- tetraenoic acid isobutylamide 	*A. paniculata* (syn. *S. acmella*)	Raner *et al*., 2007
N-phenethyl-2,3-epoxy-6,8-nonadiynamide 	*A. paniculata* (syn. *S. acmella*)	Bonen *et al*., 2010

Table 3 (Continued)

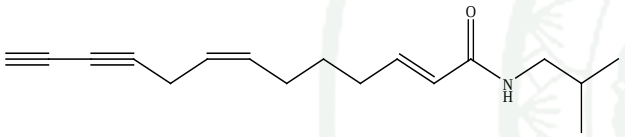
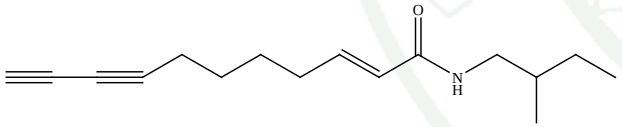
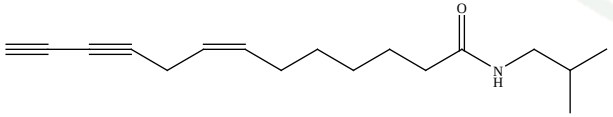
Chemical type/ Chemical compounds	Plant	Reference
(2*E,7Z)-N*-isobutyl-2,7-tridecadiene-10,12-diynamide 	*A. oleracea* *(*syn. *S. acmella* L.var. *oleracea)*	Nakatani and Nagashima, 1992
(2*E)-N*-(2-methylbutyl)-2-undecene-8,10-diynamide 	*A. oleracea* *(*syn. *S. acmella* L.var. *oleracea)*	Nakatani and Nagashima, 1992
(*7Z)-N*-isobutyl-7-tridecene-10,12-diynamide 	*A. oleracea* *(*syn. *S. acmella* L.var. *oleracea)*	Nakatani and Nagashima, 1992

Table 3 (Continued)

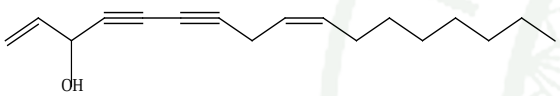
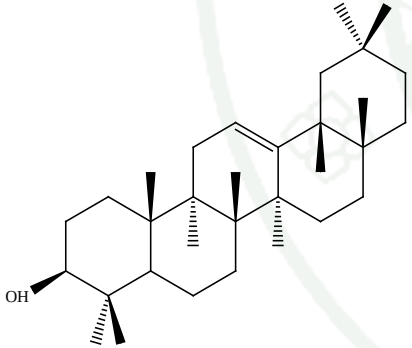
Chemical type/ Chemical compounds	Plant	Reference
(7Z,9E)-2-Oxo-Undeca-7,9-dienanyl-3,3-dimethylprop-2-enoate (acmellonate) 	*A. paniculata* (syn. *S. acmella*)	Ley *et al*., 2006
β-amyrin 	*A. paniculata* (syn. *S. acmella*)	Krishnaswamy and Prasanna, 1975

Table 3 (Continued)

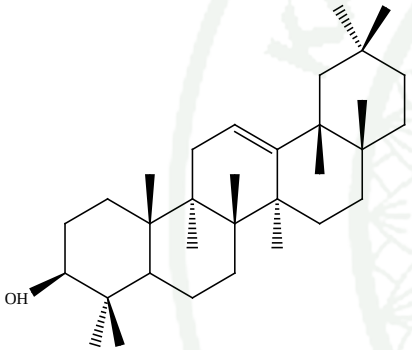
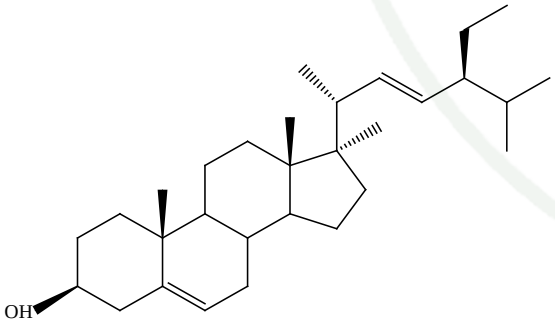
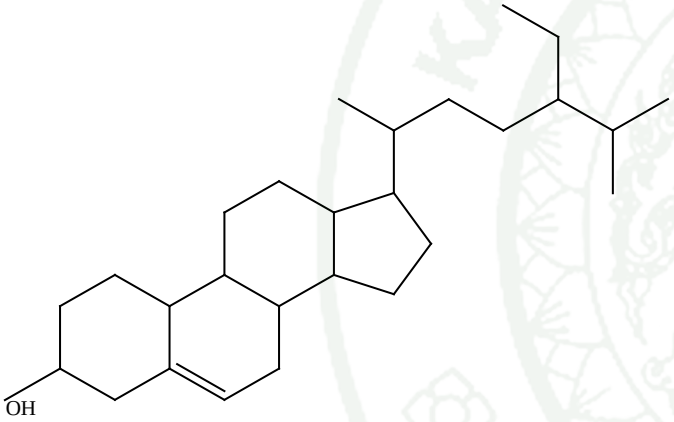
Chemical type/ Chemical compounds	Plant	Reference
β-amyrin 	*A. paniculata* (syn. *S. acmella*)	Krishnaswamy and Prasanna, 1975
Stigmasterol 	*A. paniculata* (syn. *S. acmella*)	Krishnaswamy and Prasanna, 1975

Table 3 (Continued)

Chemical type/ Chemical compounds	Plant	Reference
Sitosterol 	*A. paniculata* (syn. *S. acmella*)	Krishnaswamy and Prasanna, 1975

Malaria

Malaria is a disease cause by protozoal parasite of genus *Plasmodium*. There are four species of *Plasmodium* can cause human malaria that are *Plasmodium falciparum, P. vivax, P. ovale* and *P. malariae.* The protozoa are carried and transferred to human by infected mosquito which are in midge genera such as *Anopheles, Aedes* and *Culex*. Most infection are due to *P. vivax* and *P. falciparum.* The mainly cause of death is due to infection of *P. falciparum* (Phillips *et al.*, 2001)**.**

Life cycle of malaria disease in human starts from the bite of infected mosquito then the sporozoites attack immediately to liver cell in the first step. The sporozoites will develop and regenerate to primary schizonts in liver cell become large schizonts, however somesporozoites will be eliminate by macrophage in host body. The large schizonts can develop several thousands of merozoites. The secondary step of infection, schizonts will repture and release the parasite to invade red blood cell where merozoites multiply to infect another red blood cell (Hausmann and Hülsmann, 1996; Mandell *et al.*, 2005) (Figure 5).

In the present, Malaria remains prevalent in many tropical and subtropical regions of the world. The world Malaria report 2009 by The World Health Organization (WHO) indicated that implementation of antimalarial intervention in Thailand has scaled up leading to case reduction up to 50% from 2000 to 2008. A considerable number of against malaria researches has also been reported (World Health Organization, 2009). However, Malaria has been developed and evolved continuously although there are many effective medicines.

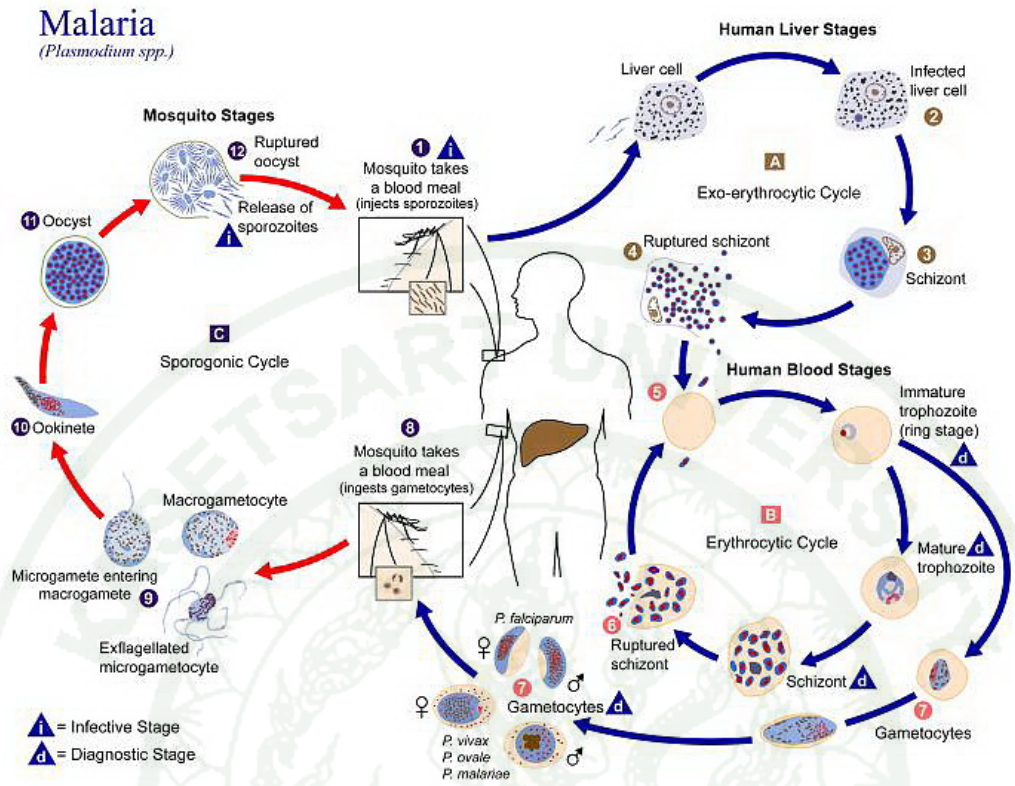


Figure 5 The life cycle of *Plasmodium*

Source: Hausmann and Hülsmann (1996**)**.

MATERIALS AND METHODS

Materials

1. Plant Material

The samples of *A. ciliata* were collected from Trat Agroforestry Research and Training Research station, Kasetsart University, Research and Development Institute, Trat province, in the eastern part of Thailand during June 2009 and July 2010. Beside, The other samples of *A. ciliata* were collected from agricultural field at Tambon bankhamyai, Ampur Muang, Ubon Ratchathani province, Thailand. Botanical identification was achieved through The Systematics of *Acmella* (Asteraceae-Heliantheae) (Jansen, 1985).

2. General Technique

2.1 Chromatography

2.1.1 Thin layer chromatography (TLC)

Technique	: one way ascending
Absorbent	: silica gel 60 F_{254} (0.2 mm thickness, 20×20 cm^2 Merck) supported on glass plate
Plate size	: 10 cm × 20 cm and 20 cm × 20 cm
Layer thickness	: 250 µm.
Solvent system	: hexane : chloroform : ethyl acetate (50 : 50 : 20)
Distance	: 17 cm
Temperature	: 25-30 °C

2.1.1 Thin layer chromatography (TLC) (continued).

Detection	: 254 nm and 365 nm UV light (Ultraviolet Radiation Obligatory eye protection: Villber Lourmal serial No. V01 5636).

2.1.2 Dry column chromatography.

Column size	: 5 cm diameter, 100 cm in height
Absorbent	: 60 g of siliga gel 60 (0.2 - 0.5 mm, 35 - 70 mesh: Merck)
Packing	: Dry packing technique
Sample loading	: 1 g of lipophilic extract was dissolved in a small amount of suitable organic solvent, mixed with small quality of absorbent, dried and then placed on the top of column.
Mobile phase	: hexane, diethyl ether and methanol from 95 : 5 : 0 to 0 : 0 : 100
Fraction volumn	: 50 ml in Erlenmeyer flask
Detection	: Fractions were investigated by TLC technique. Fractions with similar chromatographic pattern were combined.

2.1.3 Medium pressure liquid chromatography (MPLC).

MPLC technology	: ISCO Type 9 optical unit
Pump	: pump model QD-1 ssy (1-10 bar) Fluid Metering, Inc. or a chromatographic pump
Column	: glass column (400×40 mm)
Absorbent	: Lichroprep silica gel 60 (25-40 µm, 132 TA 145390)
Mobile phase	: Mixture of 5%, 10%, 15%, 30%, 50% and 70% ethyl acetate in hexane.
Flow rate	: 30 ml/min
Sample	: 1 g
Detector	: absorbance/ fluorescence detector with wave length 254 nm: ISCO UA-5
Fraction	: Collect the fraction eluted from column by chromatogram appearing.

2.1.4 High Performance Liquid Chromatography (HPLC)

HPLC technology : Agilent 1100 series

Detector : ultraviolet(UV) photodiode array detector 230 nm

Column : reverse phase C_{18}: 250x4.6 mm ChromSepher 5 Part number CP 29358

sample : 10 mg/ml of lipophilic extract filtered with 13 mm× 0.45 µm Nylon filter (Iso-disc^{TM} N-13-4)

Inject : 20 µl

Flow rate : 1.0 ml/min

Time : 30 mins

Solvent system : methanol gradient 60%-100% (HPLC grade Merck) in aqueous buffer (0.015 M tretrabutyl ammonium hydroxide ($C_{16}H_{37}NO$, AR grade Fluka) and 0.015M ortho-phosphoric acid (AR grade Merck), pH 3)

Mobile phase :

Time(Min)	MeOH	Buffer
0.00	60	40
17.00	90	10
22.00	100	0
28.00	100	0
29.00	60	40

2.2 Spectroscopy

2.2.1 Ultraviolet Spectroscopy

Ultraviolet (UV) spectra were determined from HPLC Agilent 1100 series UV photodiode array detector 230 nm wave length at Science Instrumentation Center, Faculty of Science, Kasetsart University, Bangkok, Thailand.

2.2.2 Infrared(IR) Absorption Spectra

Infrared Absorption Spectra were determined on FT-IR Spectrometer series Nicolet 6700 (Thermo Fisher Scientific) at Salaya Central Instrument Facility, Institute of Molecular Bioscience, Mahidol University, Nakhon Pathom, Thailand.

2.2.3 Mass Spectroscopy (MS)

Mass Spectroscopy were determined on Liquid Chromatography Mass Spectroscropic (LC-MS), Burker Daltonics micro TOF 72 at Department of Chemistry, Faculty of Science, Mahidol University, Payathai, Bangkok, Thailand.

2.2.4 Nuclear Magnetic Resonance (NMR) spectra

1D and 2D NMR were recorded with varian Mercury 400 NMR spectrometer and a Bruker Avance 400 at 400 MHz for ^{1}H and ^{13}C, at Department of Chemistry, Faculty of Science, Chulalongkorn University, Bangkok, Thailand.

2.3 Physical property

2.3.1 Melting point apparatus

Melting points were undertaken by using a Stuart Scientific apparatus serial number R000 100381 at Department of Chemistry, Kasetsart university, Bangkok, Thailand.

