สารต้านแบคทีเรียจากผักเสี้ยนผี *Cleome viscosa* L. และผลแตงแพะ *Gymnema griffithii* C.

นางสาวลัลธริมา พรมมิ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ANTIBACTERIAL AGENTS FROM *Cleome viscosa* L. AND *Gymnema griffithii* C. FRUITS

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biotechnology
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Thesis Title	ANTIBACTERIAL AGENTS FROM *Cleome viscosa* L. AND *Gymnema griffithii* C. FRUITS
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Field of Study	Biotechnology
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ลัลธริมา พรมมิ: สารต้านแบคทีเรียจากผักเสี้ยนผี *Cleome viscosa* L. และผลแตงแพะ *Gymnema griffithii* C. (ANTIBACTERIAL AGENTS FROM *Cleome viscosa* L. AND *Gymnema griffithii* C. FRUITS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. สุรชัย พรภคกุล, 91 หน้า.

งานวิจัยนี้ศึกษาสารยับยั้งจุลินทรีย์จากพืชสมุนไพรไทยผักเสี้ยนผี *Cleome viscosa* Linn. และผลแตงแพะ *Gymnema griffithii* Craib. ใบและลำต้น ฝัก รากและเมล็ดของผักเสี้ยนผีนำมาจากจังหวัดนครสวรรค์และราชบุรี ส่วนผลของแตงแพะเก็บจากจังหวัดเพชรบุรี สารสกัดเฮกเซนจากฝัก สารสกัดเฮกเซนจากเมล็ดและสารสกัดเอทิลอะซิเตทจากเมล็ด จากจังหวัดนครสวรรค์สามารถยับยั้ง *Staphylococcus aureus* ได้ที่ความเข้มข้น 50 ไมโครกรัม/มิลลิลิตร โดยมีบริเวณการยับยั้ง 13, 11 และ 9 มิลลิเมตร ตามลำดับ นอกจากนี้ สารสกัดเฮกเซนจากฝักที่เก็บจากนครสวรรค์และราชบุรียังสามารถยับยั้ง *Propionibacterium acnes* ได้ที่ความเข้มข้น 50 ไมโครกรัม/มิลลิลิตร โดยมีการบริเวณยับยั้งที่ปรากฏคือ 15 และ 13.5 ตามลำดับ

หลังจากการสกัดสารสกัดเมทานอลผลแตงแพะด้วยไดคลอโรมีเทน ได้นำสารสกัดไดคลอโรมีเทนและส่วนที่เหลือมาทดสอบฤทธิ์ในการยับยั้งจุลินทรีย์ จากผลการทดลองพบว่า ส่วนที่เหลือสามารถยับยั้ง *P. acnes* และ *Escherichia coli* ได้ที่ความเข้มข้น 100 ไมโครกรัม/มิลลิลิตร แต่สารสกัดไดคลอโรมีเทนสามารถยับยั้ง *P. acnes* ได้ที่ความเข้มข้น 50 ไมโครกรัม/มิลลิลิตร ในขณะที่ยับยั้ง *E. coli* ที่ความเข้มข้น 100 ไมโครกรัม/มิลลิลิตร

จากการสกัดและแยกสารออกฤทธิ์ทางชีวภาพของผลแตงแพะ สามารถแยก lupeol จากสารสกัดไดคลอโรมีเทน เมื่อนำ lupeol ที่แยกได้มาทดสอบฤทธิ์ต้านแบคทีเรียด้วยวิธี microdilution พบว่า lupeol มีฤทธิ์ต้านทานแบคทีเรีย *E. coli* และ *P. acnes* โดยมีค่า minimum inhibition concentration (MIC) เท่ากับ 25 และ 50 ไมโครกรัม/มิลลิลิตร ตามลำดับ

สาขาวิชา.....เทคโนโลยีชีวภาพ.....ลายมือชื่อนิสิต.....ลัลธริมา พรมมิ.....

ปีการศึกษา.....2553.....ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์หลัก.....

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KEYWORDS: *Cleome viscosa* Linn./ *Gymnema griffithii* Craib/ ANIMICROBIAL ACTIVITY/ MINIMUM INHIBITION CONCENTRATION METHOD

LUNTHARIMA PROMMI: ANTIBACTERIAL AGENTS FROM *Cleome viscosa* L. AND *Gymnema griffithii* C. FRUITS. ADVISOR: ASSOC. PROF. SURACHAI PORNPAKAKUL, Ph.D., 91 pp.

Antimicrobial agents from Thai medicinal plants, *Cleome viscosa* Linn. and *Gymnema griffithii* Craib. fruits were investigated in this study. Leaves and stems, pods, roots and seeds of *C. viscosa* were collected from Nakornsawan and Ratchaburi and *G. griffithii* fruits were collected from Petchaburi. The hexane extract of pods, hexane extract of seeds and ethyl acetate of seeds from Nakornsawan showed antimicrobial activity at 50 μg/ml against *Strephylococcus aureus* with inhibition zones of 13, 11 and 9 mm, respectively. The hexane extract of pods from Nakornsawan and Ratchaburi also inhibited *Propionibacterium acnes* at 50 μg/ml with the inhibition zones of 15 and 13.5 mm, respectively.