3. Chemicals

The organic solvents utilized in extraction were methanol (CH_3OH: AR grade Merck), chloroform ($CHCl_3$: AR grade Merck, Labscan) and distilled water (H_2O: 16 MΩ/cm, Millipore).

The chemicals for chromatography were ethyl acetate ($CH_3OOC_2H_5$: AR grade Labscan), hexane ($CH_3(CH_2)_4CH_3$: AR grade B & J) and diethy ether ($(C_2H_5)_2O$: AR grade B & J, Labscan)

The reagents for phytochemical screening.

- Dragendorff's reagent : (bismuth subnitrate ($BiO(NO_3)$), H_2O: AR grade Merck), glacial acetic acid (CH_3COOH: AR grade Merck), distilled water (H_2O: 16 MΩ/cm Millipore)
- potassium iodide : (KI: AR grade Merck)
- 10% Sodium hydroxide (NaOH: AR grade Mallinckrodt)
- Kedde'sreagent (3,5-dinitrobenzoic acid ($C_7H_4N_2O_6$: AR grade Fluka)), potassium hydroxide(KOH: AR grade Univar) and ethanol (C_2H_5OH: AR grade Merck)
- Raymond'sreagent (1,3-dinitrobenzene ($C_6H_4N_2O_4$: AR grade Fluka))
- acetic anhydride
- 97%sulfuric acid (H_2SO_4: AR grade Fisher Scientific)
- hydrochloric acid (HCl: AR grade Merck)

- ferric chloride ($FeCl_3$)
- anisaldehyde ($C_8H_8O_2$: AR grade Fluka)
- iodinecrystals (May & Baker LTD Dagenham England)
- chloroquine

Methods

1. Extraction and Isolation

1.1 Extraction

The samples of *A. cilliata* were collected for voucher specimen collection and extraction. The samples were separated into three parts; leaves, inflorescence and underground part then left to air dry at room temperature. The dried samples were groundto powder, macerated in methanol for 7 days at room temperature. The extracts were filtered and subsequently concentrated by using rotary evaporator at 37 °C afforded crude extract (Methanolic extract). The crude extract was separated between distilled water and chloroform in separatory funnel. The lipophilic extract was obtained from lower part of separatory funnel with chloroform, then was evaporated to dryness, and used in the following experiments (Figure 6).

1.2 Isolation

The lipophilic extracts were isolated by dry column chromatography technique. Silica gel was used as the absorbent (silica gel 60) and eluted with hexane-diethyl ether,diethyl ether, diethyl ether-methanol to give 16 fractions of approximately 50 ml each and washed down with methanol. The fractions were combined according to their TLC pattern.

The combined fractions from column chromatography were analyzed the chemical character by high performance liquid chromatography with UV photodiode

array detector 230 nm wave length. Subsequently, The combined fractions were isolated by medium pressure liquid chromatography technique.

1.2.1 Isolation of Compound BU1

The orange volatile oil was obtained from fraction B of underground parts after evaporated to dryness. The colorless needle crystals presented in the oil after left at room temperature for 1-2 hours. The crystals were separated and washed to purify by diethyl ether (Figure 7).

1.2.2 Isolation of Compound DU1

Fraction D of underground part was evaporated and left in room temperature for one days. The crystals were separated and washed by methanol. The colorless needle crystals were obtained in diethyl ether (Figure 7).

2. Preliminary Test

2.1 Thin layer chromatography (TLC)

The lipophilic extracts were spot on TLC plates. After TLC plates were developed, the constituents were analyzed by color detecting day light (DL).

The position of substance zone (spot) in a thin layer chromatogram can be described as Retardation Factor (R_f) (Hahn-Deinstrop, 2007)

$$R_f = \frac{\text{Distance of substance zone (spot) from the starting line (cm)}}{\text{Distance of the solvent front from the starting line (cm)}}$$

The TLC places were sprayed with a detecting reagent; (Table 4)

- Dragendorff's reagent for alkaloids detection
- Anisaldehyde-sulfuric acid for terpenoids detection

- Kedde's and Remond's reagent for unsaturated lactone ring detection

- 10% NaOH for coumarin detection

- 5% ethanolic solution of 95% sulfuric acid as general visualization reagent and detect color spots after warmed at 110°C till the spots appeared (in particular for sterols, steroids, bile acids and gibberellins).

For general detection, put the TLC place in chromatographic tank which saturated by iodine vapour.

For Fluorescence detection, put the TLC place in UV cabinet and investigated under 254 nm and 365 nm.

Table 4 Dyeing reagents for Thin Layer Chromatography.

Reagent	Compound group	Positive test
20% sulfuric acid	organic compounds	colorful spots
Dragendorff's reagent	Alkaloids	orange to red spot
Anisaldehyde's reagent	steroid and triterpenoid	pink or blue
Raymond's and reagent	unsaturated lactone ring	purple spot
Kedde's reagent	unsaturated lactone ring	pink spot
10% NaOH	Coumarin	fluorescence under UV 365

Source: Merck (1980).

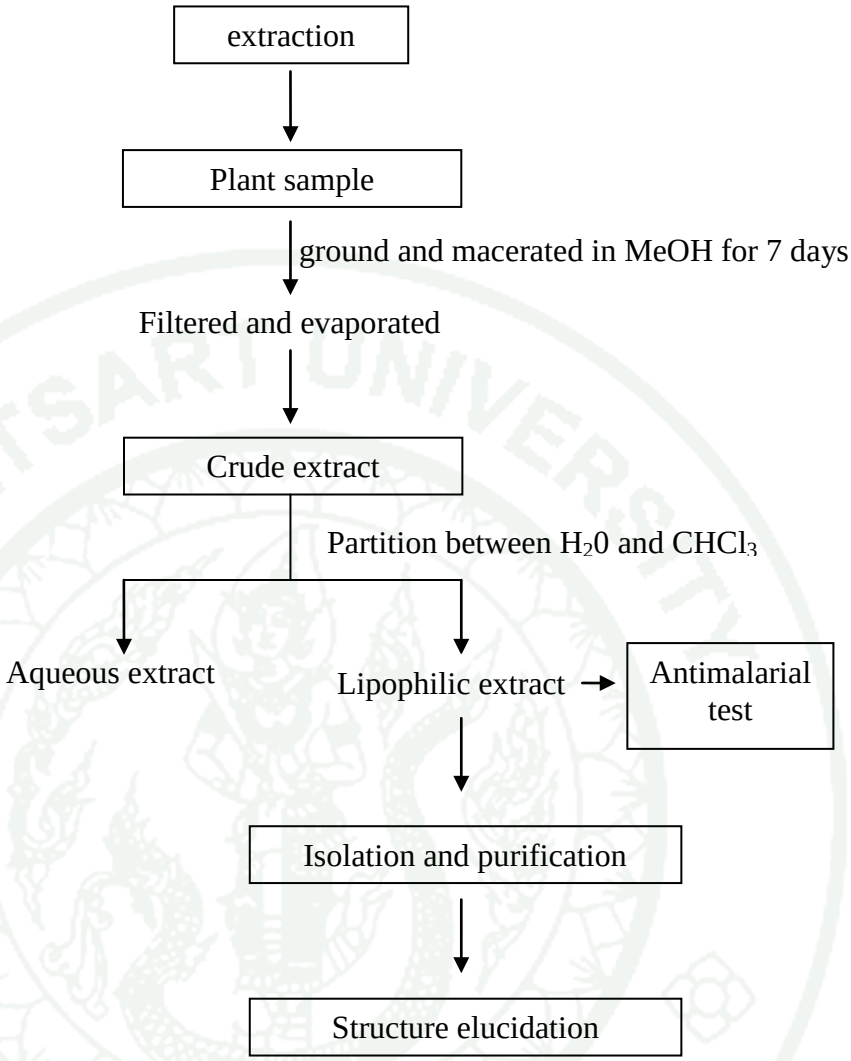


Figure 6 Extraction method of *A. cilliata*

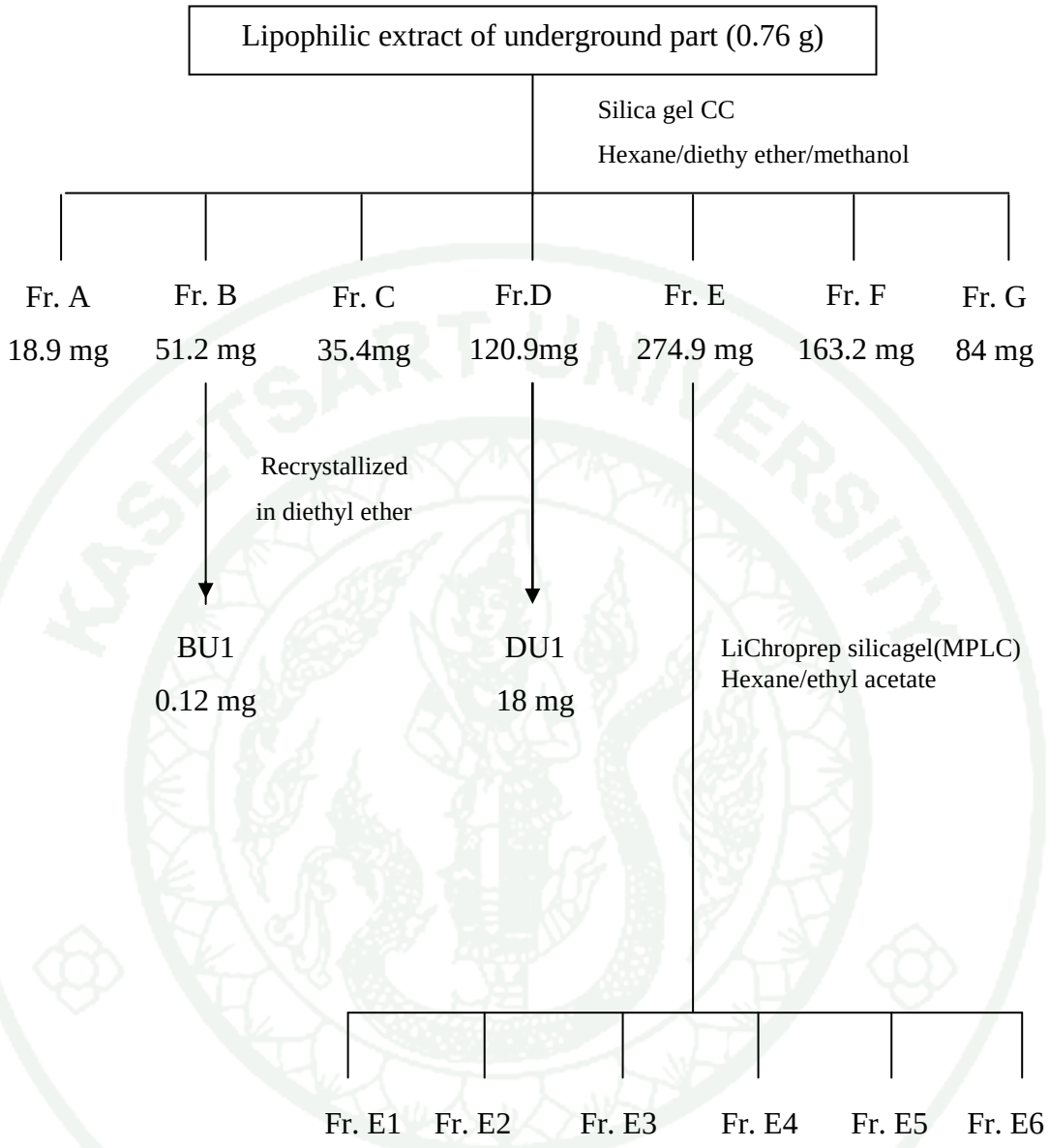


Figure 7 Separation and isolation of lipophilic extract of underground part

3. Antimalarial test

The lipophilic extract was used for antimalarial test under the cooperation of the research project “Diversity of medicinal plants for antiprotozoal which causes malaria, 2010 ” at department of medical technology, Thammasart University, Rangsit campus.

The antiplasmodial activity of the extracts was determined against the chloroquine-sensitive (3D7) and chloroquine-resistant (K1) strains of *Plasmodium falciparum.* The stock solutions of the lipophilic extract contained 100 mg dry extract/ml. The antiplasmodial activity of each extracts was tested in 96-well microtiter plates (Falcon 3070, Becton Dickinson, 8x12 wells). A suspension of parasitized erythrocytes (1% parasitaemia, 2% haematocrit) containing mainly trophozoites was added to the wells to give a final volume of 100 ml.

Places and Duration

Department of Botany, Faculty of Science Kasetsart University June 2009 – July 2012.

RESULTS AND DISCUSSTION

Botanical identification

The botanical identification had done through “The Systematic of *Acmella* (Asteraceae- Heliantheae)” (Jansen, 1985). The samples were collected from Trat Agroforestry Research and Training Research station, Kasesart University Research and Development Institute, Trat province. It was identified as *A. cilliata* Cass. which had inflorescence with radiate, yellow – yellow to orange. Stem is decumbent and rooting at green to purple nodes (Figure 8). The ray florets are sometimes shorter than or only barely exceeding the phyllaries which are biseriate (Figure 9). Ray florets are yellow, tube 0.9-2 mm long, 0.2 – 0.9 mm in dimeter, sparsely to densely pilose (Figure 10). The mature achenes have an evident, stramineous, moderately to densely ciliated with straight-tipped hairs with cork-like margin, lacking shoulder (Figure 11). Pappus usually are a well-developed of 2 bristles.

Figure 8 Stem is cylindrical, succulent, rooting at green to purple nodes.

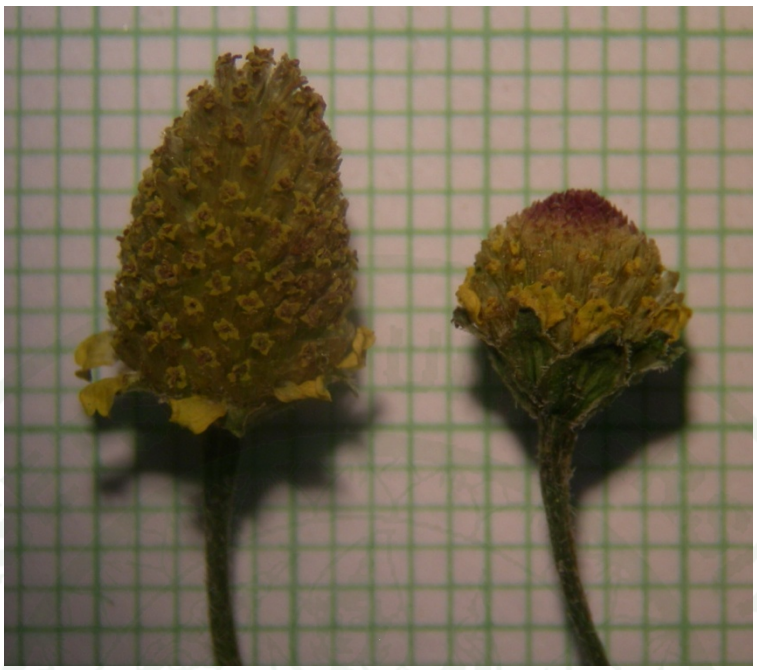


Figure 9 The inflorescence with ray and disc florets

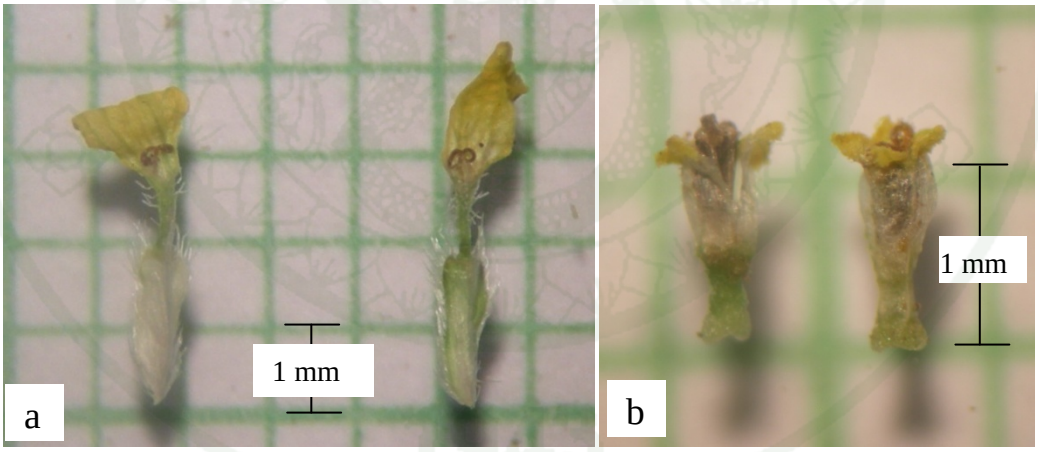


Figure 10 Ray florets (a) and disc corollar tubes of disc floret (b) of *A.ciliata*

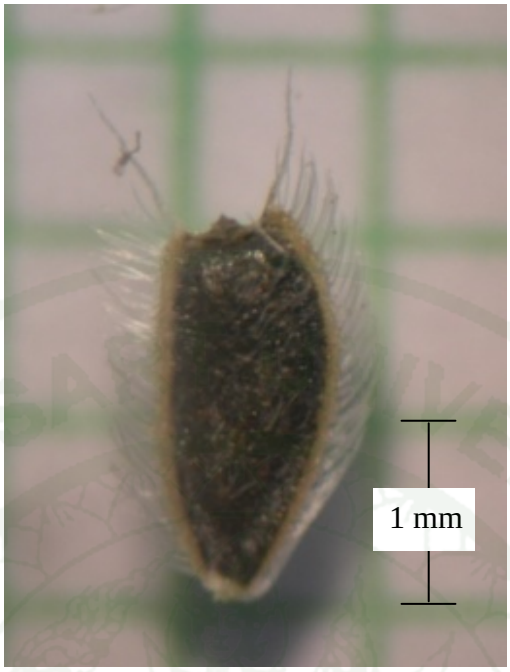


Figure 11 Fruit is achene with evident, cork-like margin.

Preliminary Test

Preliminary phytochemical results of lipophilic extracts from different plant parts; leaves, inflorescence and underground were investigated on the basis of their TLC profiled. The chemical constitutes were separated with color and colorless in day-light. The TLC plate was detected by different reagents in order to investigate the chemical constituents, the results showed in the Table 5. The positive test for terpenoids, steroids and triterpenoids presented in all parts of *A.ciliata*. Coumarins were detected only in the lipophilic extract of underground parts. The colorless of chemical constituents were detected by visualization under UV light at wavelength 254 nm and 365 nm (Figure 12).

Table 5 Preliminary phytochemical analysis of different parts from *A.ciliata*

Chemical constituents	leaves	inflorescence	underground part
All compound (Iodine vapors)	+	+	+
Organic compounds	+	+	+
Alkaloids	-	-	-
Terpenoids	+	+	+
Steroids and triterpenoids	+	+	+
Unsaturated lactone ring	-	-	-
Coumarins	-	-	+

Remark + = positive
- = negative

Comparison for three plant parts; leaves, inflorescence and underground part could be done. The Figure 12, TLC plate observed under UV 254 nm showed of dark spots that appeared at the same at R_f values 0.53, 0.57, 0.65, 0.68 and 0.80 in all three parts. However when observed TLC plate under UV 365 nm, the blue fluorescent spots appeared at R_f values 0.25 in both inflorescence and underground part. Moreover, there are four fluorescent spots at the R_f values 0.39, 0.57, 0.63 and 0.77 respectively in underground part extract (Figure 12).

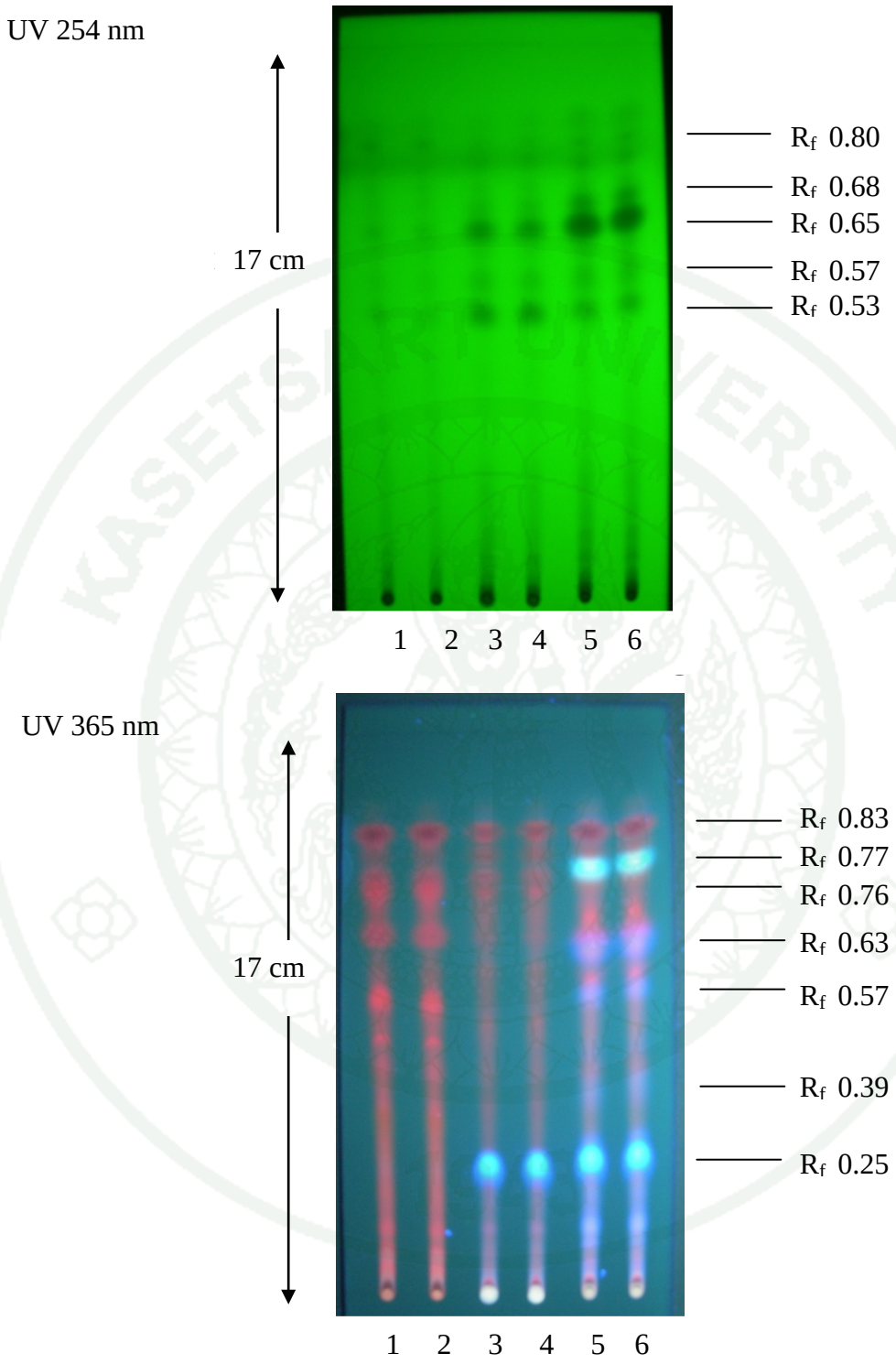


Figure 12 TLC pattern under UV 254 nm and UV 365 nm

1, 2 leaves extract

3, 4 inflorescence extract

5, 6 underground part extract

Iodine vapors was used to detect general compounds. The TLC place was showed as Figure 13 that all R_f values could be detect by iodine vapors. The underground parts showed dominant detection. However, this method worked on several compounds but not very sensitive.

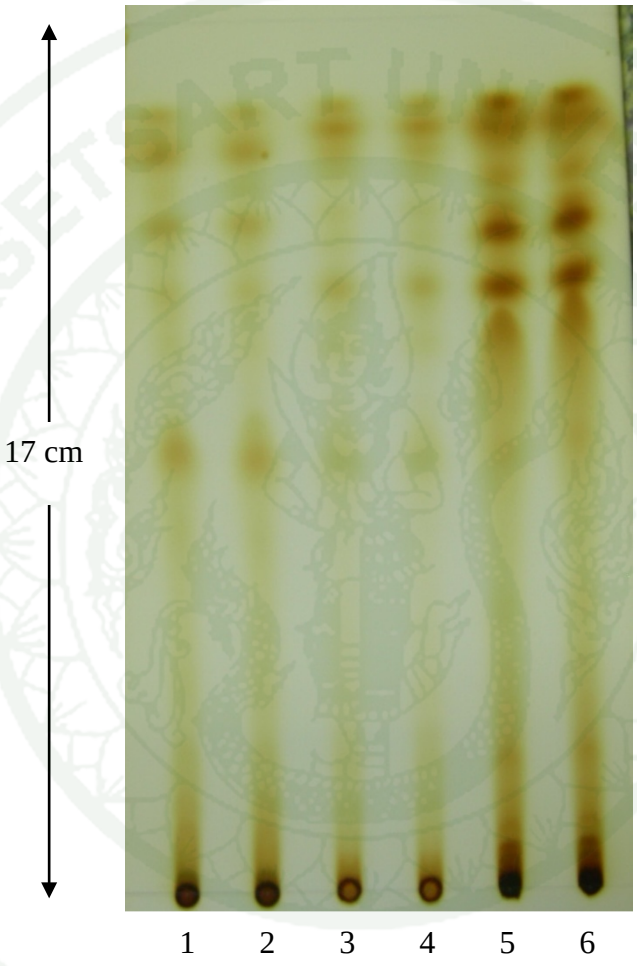


Figure 13 TLC pattern detected by Iodine vapors
1, 2 leaves extract
3, 4 inflorescence extract
5, 6 underground part extract

.

After spraying developed TLC plate with anisaldehyde-sulfuric acid reagent for terpenoids detection. Color range from violet (lichens constituents), blue (phenol), red (terpenes) and gray-green (sugar and steroids) (Merck, 1980). The result was shown in the Figure 14, all lipophilic extracts gave a color as red at R_f 0.39 indicated that all lipophilic extract contained terpenes. The violet color at R_f 0.76 and blue color at R_f 0.68, 0.83 indicated that all plant parts contain lichen constituents and phenols, respectively.

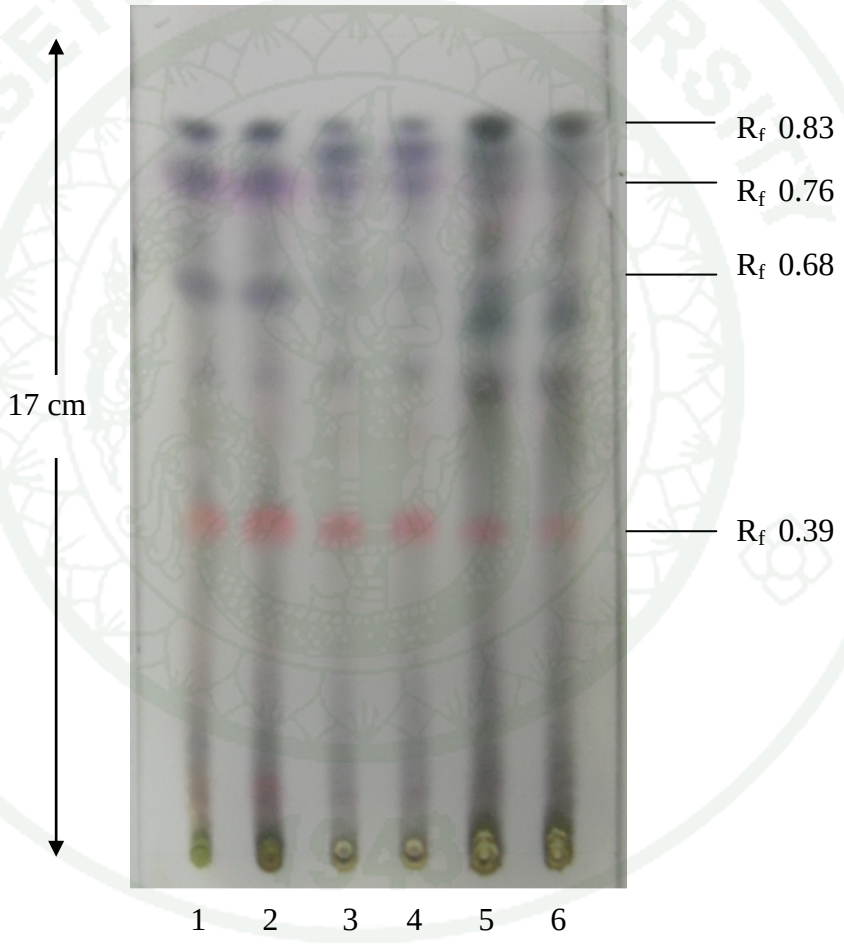


Figure 14 Terpenoids detection, after spraying developed TLC plate with anisaldehyde – sulfuric acid from
1, 2 leaves extract
3, 4 inflorescence extract
5, 6 underground part extract

To examine the coumarins, the TLC place was sprayed with 10%NaOH and investigated under UV light at wavelength 365nm. The result was shown in the Figure 15, there were positive test in leaves and underground part extracts which gave a bright blue fluorescence under UV light at the same R_f values as 0.24 (Figure 15B). The others coumarins were detected in underground part at R_f values 0.39 and 0.63. The results indicated that leaves and underground part contained coumarins. However the coumarins could not detected in inflorescence.