After partition of methanol extract of *G. griffithii* fruits by dichloromethane. The dichloromethane extract and residue of *G. griffithii* were tested for antimicrobial activity. The results showed that the residue could inhibit *P. acnes* and *E. coli* at 100 μg/ml. But the dichloromethane extract could inhibit *P. acnes* at 50 μg/ml while inhibited *E. coli* at 100 μg/ml.

Isolation the antimicrobial compound from dichloromethane extract of *G. griffithii* fruits gave lupeol. Antimicrobial activity of lupeol was examined and it exhibited antimicrobial activity against *E. coli* and *P. acnes* with the minimum inhibition concentration (MIC) value of 25 and 50 μg/ml rescpectively.

Field of Study:.....Biotechnology.....Student's Signature.....Luntharima Prommi.....
Academic Year:....2010.....Advisor's Signature.....S. Pornpakakul.....

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CONTENTS

Page

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF SCHEMES

LIST OF ABBREVIATIONS

$[\alpha]_D$	optical rotation
^oC	degree Celsius
cm^{-1}	reciprocated centimeter (unit of wave number)
^{13}C NMR	carbon-13 Nuclear Magnetic Resonance
COSY	Correlated Spectroscopy
d	doublet (NMR)
dd	doublet of doublet (NMR)
ddd	doublet of doublet of doublet (NMR)
DEPT	Distortionless Enhancement by Polarization Transfer
EI	Electron Impact
g	gravity (NMR)
h	hour
HMBC	Heteronuclear Multiple Bond Correlation
^{1}HNMR	Proton Nuclear Magnetic Resonance
HSQC	Heteronuclear Single Quantum Correlation
Hz	Hertz
IR	Infared spectroscopy
J	coupling constant
m	multiplet (NMR)
m	medium (IR)
M^+	molecular ion
MHz	megahertz
MS	Mass Spectroscopy
m/*z*	mass to change ratio
nm	nanometer
NOSEY	Nuclear Overhauser Enhancement Spectroscopy
ppm	part per million
q	quartet (NMR)
s	singlet (NMR)
t	tripet (NMR)

TLC	Thin Layer Chromatography
TOCSY	Total Correlation Spectroscopy
UV	Ultraviolet
µg	microgram
µl	microliter
δ	chemical shift
λ_{max}	the wavelength at maximum absorption (UV)
υ_{max}	wave number at maximum absorption (IR)

CHAPTER I

INTRODUCTION

Human have always relied on natural products and have continually explored their application to improve various aspects of our lives such as flavoring agents, perfumes, cosmetics, and dyes. Antimicrobial agents were also studied due to problems from resistant antibiotic drug action. Thus scientists are interested to discover new compounds from the new origin of natural sources products.

Plants have always been a rich source of lead compounds (e.g. alkaloids, quinine, tubocurarine, nicotine, and muscarine). Many of these lead compounds are useful drugs themselves and others have been the basis for synthetic drugs. Clinically useful drugs which have been recently isolated from plants include paclitaxel (taxol), anticancer agent from the yew tree, and artemisinin, an antimalarial agent from *Artemisia annua* (Steven et al., 2008).

Thailand has rich diversity of medicinal plants distributed in different geographical and environmental conditions. Many medicinal plants were interesting for many phamarcological activities including antimicrobial activity.

Cleome viscosa Linn. (Family: Capparidaceae) is a sticky herb with yellow flowers and long slender pods containing seeds. It has strong penetrating odour and various pharmacological activities. Whole plants are used as antiflatulent, antidiarrheal, antipyretic and fire element tonic and for treatment of abdominal pain, abscess or infected inflammation, tumor and cancer in lung, intestine or liver. Leaves were used as disfunction urine (Wongsatit et al., 1997) and useful in healing the wounds and ulcers externally. The juice of the plant diluted with water was used for fever. (Asolkar et al., 1992; Kirtikar and Basu, 1975; Nadkararni and Nadkarani, 1976). Flowers are used as antiseptic. Fruits are used as anthelmintic. Moreover, roots are used for treatment of uterine infection for post-labor (Wongsatit et al. 1997).

Gymnema is herb, twining shrubs, lianas or rarely trees but notably also contains a significant number of leafless stem succulents. It was found in tropics to subtropics forest in northern and south-western Thailand. *Gymnema griffithii* Craib is

one of plants in Family Gymnema. It is a climber twisting to the right side with the elliptic or oval leaves. There are few reports involving bioactive compounds from *C. viscosa* and *G. g riffithii*. T herefore, it is in teresting to in vestigate th e bioactive compounds from local plants.

In t his r esearch, l eaves a nd s tems, pods , r oots a nd s eeds of *C. viscosa* and fruits of *G. griffithii* were used for extract and investigate for the compounds.

Objectives

1. To extract and separate antibacterial agents against *Propionibacterium acnes* and *Strephylococcus aureus* from *Cleome viscosa* Linn. and *Gymnema griffithii* Craib.