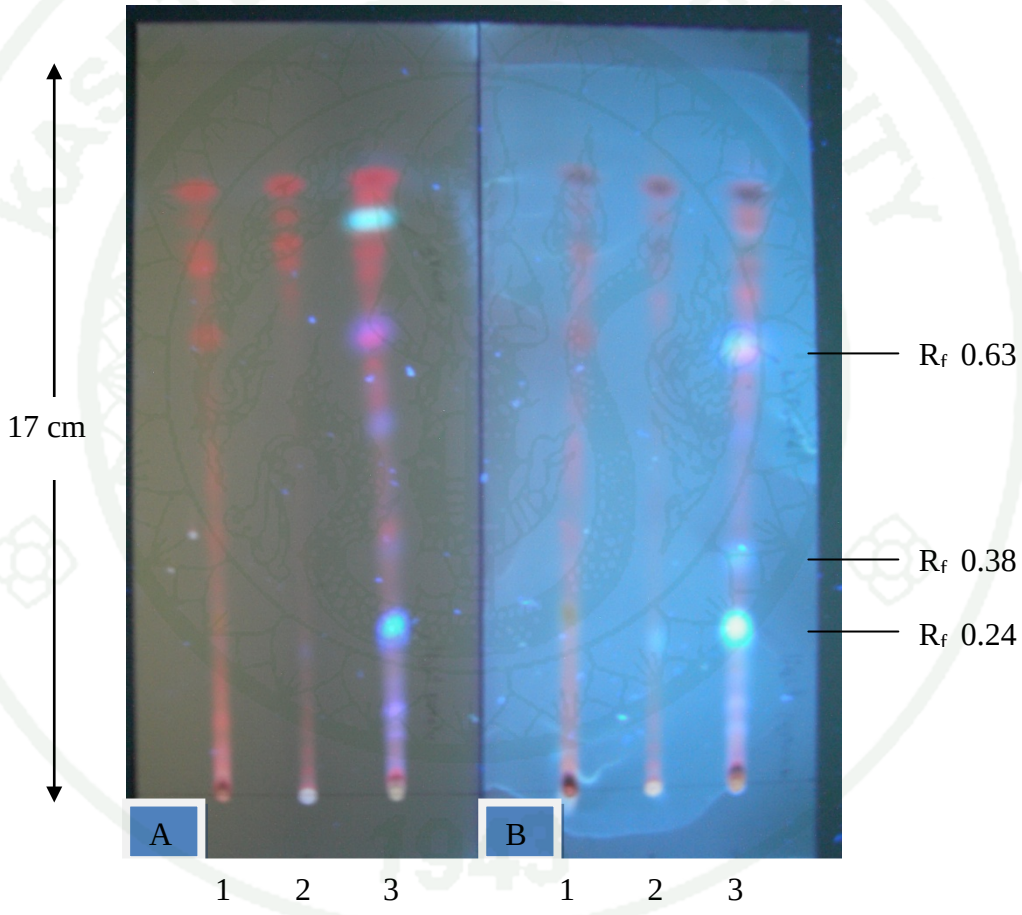


Figure 15 Coumarins detection under UV light at the wavelength 365nm

A. before spraying with 10%NaOH.

B. after spraying detected TLC plate with 10%NaOH.

1, 2 leaves extract

3, 4 inflorescence extract

5, 6 underground part extract

The 5% ethanolic solution of sulfuric acid as general visualization reagent, was sprayed on developed TLC plate and heat to 110 °C until maximal visualization of the spots. Cholesterol and vitamin A, their esters and many isoprenoid lipids show characteristic colours. Cholesterol and esters first turn red, then red- violet and brown while vitamin A and ester first turn blue. Most compounds may be subsequently charred, yielding dark spots (Merck, 1980). The results were shown in the Figure 14 that all components in lipophilic extracts first turned blue and brown at R_f value 0.59 ,0.83 respectively. The lipophilic extract of underground part turn brown at R_f value 0.63. The components in both inflorescence and underground part extracts turned brown at R_f value 0.8 (Figure 16).

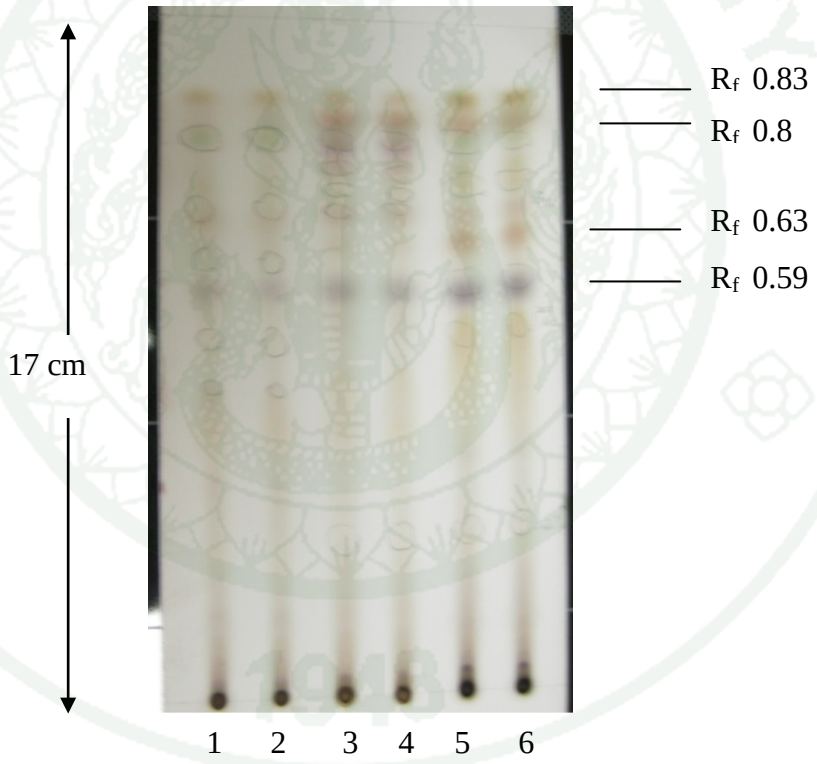


Figure 16 Sterols and steroids detection, after sprayed developed TLC plate with 5% ethanolic solution of sulfuric acid and heated at 110 °C.
1, 2 leaves extract
3, 4 inflorescence extract
5, 6 underground part extract

Dragendorff is reagent, was sprayed onto TLC plate in order to detect alkaloids (Farnsworth, 1966). The positive test was orange to red precipitation becomes, heavy metal containing in the reagent (Bismust (III)) would react to nitrogenous base in alkaloids and form insolutable complex heavy metal salt. After sprayed developed TLC plate with Dragendorff's reagent, there were negative test in all extracts.

In addition, The lipophilic extract of leaves, inflorescence and underground parts were analyzed by HPLC technique. The HPLC system gives out two types of data images. One is called a chromatogram, and the other is UV spectrum. The chromatogram is graph that monitors the signal in the detector over time. As chemicals are detected by the instrument, the signal increases and the chromatogram displays a "peak". Each peak in the chromatrogram indicates the presence of a chemical in the sample. The peak is labeled with retention time (Rt) which indicates how long it takes for a compound come out of the HPLC column. This time is measured from the time at sample is injected to the point at which the display shows a maximum peak height for that compound. The UV spectrum is signatures produced by each chemical in the detector.

Firstly, The lipophilic extracts of leaves, inflorescence and underground part were analyzed, chromatogram and UV spectrum of three extracts could be compared. In the Figure 17 and Table 6, the result showed the chromatograms of lipophilic extracts from leaves, inflorescence and underground part were different. Although, the chromatogram of inflorescence and underground parts showed the dominant peak at Rt 12.852 and 12.976 respectively, however the UV spectrum of both compounds were different (Figure 17). The lipophilic extract of leave showed several peaks which were not sharp, so they might not be pure compounds.

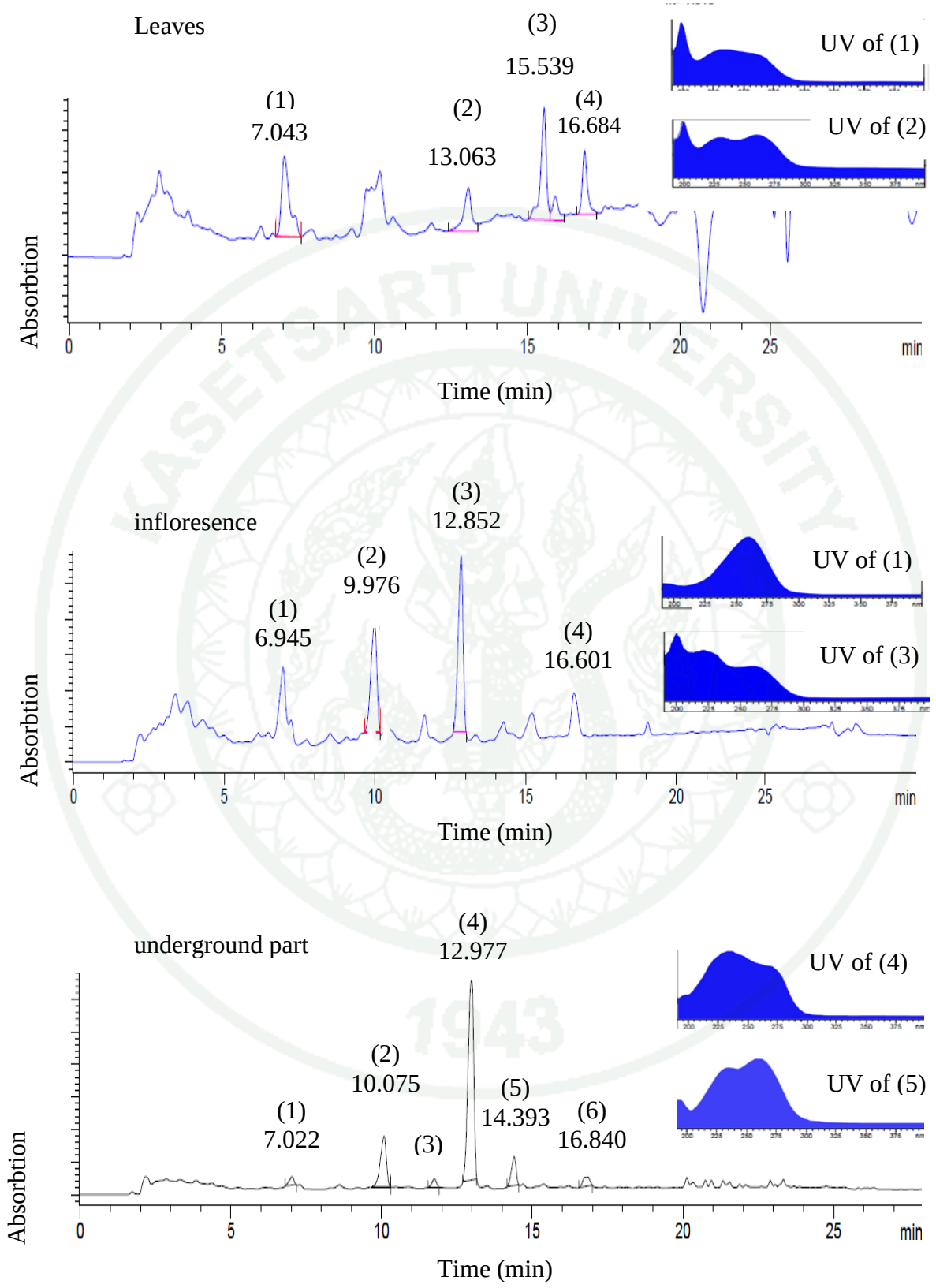


Figure 17 HPLC chromatogram and UV spectrum of lipophilic extracts from leaves, inflorescence and underground part.

Table 6 Retention time (Rt) and area of chromatogram of lipophilic extracts from leaves, inflorescence and underground part**.**

Leaves			Inflorescence			Underground part		
Peak No.	Rt (min)	Area (%)	Peak No.	Rt (min)	Area (%)	Peak No.	Rt (min)	Area (%)
1	7.043	16.0086	1	6.945	7.8423	1	7.022	1.1517
2	13.063	8.8388	2	9.976	13.0759	2	10.075	8.7810
3	15.539	15.7237	3	12.852	15.6598	3	11.751	1.1914
4	16.864	8.0011	4	16.601	8.9426	4	12.977	35.646
						5	14.393	3.9317
						6	16.840	1.9118

Secondly, it was due to the result of *In vitro* antiprotozoal test that underground part extract showed the highest activity against resistance strain of *Plasmodium falciparum*. The underground part of *A. ciliata* were collected from Trat and Ubon Ratchathani province. The samples were compared by HPLC technique showed similarity of both sample (Figure 18). However, when compared each peak at the same retention time by UV spectrum, it was shown that those two peak (4 and 2) were not the same compound. From Table 7 there was only peak no. 5 of Trat and peak no. 3 of Ubon Ratchathani at Rt 14.044 and 14.392 respectively that showed similar UV spectrum and it could be the same compound. The lipophilic extract of underground part was isolated in order to get pure compound.