2. To investigate antimicrobial from *C. viscosa* and *G. griffithii*

CHAPTER II

LITERATURE REVIEWS

2.1 Natural products

The natural product is a chemical compound or substance derived from a living organism. It is widely found in nature and has a pharmacological or biological activity for the purpose of pharmaceutical discovery and drug design. Perhaps it is extracted from terrestrial plant tissues, marine organisms or microorganism fermentation broths.

The untreated crude extracted from any of these sources normally contains novel and structurally diverse chemical compounds, which can probably be found in the natural environment. The chemical diversity in nature is based on the biological and geographical diversity, therefore researchers who travel around the world obtain samples to analyze and evaluate in drug discovery screens or bioassays.

Most biologically active natural product compounds are secondary metabolites with extremely complex structures. In spite of their advantage in that they are extremely novel compounds; their complexity makes synthesis of many lead compounds difficult. Also, the compound often has to be extracted from its natural source with its slow, expensive and inefficient process. Consequently, an advantage may usually be found in designing simpler analogues.

There has always been a rich source of lead compounds (e.g. alkaloids, morphine, cocaine, digitalis, quinine, tubocurarine, nicotine, and muscarine) in plants. Indeed, many of these lead compounds are useful drugs in themselves (e.g. alkaloids, morphine and quinine) and others have been used as the basis for synthetic drugs (e.g. local anaesthetics developed from cocaine). Clinically useful drugs that have been recently isolated from plants include the anticancer agent paclitaxel (taxol) from the yew tree, and the antimalarial agent artemisinin from *Artemisia annua* (Sam, 1993).

Plants provide a large bank of rich, complex and highly various structures which are unlikely to be synthesized in laboratories. In addition, evolution has already carried out a screening process itself by which plants are more likely to survive if they contain potent compounds that deter animals or insects from eating them. Even the present, the number of plants that have been widely studied is relatively very few and the vast majority has not been studied at all. The use of herbal and other natural medicines has a dramatic history. Nonetheless, the utilization of the whole plant or other crude preparations for therapeutic or experimental reasons can have many disadvantages including:

1. Variation in the amount of the active constituent with geographic areas, from one season to another, with different plant parts and morphology, and with climatic and ecological conditions.

2. Co-occurrence of undesirable compounds causing synergistic, antagonistic, or other undesirable, and possibly unpredictable, modulations of the bioactivity.

3. Changes or losses of bioactivity due to variability in collection, storage, and preparation of the raw material (Steven and Russell, 2008).

Thai medicinal plants were also studied for bioactive compounds for many years. There were many family of plants which exhibit the antimicrobial activity, including family Cappadiceae and Asclepiadoidae

2.2 Family Cappadiceae

Capparaceae is a medium-sized family of approximately 40–45 genera and 700–900 species, whose members present considerable diversity in habit, fruit, and floral features (Cronquist, 1981; Heywood, 1993; Mabberley, 1997). ''The flowers of Capparaceae are ecologically versatile and aesthetically exciting'' (Endress, 1992). Floral variation in Capparaceae includes both actinomorphy and zygomorphy, a wide range of stamen number (1–0.25), and pronounced basal intercalary elongation zones that may produce gynophores, androgynophores, and elongated stamens (Endress, 1992). Bees, hummingbirds, hawkmoths, and bats are involved in pollination

(Endress, 1992). Capparaceae are pantropical in distribution, being the most conspicuous in tropical seasonally dry habitats.

2.2.1 *Cleome viscosa* Linn.

Cleome viscosa Linn. (Family: Capparidaceae) called in Thai "Phak Sian Phee" (Figure 2.1), is annaual herb, up to 1 meter high; densely glandular pubescent and viscid throughout. Leaves are palmately compound, altermate; leaflets 3-5, obovate or ovate, 1-1.5 cm wide, 1.5-4.5 cm long. Inflorescence in terminal raceme; flowers are yellow. Fruit silique is dehiscing from base (Wongsatit et al., 1997).

Figure 2.1 *Cleome viscosa* Linn.

2.2.2 Medicinal uses

In the Ayurvedic system of medicine, this plant is used to cure fever, inflammations, liver diseases, bronchitis, infantile convulsions and diarrhea (Chatterjee and Pakrashi, 1991). The whole plant is used as antiflatulent, antidiarrheal, antipyretic, fire element tonic; the treatment of abdominal pain, abscess or the infected inflammation, tumor and cancer in lung, intestine or liver. Leaves were used as disfunction urine (Wongsatit et al., 1997).

1) Anthelmintic

Kirtikar and Basu (1975) reported that seeds were used as anthelmintic as well as Asolkar et al. (1992).

2) Anti- diarrhea

Parimala Devi et al. (2002) found that the methanol extract of the entire plant of *Cleome viscosa* L. had the anti-diarrhea potential against some of the experimental models of diarrhea in rats. The methanol extract of *C. viscosa* (CVME) showed a significant inhibitory activity against castor-oil-induced diarrhea and PGE2-induced enteropooling in rats. The extract also showed a significant reduction in gastrointestinal motility in the charcoal meal test in rats. The results obtained to establish the efficacy and substantiate the folklore claim as an anti- diarrhea agent. CVME extract at doses of 200, 400 and 600 mg/kg body weight significantly inhibited frequency of the defecation and wetness of fecal droppings, like the standard antidiarrhea agent (Diphenoxylate) as compared to untreated control rats.

3) Antipyretic

In 1975, Kirtikar and Basu found that the juice of the plant diluted with water and given internally in small quantities in fever. Parimala Devi (2003b) also reported that the *C. viscosa* (CVME), at doses of 200, 300, and 400 mg/kg body weight, showed a significant reduction in normal body temperature and yeast-provoked elevated temperature in a dose-dependent manner. The effect also extended up to 5 h after the drug.

4) Analgesic

The methanolic extract of *C. viscosa* of the whole plant given by oral route in mice showed a significant and dose-dependent analgesic activity in all the four tests used with an ED_{50} of approximately 200 mg/kg (Parimala Devi 2003a).

5) Healing wounds

Asolkar et al. (1992) reported that the leaves of *C. viscosa* crushed fresh were useful for healing wounds and ulcer.

6) Hepatoprotective

The ethanolic extract of leaves offer hepatoprotection, the higher dose (200 mg/kg body weight) had more effectively evaluated the hepatoprotective activity of

the ethanolic extract of *C. viscosa* L. against carbon tetrachloride (CCl_4) induced hepatotoxicity in experimental animal models (Gupta and Vinod, 2009).

7) **Anti-inflammations**

The whole plant was efficacious as a counterirritant in chronic painful joints. Juice was used to remove pus from ear (Nadkararni and Nadkarani, 1976).

8) Hepatoprotective

The ethanolic extract of leaves offer hepatoprotection, the higher dose (200 mg/kg body weight) was more effective to evaluate the hepatoprotective activity of ethanolic extract of *C. viscosa* L. against carbon tetrachloride (CCl_4) induced hepatotoxicity in experimental animal models (Gupta and Vinod, 2009).

9) Anti-inflammations

Whole plant was efficacious as a counterirritant in chronic painful joints. Juice was used to remove pus from ear (Nadkararni and Nadkarani, 1976).

2.2.3 Other uses

1) Antimicrobial activity

The hexane extract of leaves and stems was studied for antimicrobial activity by Williams et al. (2003). The results showed that *C. viscosa* was contained anti-bacterial agent against *Bacillus subtilis* (Gram-positive) and *Pseudomonas fluorescens* (Gram-negative). The ethanolic extracts of the leaves and flowers of *C. viscosa* were also inhibited *Escherichia coli*, *Proteus vulgaris* and *P. aeruginosa* (Sudhakar et al., 2006).

2) Insecticidal activity

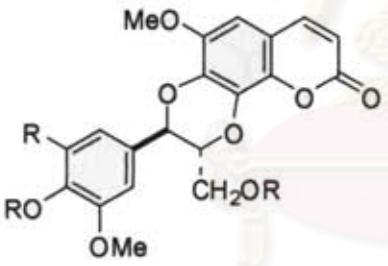
Williams et al. (2003) found that the hexane extract of leaves and stems of *C. viscosa* also could inhibit *Cylas formicarius elegantulus* Summer (Coleoptera: Curculionidae), one of the most destructive pests of the sweet potato plant as same as Dabire et al. (2008), their report showed that the fresh crushed leaf of *C. viscosa* could

inhibit the embryonic development of *Callosobruchus maculatus*'s eggs exposed for 48 h. Moreover, They found that the crushed plant of *C. viscosa* could reduce the number of bruchids in cowpea for 120 days storage.

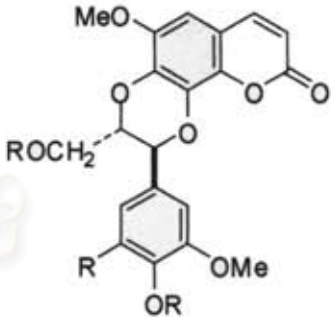
2.2.4 Bioactive compounds of *C. viscosa*

There were a few reports involving the constituents which isolated from *C. viscosa* (Figure 2.2). But only some showed the efficiency of the constituents.

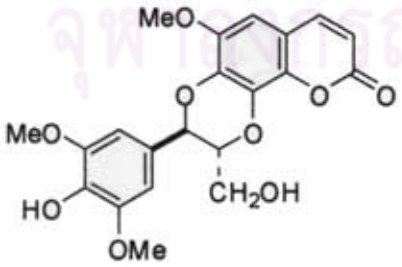
Cleomiscosin A, B and C isolated from roots by Chattopadhyay et al. (1999), had the hepatoprotective activity. Bawankule et al. (2008) also found the hepatoprotective activity of cleomiscosin A, B and C with dose 12, 30 and 100 mg/kg body weight in female albino mice. On the other hand, cleomiscosin A and B were also isolated from seeds (Kumar et al., 1987).