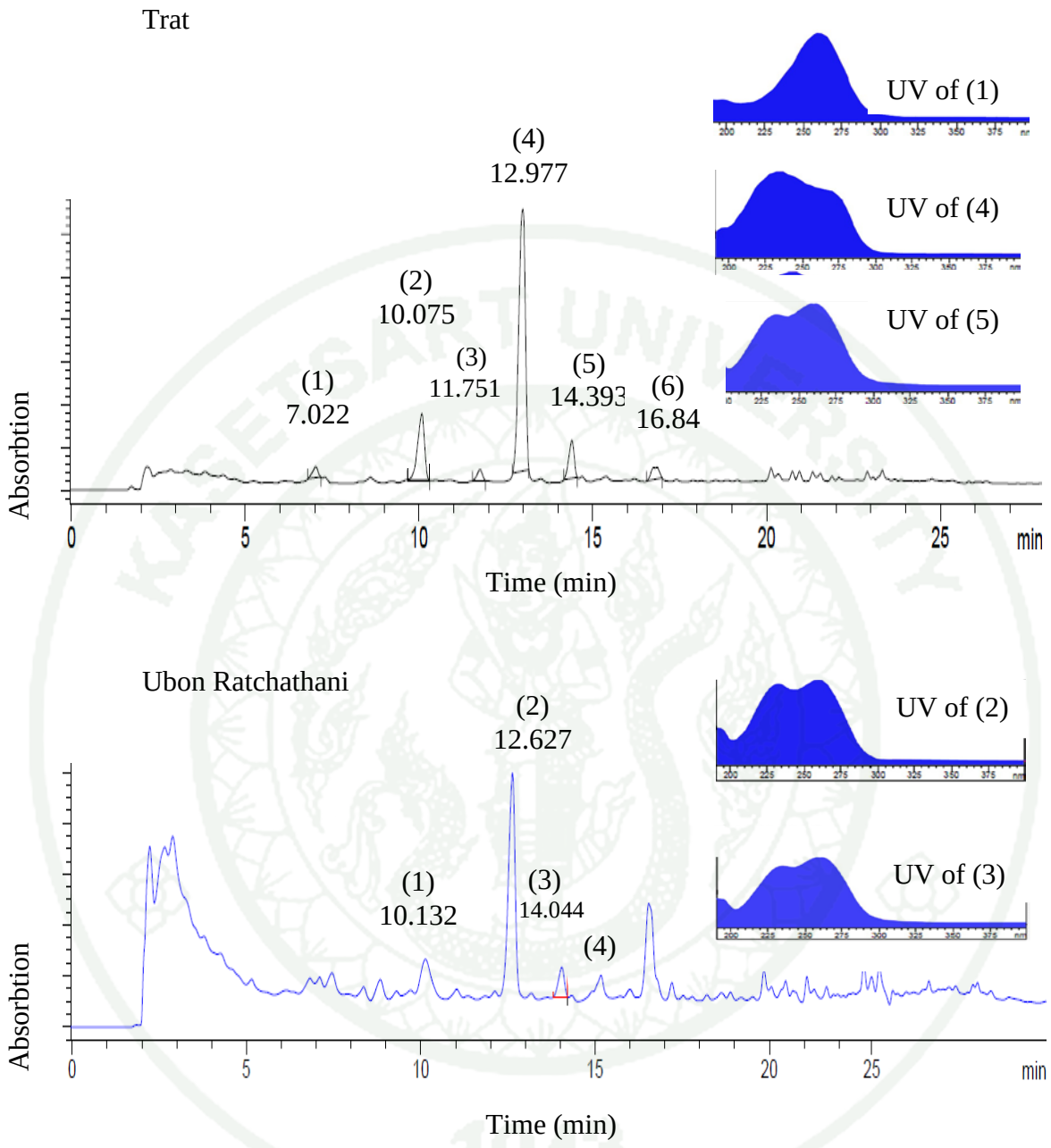


Figure 18 Comparison of HPLC chromatogram and UV spectrum of underground part extracts from *A. ciliata* collected from Trat and Ubon Ratchathani province.

Table 7 Retention time (R_t) and area of chromatogram of underground part which were compared between samples from Trat and Ubon Ratchathani province.

Trat			Ubon Ratchathani		
Peak No.	Rt (min)	Area (%)	Peak No.	Rt (min)	Area (%)
1	7.022	1.1517	1	10.132	8.3989
2	10.075	8.7810	2	12.627	15.2313
3	11.751	1.1914	3	14.044	4.4512
4	12.977	35.6476	4	15.168	7.2960
5	14.393	3.9317	5		
6	16.840	1.9118	6		

Isolation and purification

The lipophilic extract of underground part was isolated using dry column chromatography and were eluted by hexane, diethylether and methanol with various gradations, eighteen fractions were collected. Based on the TLC analysis of the separated fraction (Table 8 and Appendix 1-3), the fractions containing compounds with similar R_f values were combined into A (fraction 1I-1II), B (2I-2II), C (3I), D (3II-4I), E (4II-5II), F (6I-7I), G (7II-9II), respectively.

Combined fraction E was concentrated to dryness. Fraction E was isolated by MPLC technique, however the crystals were not obtained.

Table 8 Column chromatography information of lipophilic extract of underground part.

Fraction number	Solvent hexane:diethy ether:methanol	Combined fraction	Remark
1I-1II	95:5:0	A	non fluorescence
2I-2II	90:10:0	B	green fluorescence under UV light (254 nm)
3I	75:25:0	C	non fluorescence
3II-4I	75:25:0–50:50:0	D	violet, red and aqua fluorescenceunder UV light (365 nm)
4II-5II	50:50:0 - 0:25:75	E	Aqua, blue,violet, pink and red fluorescence under UV light(365 nm)
6I-7I	0:50:50 – 0:10:90	F	violet, blue and red fluorescence under UV light (365 nm)
7II-9II	0:10:90 – 0:0:100	G	non fluorescence

Structure determination of compound isolated from *A. cilliata*

1. Characteristics of compound BU1

Compound BU1 was isolated as colourless needle crystals (Figure 19). After purification by recrystallization few amount of pure compound could be obtained but not enough for structure elucidation.

Melting point : 263-264 °C

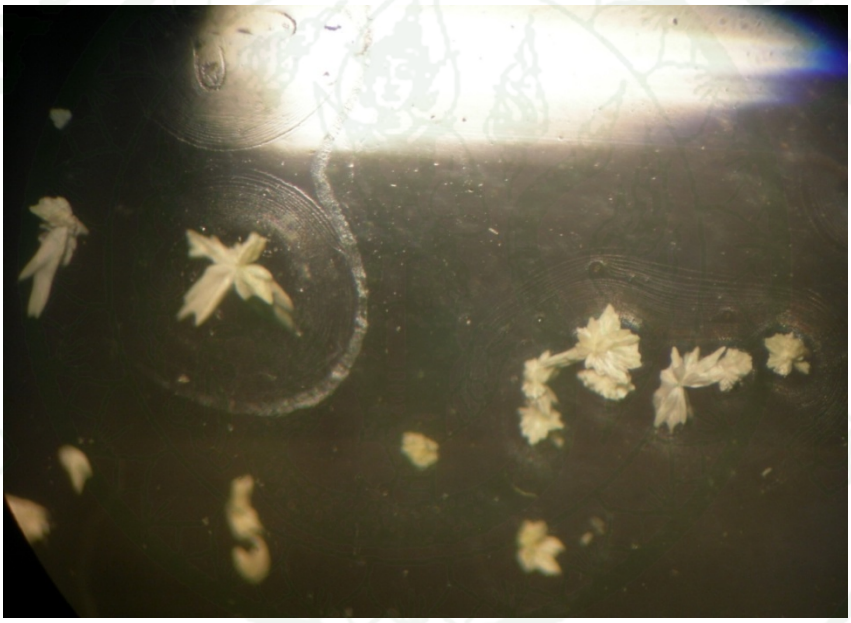


Figure 19 Colorless needle crystals of compound BU1.

2. Characteristics of compound DU1

The underground part samples 231 g, were isolated and obtained compound DU1 which was colorless needle crystals (Figure 20) and detected by TLC technique spraying with anisaldehyde-sulfuric acid reagent. A single violet spot was present.

Solubility : soluble in isopropanol, chloroform, diethyl ether and hexane.

R_f values : 1) 0.35 in hexane: chloroform: ethyl acetate (5:5:2) (Appendix Figure.4)
2) 0.65 in hexane: chloroform: methanol (4:6:2) (Appendix Figure.5)
3) 0.31 in chloroform (Appendix Figure 6)
4) 0.56 in ethyl acetate (Appendix Figure.7)

Melting point : 161-162 °C

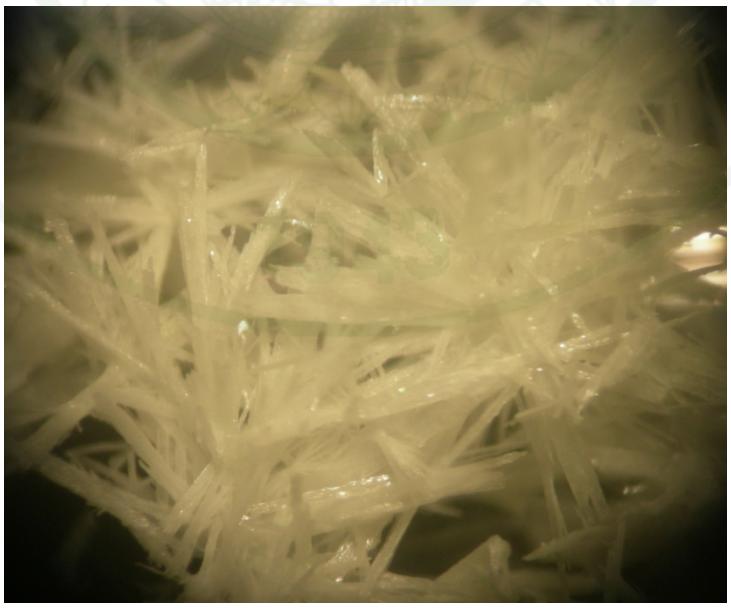


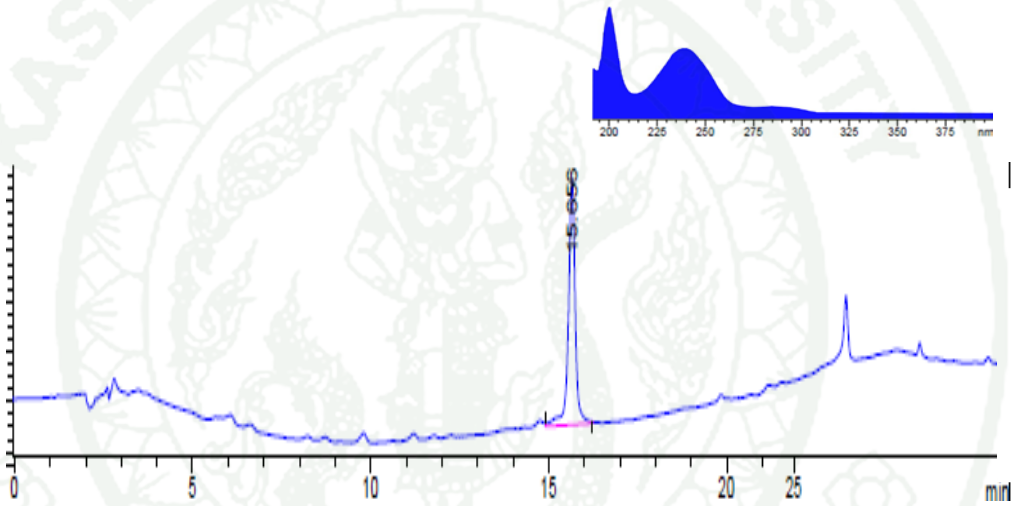
Figure 20 Colorless needle crystals of compound DU1

2.1 Ultraviolet spectrometer (UV)

UV spectrum of DU1 presented at retention time (Rt) 15.655 min in mixed solvent system of 0.1 N Ortho-phosphoric acid in acetonitile and 0.1 N Ortho-phosphoric acid in ultrapure water (Figure 21).

UV spectrum: UV λ max ^{Isopropanol} (nm) 200, 240, 285

(Appendix Figure 8)



Time (min)

Figure 21 HPLC chromatogram and UV spectrum of compound DU1

2.2 Fourier Transform Infrared Spectrometer (FT-IR) FT-IR V max cm^{-1}(Appendix Figure.9)

FT-IT, ν_{max} (cm^{-1}) = 3339.64 cm^{-1} (O-H); 2935.41 cm^{-1} and 2864.20 cm^{-1}(aliphatic C-H); 1666.57 cm^{-1} (C=C); 1462.03 cm^{-1} (CH2); 1332.13 cm^{-1} (OH def); 1046.73 (cyclohexane)

2.3 Mass spectrometer (MS)

EI mass spectroscopy (Appendix Figure 10) showed molecular ion peak at *m/z* 412.36 and 414.38, suggesting the molecular formula of $C_{29}H_{48}O$ and $C_{29}H_{50}O$, Respectively.

2.4 Nuclear Magnetic Resonance Spectrometer (NMR)

2.4.1 ^{1}HNMR (δ ppm, 400 MHz $CHCl_3$) (Appendix Figure 11)

^{1}HNMR had give the signals at δ 3.2 (1H, m, H-3), 5.26 (1H, m, H-6), 5.19 (1H, m, H-23), 4.68 (1H, m, H-22), 3.638 (1H, m, H-3), 2.38(1H, m, H-20), 1.8-2.0 (5H, m) ppm. Other peaks were observed at δ 0.76-0.89 (m, 9H), 0.91-1.05 (m, 5H), 1.35-1.42 (m, 4H), 0.69-0.73 (m, 3H), 1.8-2.0 (m, 5H), 1.07-1.13 (m, 3H), 1.35-1.6 (m, 9H)

2.4.2 ^{13}CNMR (δ ppm, 400 MHz $CHCl_3$) (Appendix Figure 12)

Further comparison of the ^{13}CNMR spectral data of DU1 with the data reported in the literature (Rubinstien *et al*., 1976) (Pateh *et al*., 2009) (Kamboj and Saluja, 2011) confirmed that this compound was a mixture of stigmasterol (Figure22) and β-sitosterol (Figure 23) (Table 9).

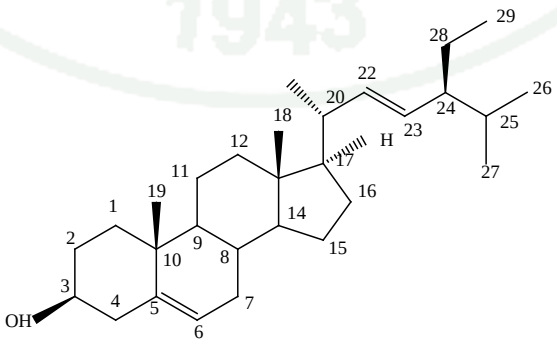


Figure 22 Chemical structure of stigmasterol.

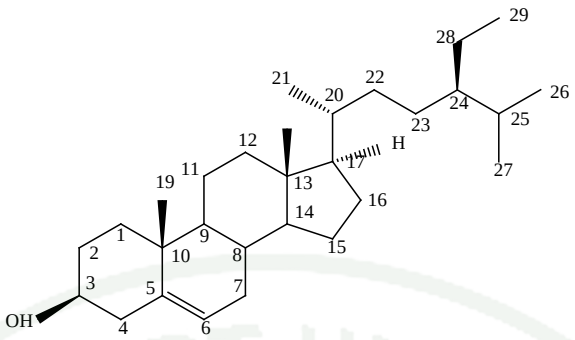


Figure 23 Chemical structure of β–sitosterol.

Table 9 Comparison of the ^{13}CNMR spectral data of stigmasterol and β–sitosterol and compound DU1 (a mixture of DU1A and DU1B) ($CDCl_3$, 400 MHz).