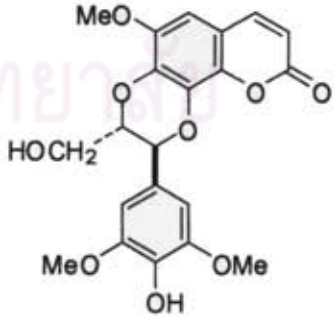
Cleomiscosins A



Cleomiscosins B

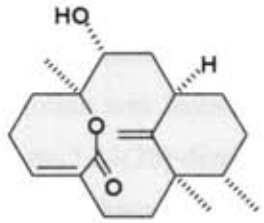


Cleomiscosin C

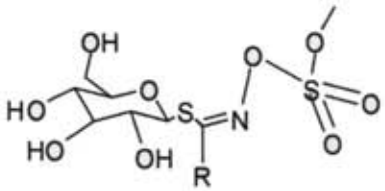


Cleomiscosin D

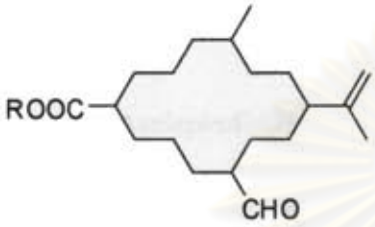
Figure 2.2 Cleomiscosins isolated from *C. viscosa* roots and seeds



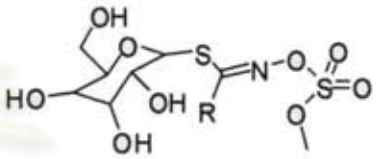
Cleomeolide



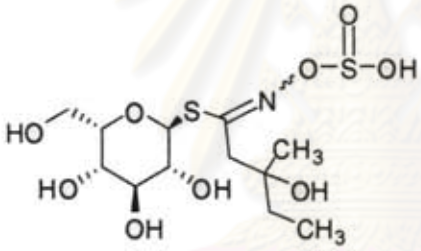
Glucosinolate



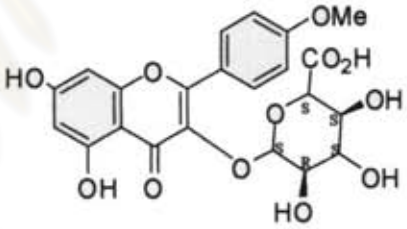
Cleomaldeic acid



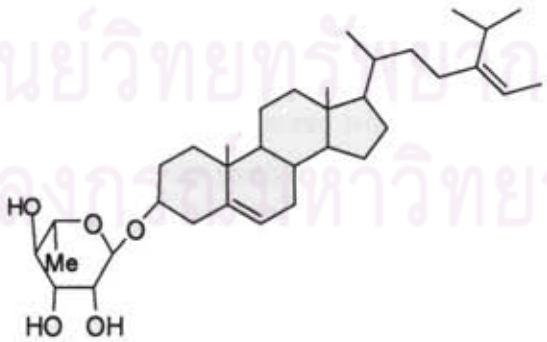
Glucocapparin



Glucocleomin



Kaempeferide-3-glucuronide



Stigmasta-5,24(28)-diene-3-*O*-rhamnoside

Figure 2.3 Constituents isolated from *C. visosa*

Glucosinolates were isolated from seeds (Songsak and Lockwood, 2002) and cleomeolide was isolated from leaves and stems (Paquette et al., 1993). Moreover, stigmasta-5,24(28)-diene-3-*O*-rhamnoside was isolated from whole plants (Srivastava, 1980) while kaempeferide-3-glucuronide was isolated from ethanolic extract of roots (Chauhan et al., 1979) and naringenin glycoside was isolated from whole plants by Srivastava et al.(1979). The constituensts of *C. viscosa* were shown in Figure 2.3.

2.3 Family Asclepiadoideae

This family was formed a group of perennial herbs, twining shrubs, lianas or rarely trees but notably also contained a significant number of leafless stem succulents. The plants in this family are mainly located in the tropics to subtropics, especially in Africa, South America and also in Asia (Ping-tao et al., 1995).

2.3.1 *Gymnema griffithii* Craib

Kingdom: Plantae
Order: Gentianales
Family: Asclepiadaceae
Genus: *Gymnema*
Species: *griffithii*

The name "Gymnema" is from two words in Greek. The first one is "gymnos" which means bare and the word "thread" means yarn which refers to the hairless stamen. The word "*griffithii*" be honored with William Griffith, an English botanist who collected plants around the south eastern India through southern Myanmar.

In Thai, *G. griffithii* is known as "Taeng Phae" for the common name and "Pra song kaw" for the local name. It is found in northern and south-western Thailand, and also found in Myanmar

G. griffithii is a climber twisting to the right side. Leaves are elliptic or oval 4-6 cm in width, 5-7 cm in length, simple and opposite. A tip of leaves is usually sharp and the base is round or heart shaped. There are 4-5 branch-line forms. A stalk

is 1.5-2 cm in length with hair rooting down to its stalk. The flower is 5-lobed and has 5 sepals, wide round with 3 mm in length, sharp tips. The outside is slightly covered by hair. Its petal bases merge as tube around 3 mm in length with 5 separated edges like oval, which are parallel to each other with 2 mm width and 7 mm length. The round tip usually has eccentric burs at its base with a cluster of hair inside.

Figure 2.4 Fruit and flower of *Gymnema griffithii* Craib

2.3.2 Medicinal uses

Members of *Gymnema* have been used for the treatment of diabetes for many years (Shanmugasundaram et al., 1981). The leaves were also used for stomach ailments, constipation, water retention and liver disease (Chattopadhyay, 1999). Few *Gymnema* species have been pharmacologically evaluated by researchers. There are reports that some *Gymnema* species (*G. sylvestre*, *G. inodorum*, and *G. yunnanense*) help to repair or regenerate the pancreatic β-cells, which play a crucial role in the production and secretion of insulin (Persaud et al., 1999; Shimizu et al., 1997; Xie et al., 2003).

Gymnema was reported to increase glucose uptake and utilization and improve the function of pancreatic beta cells. *Gymnema* may also decrease glucose absorption in the gastrointestinal tract (Shanmugasundaram et al., 1981; Sharma et al., 2006).

2.3.3 Antimicrobial activity

Even *Gymnema* was well known as antidiabetes for many years but there were still few studies about antimicrobial activity.

Satdive et al., (2003) found that the ethanol extract of *G. sylvestre* leaves demonstrated the antimicrobial activity against *Bacillus pumilis*, *B. subtilis*, *Pseudomonas aeruginosa* and *Strephylococcus aureus*.

2.3.4 Study of bioactive compounds of *Gymnema*

1) *G. sylvestre*

Phytochemically the plant has been reported to contain gymnemagenin the sapogenin, gymnemic acid-III, -IV, -V, -VIII, and -IX, isolated in pure states from the hot water extract of leaves of *G. sylvestre* (Liu et al., 1992).

Dihydroxy gymnemic triacetate (Figure 2.5), an antidiabetic agent was isolated from the acetone extract of leaves of *G. sylvestre*, one of plants in this Genus. It was an effective compound, against streptozotocin (STZ) induced diabetic rats. The increased levels of plasma glucose in STZ-induced diabetic rats were lowered by the administration of *G. sylvestre* crude extracts and its isolated compound, dihydroxy gymnemic triacetate in dose, 20 mg/kg body weight (Daisy et al., 2009; Pitchai et al., 2009).

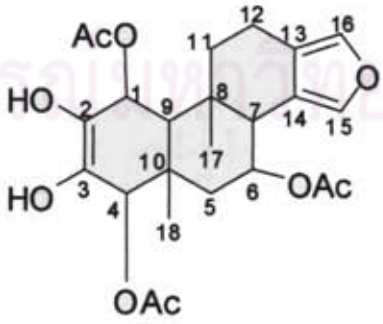


Figure 2.5 Structure of dihydroxy gymnemic triacetate

Hong-Min et al (1992) were isolated the antisweet agent, diacetonides, gymnemagenin, deacylgymnemic acid and gymnemagenin glucuronide from *G. sylvestre* (Figure 2.6-2.9).