Position	DU1A	Stigmasterol	DU1B	β–sitosterol
1	37.3	37.2	37.3	37.2
2	31.7	31.7	31.7	31.7
3	71.8	71.8	71.8	71.7
4	42.3	42.4	42.3	42.3
5	140.8	140.8	140.8	140.8
6	121.7	121.7	121.7	121.7
7	32.1	32.0	31.9	31.9
8	32.1	32.0	31.9	31.9
9	50.2	50.2	50.1	50.1
10	36.5	36.6	36.5	36.5
11	21.1	21.1	21.1	21.1
12	39.7	39.7	39.8	39.8
13	42.3	42.4	42.2	42.3
14	56.9	56.9	56.8	56.8
15	24.4	24.4	24.3	24.3
16	29.2	29.0	28.2	28.2

Table 9 (Continued)

Position	DU1A	Stigmasterol	DU1B	β–sitosterol
17	56.1	56.1	56.1	56.0
18	12.1	12.1	11.9	11.9
19	19.4	19.4	19.4	19.4
20	40.5	40.5	36.1	36.1
21	21.1	21.1	18.8	18.8
22	138.2	138.0	33.9	33.9
23	129.3	129.3	26.1	26.1
24	51.2	51.3	45.8	45.8
25	32.0	32.0	29.2	29.1
26	21.2	21.3	19.8	19.8
27	19.0	19.0	19.0	19.0
28	25.4	25.4	23.1	23.1
29	12.2	12.3	11.9	11.9

Source: Rubinstien *et al.* (1976); Pateh *et al.*,(2008); Kamboj and Saluja (2011)

The main sterols in plants, fungi and algae are characterized by the extra one - carbon or two – carbon substitution on the side-chain attached at C-24. Stigmasterol is one of a group of phytosterol or plant sterols which is an unsaturated plant sterol occurring in the plants fats or oils of soybean, calabar bean, and rape seed, and in a number of medicinal herbs (Paul, 2001).

Stigmasterol, isolated from bark of *Butea monosperma* can reduced serum triiodothyronine (thyroxin) and glucose concentrations as well as the activity of hepatic glucose – 6- phosphatase with a concomitant increase in insulin indicating its thyroid inhibiting and hypoglycemic properties (Panda *et al.*, 2009). The antimicrobial activity was tested by using the following clinical microbes to determine bioactivity of stigmasterol, isolated from *Spilanthes acmella* (syn. *Acmella*

paniculata). The inhibited zone was observed between 20 to 24 mm with *Bacillus subtilis* having the largest zone of inhibition of 24 mm (Yinusa *et al*., 2014).

***In vitro* antiprotozoal test**.

The lipophilic extract of inflorescence and underground part were subjected to *in vitro* screenings for their antimalarial activity against protozoal which caused malaria, *P. falciparum*. Both of inflorescence and underground part extracted exhibited the most active antimalarial activity against *P. falciparum* to chloroquine sensitive strain 3D7 and resistance strain K1. The inflorescence extract showed the most active antimalarial activity against *P. falciparum*, 3D7 and K1 strain at the IC_{50} values of 5.185 and, 9.656, respectively. The underground part showed antimalarial activity, 3D7 and K1 strain at the IC_{50} values of less than 7.8125 and 8.613, respectively (Table 10).

The antimalarial activity of inflorescence and underground part of *A. ciliata* showed high effective activity to chloroquine resistance strain of *P. falciparum*. The antimalarial activity result of *A. ciliata* relate to the antimalarial activity of *A. paniculata* (*S. acmella*) to inhibit that was isolated the purify as alkylamide, spilanthol and undeca-2E-ene-8, 10-diynoic acid butylamide (Spelman *et al.,* 2011).

The underground part extract showed more effective than the inflorescence extract.The result was consistent to another experiment, that the root extract of *Acmella oleraceae* which expressed larvicidal activity against both malarial mosquito vectors (*Anopheles stephensi* Liston and *Culex quinquefasciatus* Say) (Pandey *et al*., 2007)

Table 10 The antimalarial acitvity of lipophilic extracts of *A. cilliata*

Source	Local name	Parts of used	Antimalarial activity: IC_{50} (µg/ml)	
			3D7	K1
A.ciliata	Para cress, toothache plant	leaves	nd	nd
		Inflorescence	5.185	9.656
		Underground parts	7.8125	8.613
Chloroquine			0.004	0.51

nd = not determined

3D7 = chloroquine sensitive strain of *Plasmodium falciparum*

K1 = chloroquine resistance strain of *Plasmodium falciparum*

$IC_{50} < 10$ µg/ml = the most active, $10 < IC_{50} \leq 50$ µg/ml = high moderately active, $50 < IC_{50} \leq 100$ µg/ml = moderately active, $100 < IC_{50} \leq 500$ µg/ml = less active $IC_{50} > 500$ µg/ml = none active.

CONCLUSION AND RECOMMENDATION

Conclusion

1. The sample was identified as *Acmella ciliata*

2. The lipophilic extract of leaves, inflorescence and underground part of *Acmella ciliata* were investigated that contained terpenoids, steroids and triterpenoids. There was only the extract of underground part contained coumarins.

3. HPLC chromatogram among three samples (leaves, inflorescence and underground parts) showed different profiles. The underground part which were collected from different place, (Trad and Ubon Ratchathani) showed similar profile of HPLC chromatogram.

4. The crystals from underground part were identified as stigmasterol and β-sitosterol by using spectroscopic data that included UV, FT-IR, MS, ^{1}H and ^{13}C NMR

5. The underground part showed the most active antimalarial activity to both chloroquine sensitive and resistance strain of *Plasmodium falciparum*

Recommendation

1. Sample of underground part from different places could be compared antimalarial activity to observe the suitable environment for produced secondary metabolites.

2. Some fractions were isolated by dry column chromatography, contained volatile oil that should be investigated by gas chromatography technique.

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APPENDIX

Dyeing reagents for TLC

Dragendorff's reagent

Solution A:

Bismuth sub nitrate ($BiO(NO_3)$): AR grade Merck	0.85 g
Glacial acetic acid (CH_3COOH): AR grade Merck	10.0 ml
Distilled water (H_2O): 16 MΩ/cm Millipore	40.0 ml

Solution B:

Potassium iodine (KI): AR grade Merck	8.0 g
Distilled water (H_2O): 16 MΩ/cm Millipore	20.0 ml

Solution C:

Glacial acetic acid (CH_3COOH): AR grade Merck	20.0 ml
Distilled water (H_2O): 16 MΩ/cm Millipore	80.0 ml

Preparation, 5 ml of solution A was mixed with 5 ml of solution B and 100 ml of solution C. Amines and basic heterocycles like pyridine produce brown-orange spot at retention time. Phosphines and crow ethers are also detected (Farnsworth, 1966).

Anisaldyhyde-sulfuric acid

The reagent was prepared freshly before use by mixture of 0.5 mlanisaldehyde ($C_8H_8O_2$: AR grade Fluka) in 50 ml Glacial acetic acid and 1 ml 97% sulfuric acid (H_2SO_4: AR grade Fisher Scientific) following by heating on plate heater at 100 to 105 °C until maximal visualization of the spots. Variously colored spots appeared on a bright background: terpene (red), sugar (grey), steroid (green), phenol (blue) and lichen constituents (violet) (Merck, 1980).

Kedde's reagent

The reagent was freshly prepared by mixture of Kedde I, 5 mlof 3% ethanolic-3,5-dinitrobenzoic acid ($C_7H_4N_2O_6$: AR grade Fluka) and Kedde II, 5 ml of 2M ethanolic potassium hydroxide (KOH: AR grade Univar) and evaluated in the air.

The reagent was useful for detected of C_{17} unsaturated lactone ring like cardiac glycoside. The present of purple color was assessed as positive result (Farnsworth, 1966).

Raymond's reagent

The reagent was freshly prepared by Raymond I, 5ml of 2% ethanolic-1,3-dinitrobenzene ($C_6H_4N_2O_4$: AR grade Fluka) mixed with Raymond II, 5ml of 5% ethanolic potassium hydroxide and evaluated in the air.

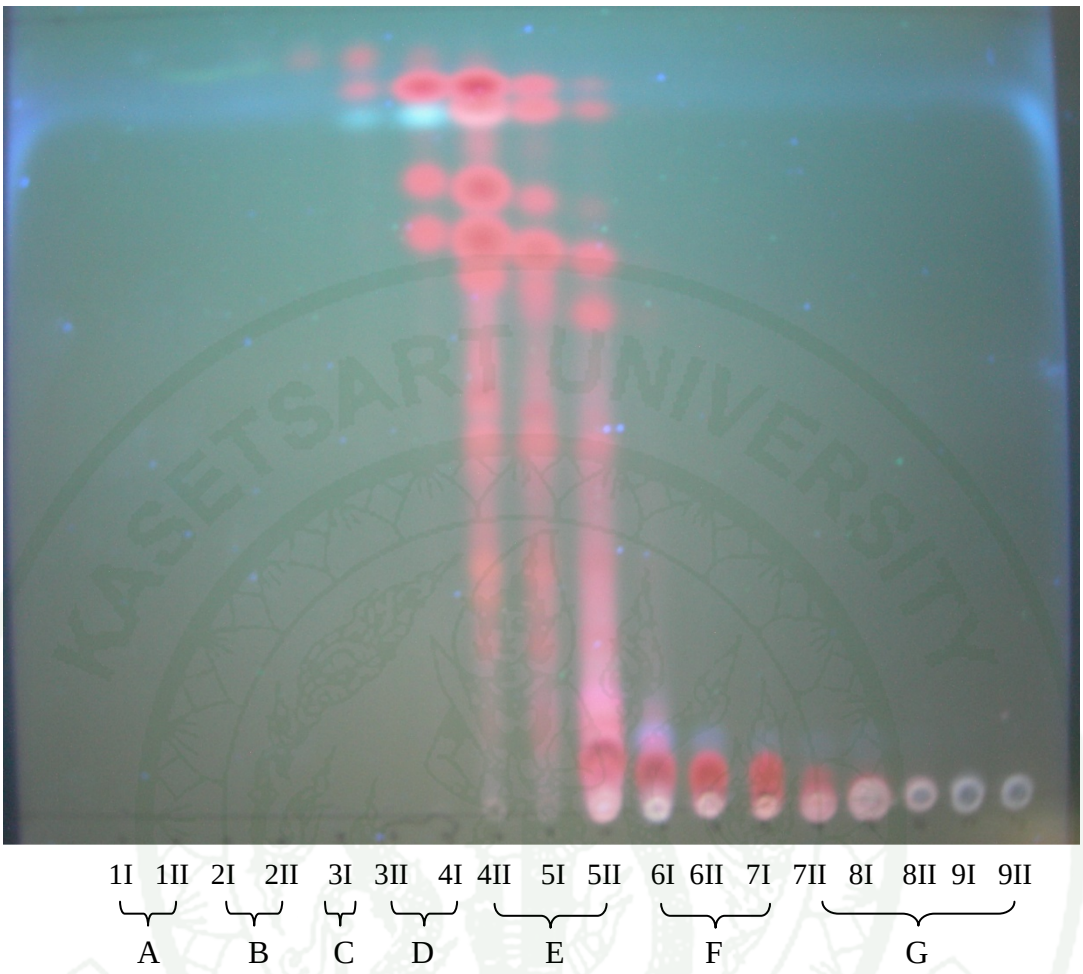
The reagent was useful for detection of C_{17} unsaturated lactone ring like cardiac glycoside. The present of blue color was assessed as positive result (Farnsworth, 1966).

2N sodium hydroxide

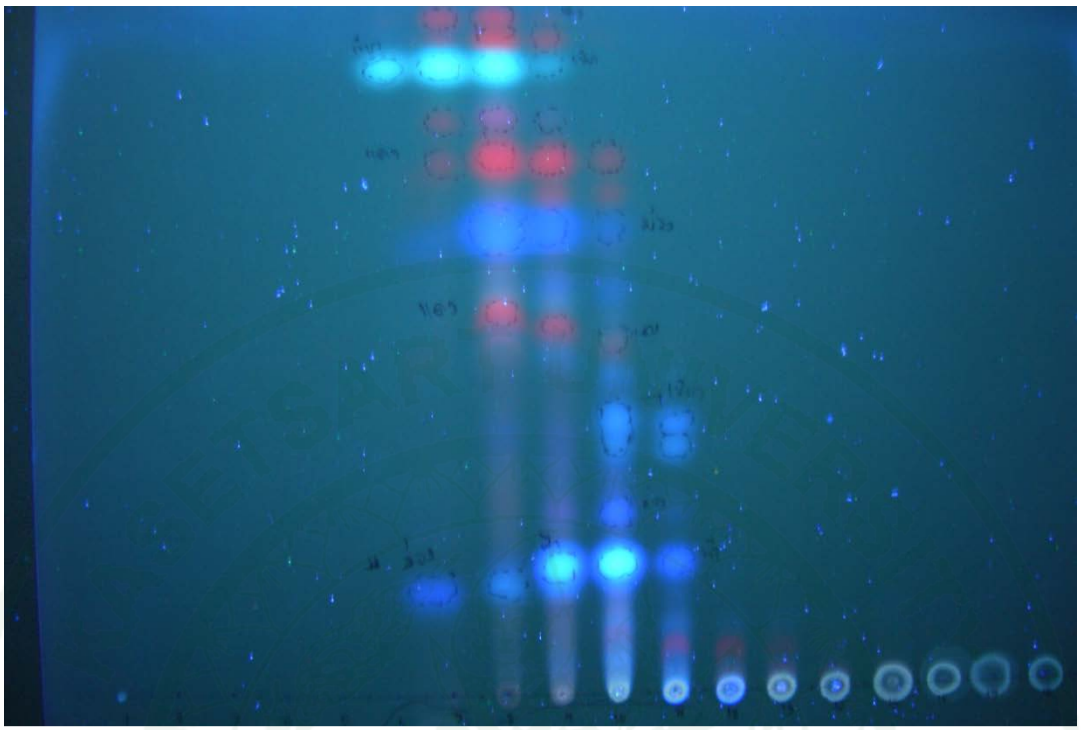
Dissloved 8 g of pellets sodium hydroxide (NaOH: AR grade Mallinckrodt) in 100 ml ethanol. After spraying, evaluated in the air, The chemical constituent was detected as blue fluorescence by visualization under UV light at 365 nm (Farnsworth, 1966).