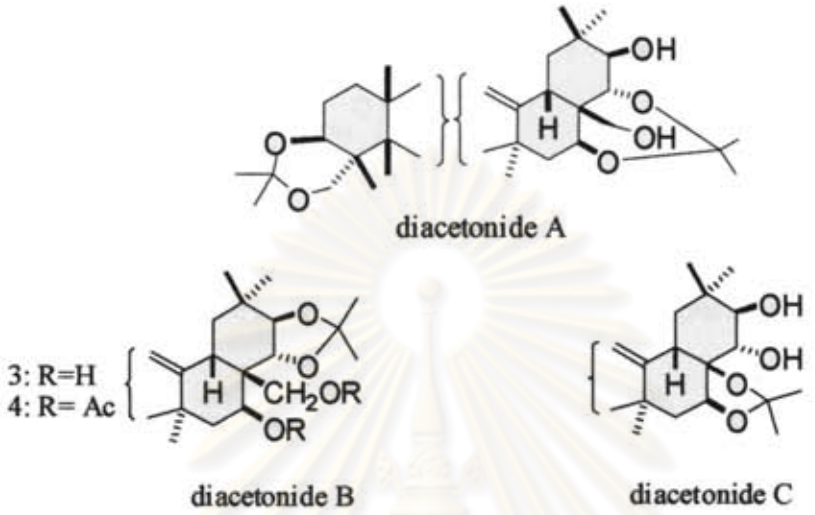


Figure 2.6 Structure of diacetonides

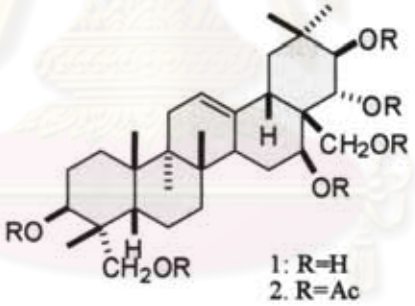


Figure 2.7 Structure of gymnemagenin

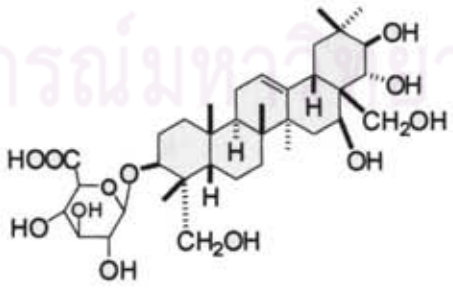


Figure 2.8 Structure of deacylgymnemic acid

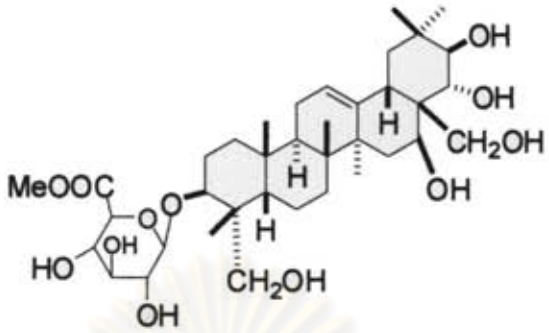


Figure 2.9 Structure of gymnemagenin glucuronide

A methanol extract of *G. sylvestre* leave and callus also showed anti-diabetic activities (alloxan-induced diabetic) through regenerating β–cells in Wistar rats. Optimum callus was developed under stress conditions of blue light with 2,4-dichlorophenoxy acetic acid (2,4-D) (1.5 mg/l) and Kinetin (KN) (0.5 mg/l), which induced maximum biomass of green compact callus at 45 days. Leaf and optimum callus extracts contain gymnemic acid (Bakrudeen Ali Ahmed et al., 2010).

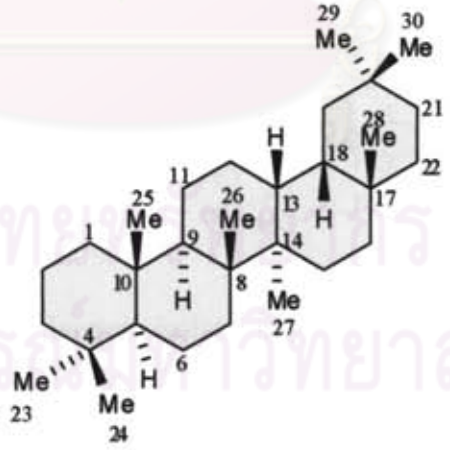


Figure 2.10 Structure of oleanane

Oleanane saponins (Figure 2.7-2.8), known as anti-sweetening agent, isolated from *G. sylvestre*, was shown to be able to inhibit glucose absorption in the small

intestine and to suppress elevated glucose levels in blood following the administration of sucrose in rats (Shi-mizu et al., 1997; Wen-Cai, Y., 1999).

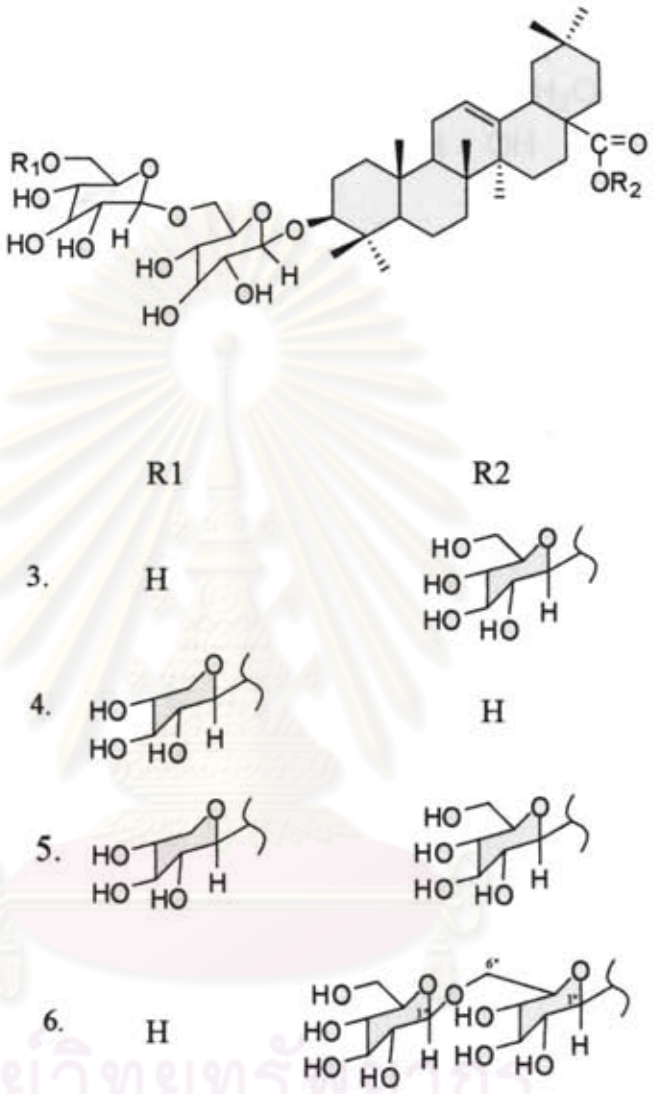


Figure 2.11 Saponin isolated from *G. sylvestre*

In 2000, Ye et al. separated the active principles from the ethanolic extract of *G. sylvestre* as characterized as 21*β*-*O*-benzoylsitakisogenin-3-*O*-*β*-D-glucopyranosyl (1→3)-*β*-D-glucuronopyranoside (antisweet agent, Figure 2.9), potassium salt of longispinogenin 3-*O*-*β*-D-glucopyranosyl (1→3)-*β*-D-glucuronopyranoside, potassium salt of 29-hydroxylongispinogenin-3-*O*-*β*-D-glucopyranosyl(1→3)-*β*-D-glucuronopyranoside and alternoside II (antisweet agent, Figure 2.10).