Iodine

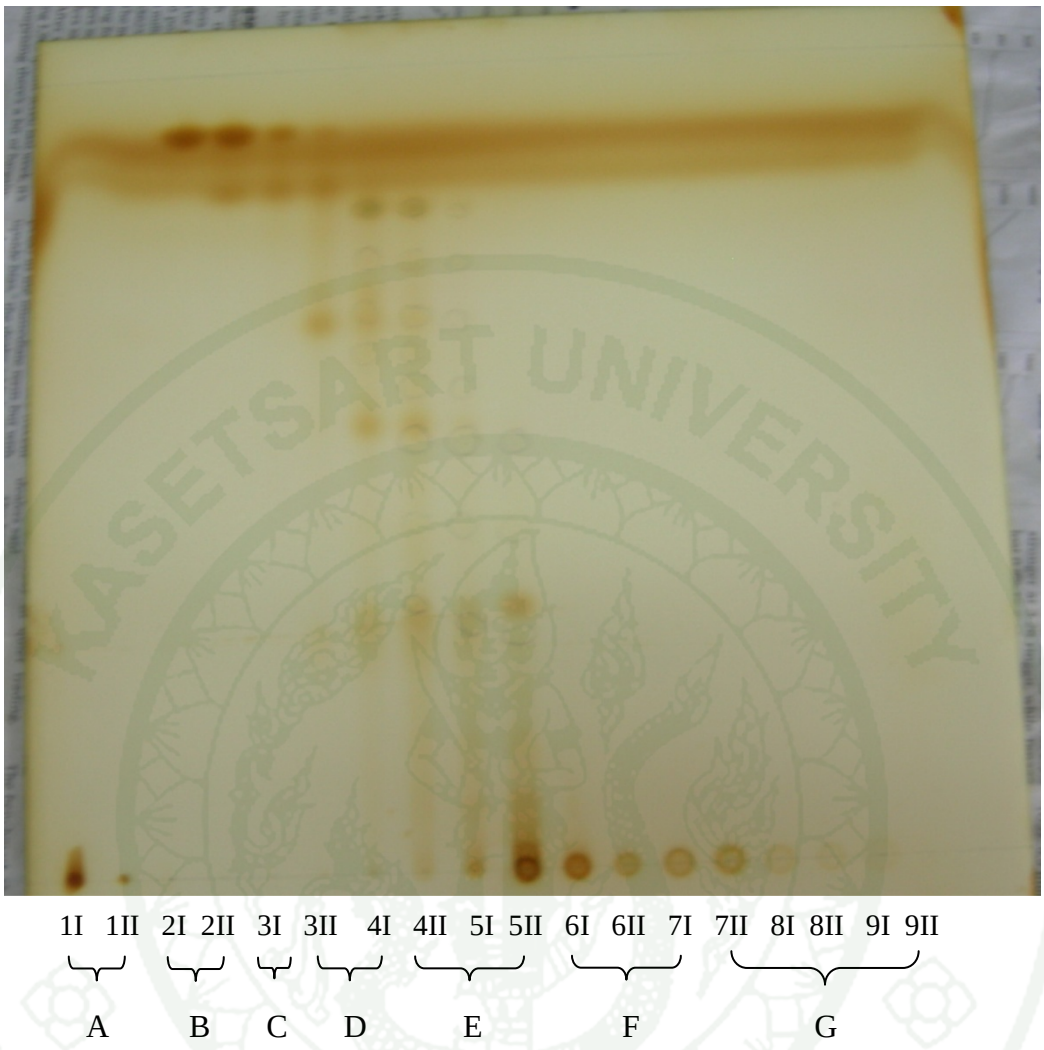
Iodine vapor chamber is made from a TLC jar by adding iodine crystals (May & Baker LTD Dagenham England). Dry TLC plate was placed in the chamber. The organic compounds were detected and presented as brown spots. This technique works on variety compounds but often not very sensitive. Iodine stained TLC can be developed subsequently with other stains (Merck, 1980)



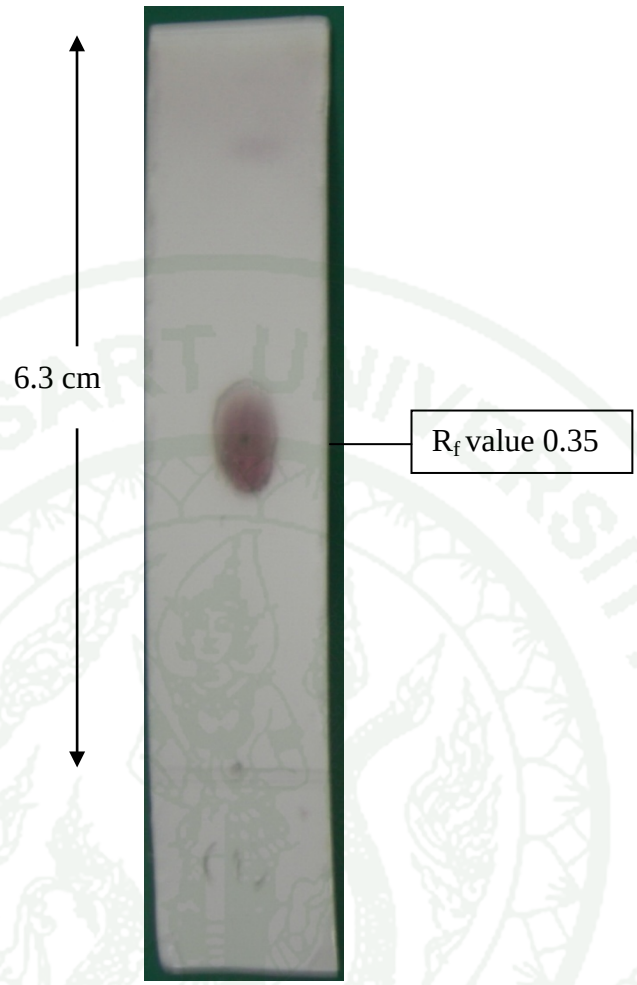
Appendix Figure 1 TLC information (under UV light wavelength 254 nm) of eighteen fractions collected from column chromatography.



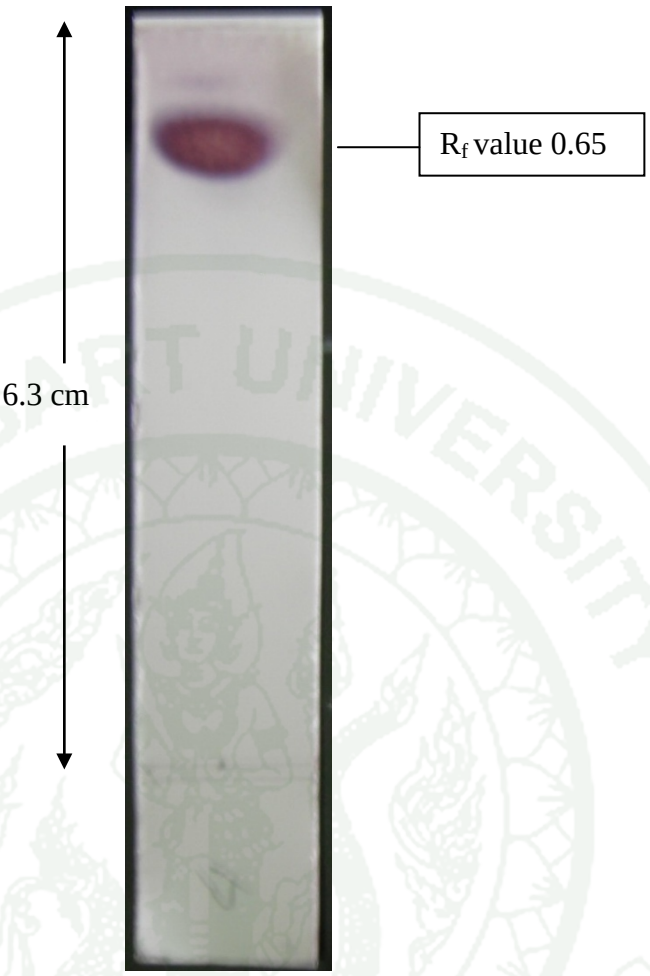
Appendix Figure 2 TLC information (under UV light wavelength 365 nm) of eighteen fractionscollected from column chromatography.



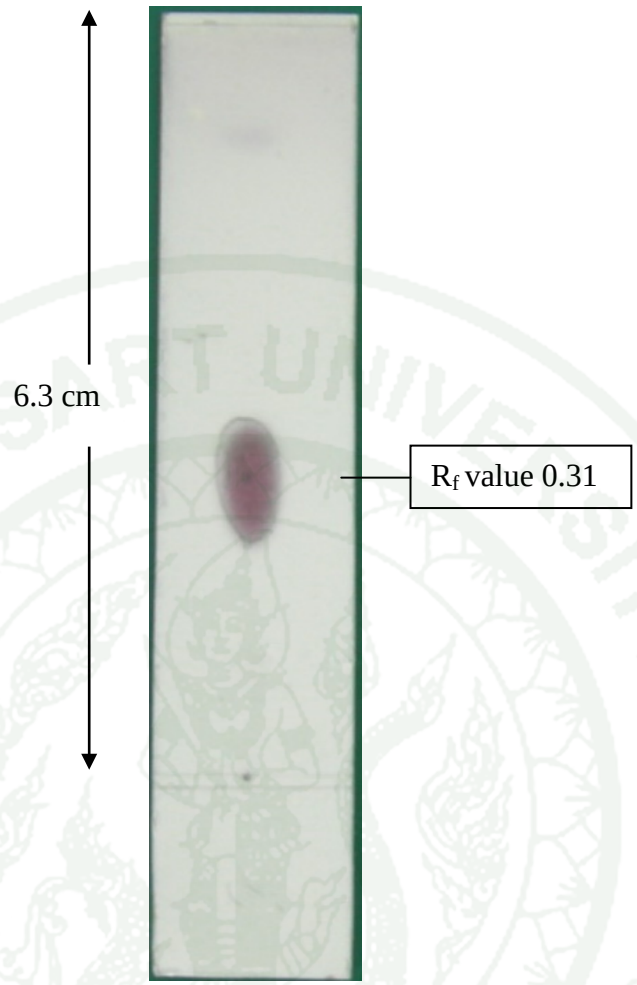
Appendix Figure 3 TLC information of eighteen fractions after treated with iodine vapor.



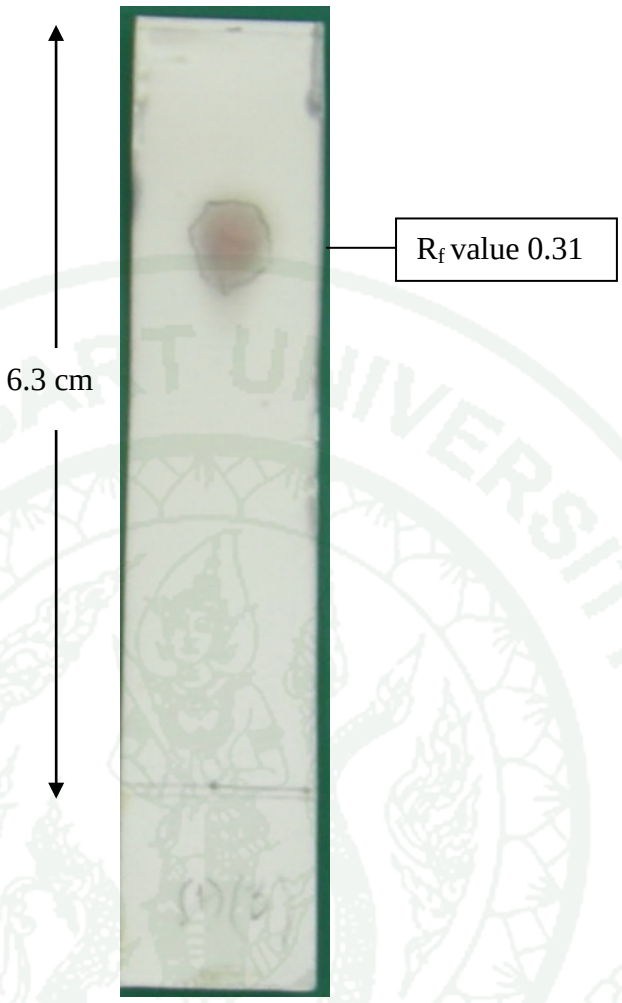
Appendix Figure 4 TLC chromatogram of crystals from fraction D developed in hexane: chloroform: ethyl acetate (5:5:2) solvent system.



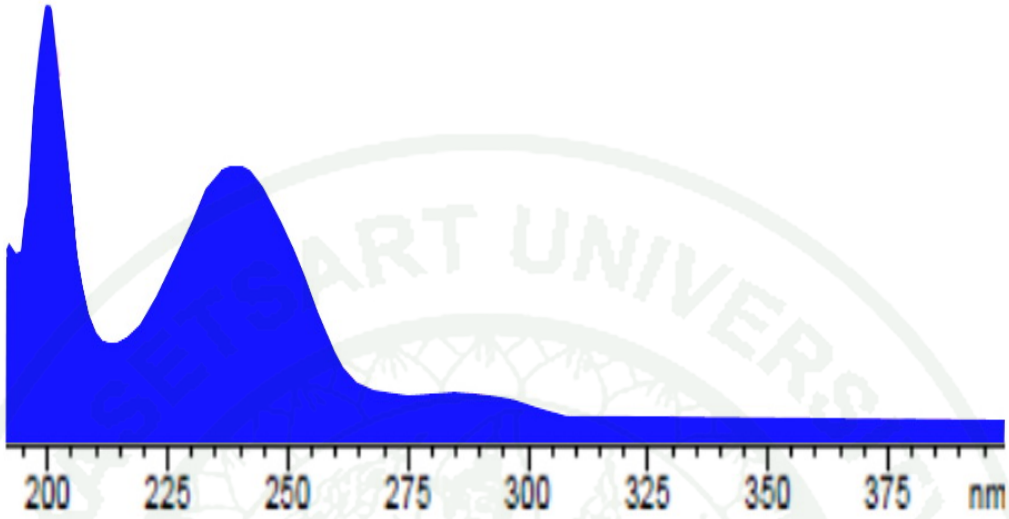
Appendix Figure 5 TLC chromatogram of crystals from fraction D developed in hexane: chloroform: methanol (4:6:2) solvent system.



Appendix Figure 6 TLC chromatogram of crystals from fraction D developed in chloroform solvent system.

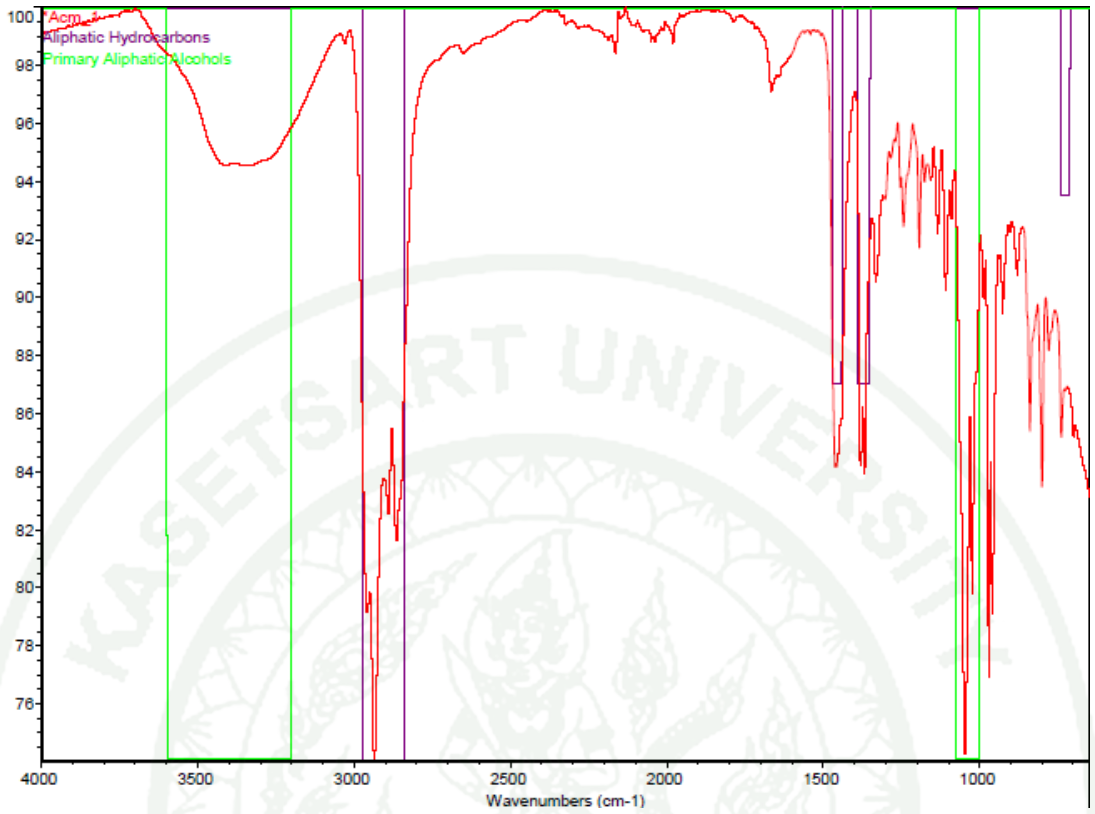


Appendix Figure 7 TLC chromatogram of crystals from fraction D developed in ethyl acetate solvent system.

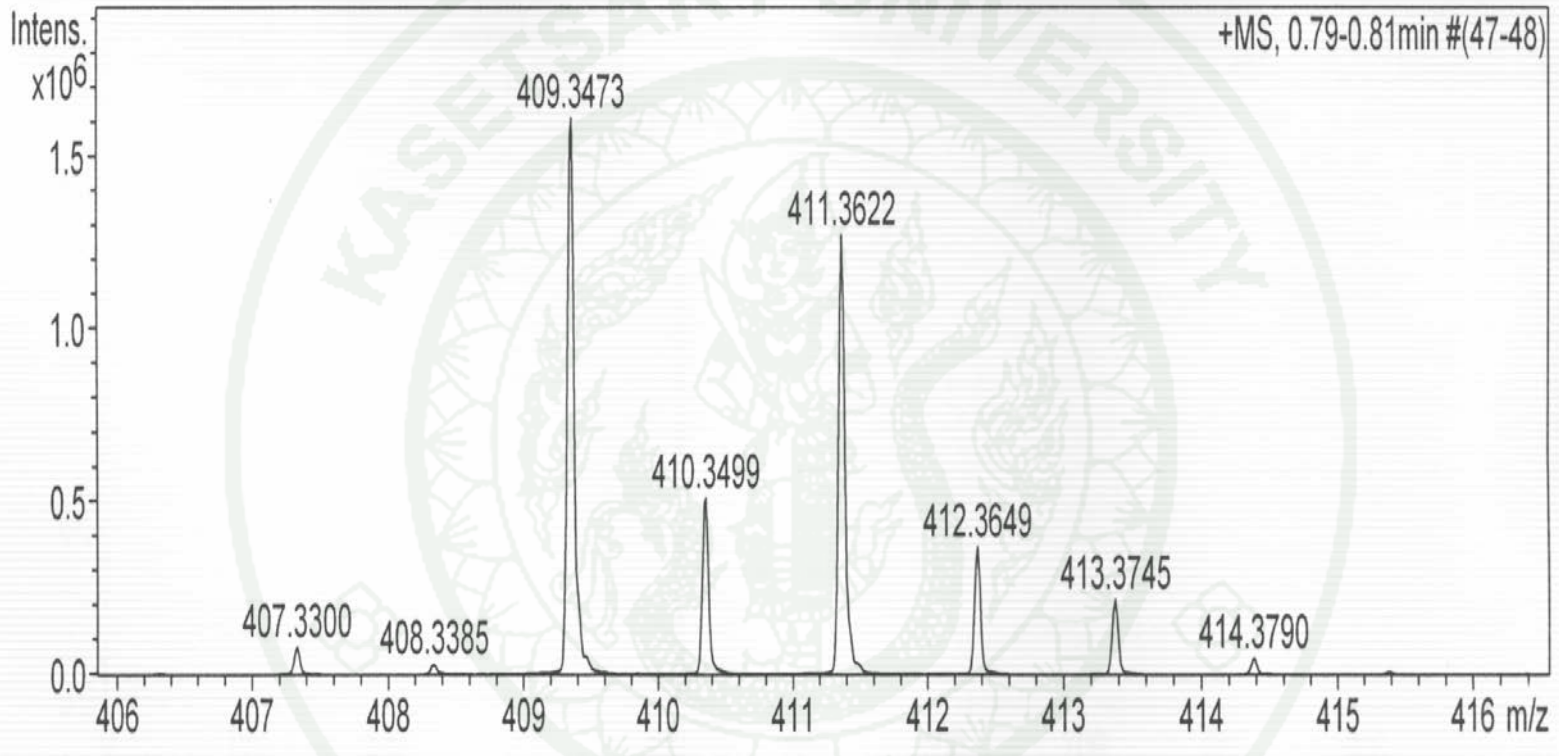


Appendix Figure 8 UV spectrum of compound DU1

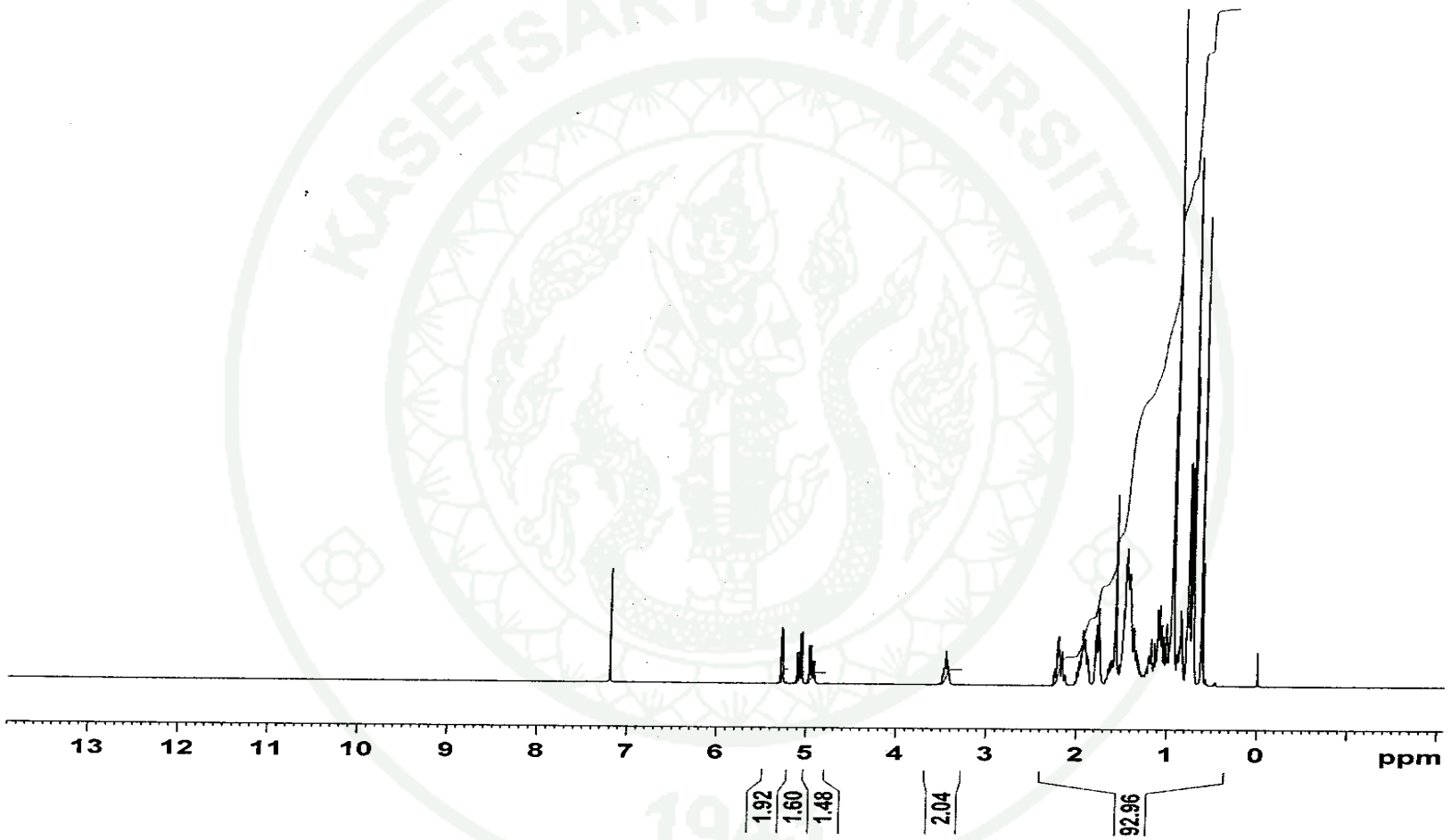
UV λ max ^{Isopropanol} (nm) 200, 240, 285



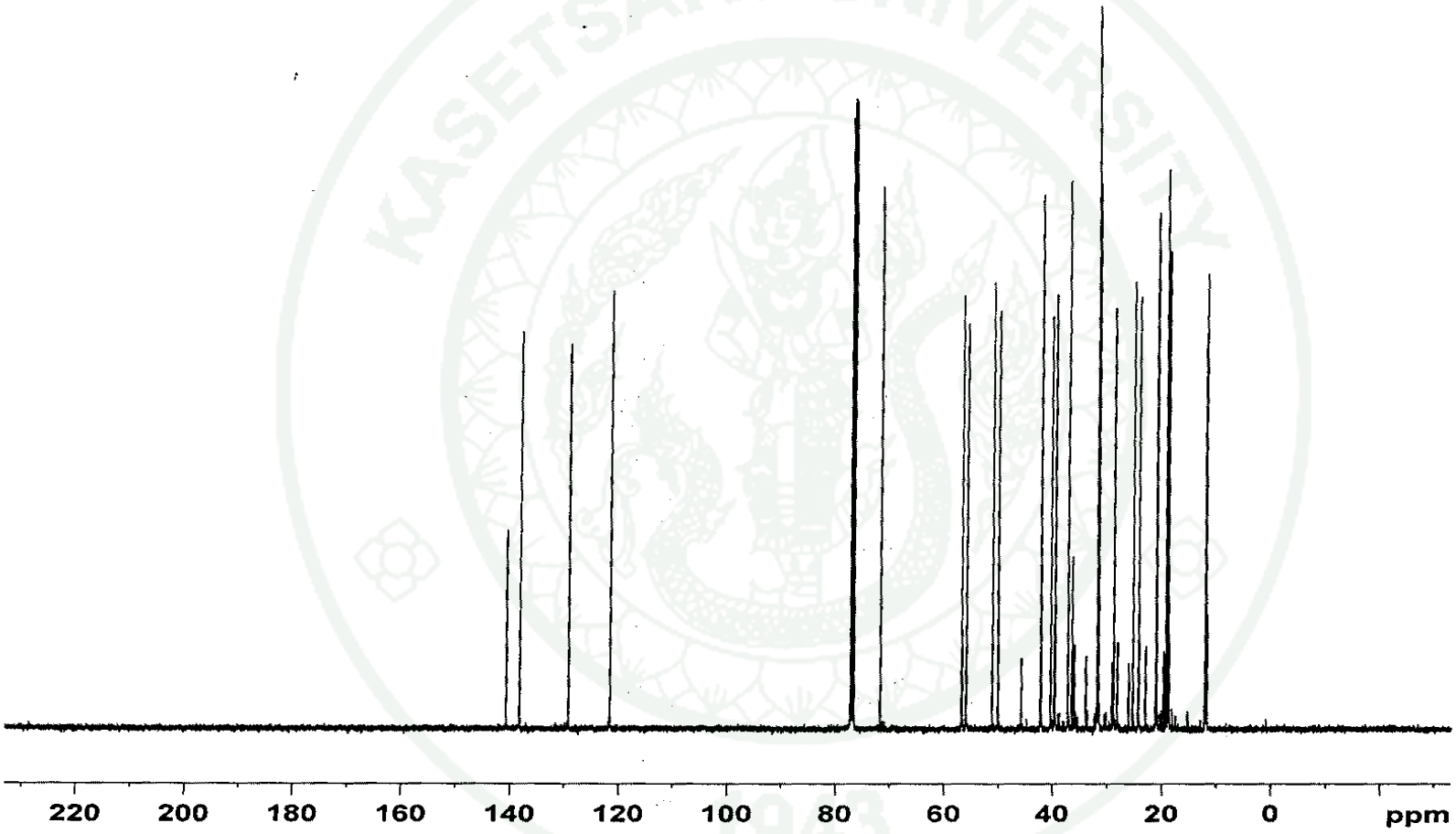
Appendix Figure 9 FT-IR spectrum of compound DU1



Appendix Figure 10 Mass spectrum of compound DU1



Appendix Figure 11 ^{1}H NMR (400 MHz) of spectrum of compound DU1 ($CDCl_3$)



Appendix Figure 12 ^{13}C NMR (400 MHz) of spectrum of compound DU1 ($CDCl_3$).

CURRICULUM VITAE

NAME : Ms. Chonchawan Jankam

BIRTH DATE : December, 14, 1983

BIRTH PLACE : Ubon Ratchathani, Thailand

EDUCATION :

YEAR	**INSTITUTE**	**DEGREE/DIPLOMA**
2006	Kasetsart Univ.	B.Sc. (Biology)

POSITION : -

WORK PLACE : -

SCHOLARSHIP/AWARD :The Graduate School, Kasetsart University, Bangkhen