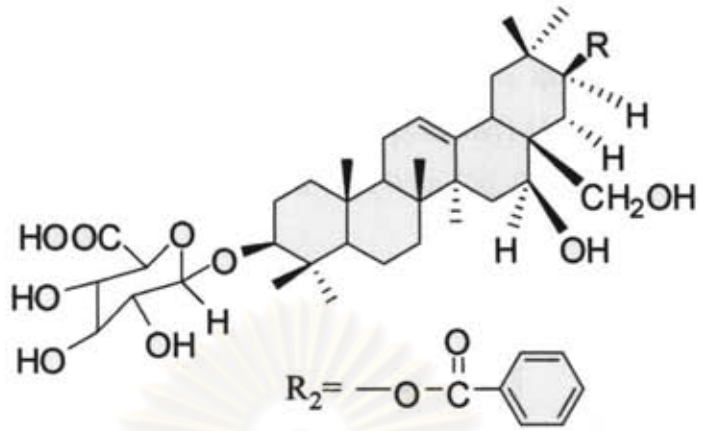
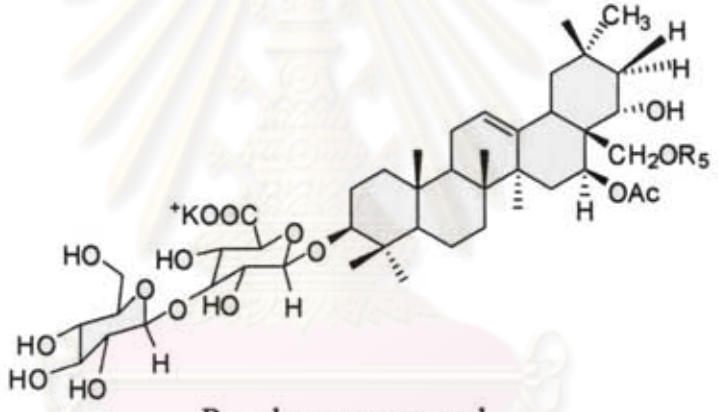


Figure 2.12 21*β*-*O*-benzoylsitakisogenin-3-*O*-*β*-D-glucopyranosyl (1→3)-*β*- D-glucuronopyranoside



R_5= rhamnopyranosyl

Figure 2.13 Alternoside II

There were many compounds isolated from ethanol extract of *G. sylvestre* leaves such as kaempferol 3-*O*-robinobioside, 3 rutin, quercetin 3-*O*-robinobioside, tamarixetin and kaempferol 3-*O*-β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside (Figure 2.11) (Wen-Cai, 2000).

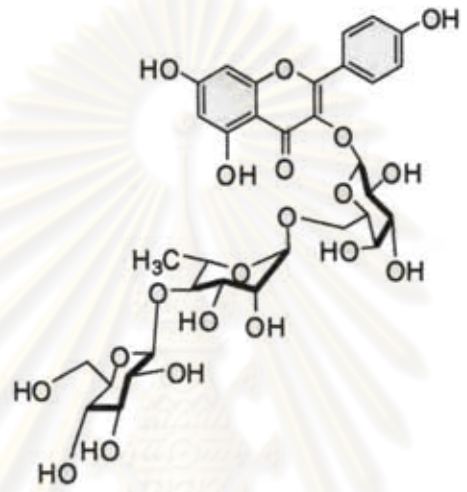


Figure 2.14 Kaempferol 3-*O*-*β*-D-glucopyranosyl-(1→4)-*α*-L-rhamnopyranosyl-(1→6)-*β*-D-galactopyranoside

2) *G. montanum*

G. montanum was found to be rich in phenolics. Leaves had the modulated effects on glycoprotein levels in alloxan-induced diabetic rats. The ethanol extract of *G. montanum* leaves was administered orally (200 mg/kg of body weight) for 3 weeks. Comparing to a reference drug, glibenclamide (600 mg/kg of body weight), the ethanol extract of *G. montanum* could increase the levels of blood glucose and plasma glycoproteins (Kunga et al., 2007). Levels of plasma insulin and sialic acid in diabetic rats were decreased by *G. montanum*. Moreover, *G. montanum* showed the efficiency of antioxidant activity, related to high level of phenolics in leave (Ramkuma et al., 2005).

CHAPTER III

MATERIALS AND METHODS

3.1 Instruments

3.1.1 Nuclear Magnetic Resonance Spectrometer (NMR)

^{1}H and ^{13}C NMR data were performed on Varian model Mercury +400 at 400 MHz for ^{1}H and 100 MHz for ^{13}C. Deuterated solvents chloroform-*d* ($CDCl_3$), methanol-d_4 (CD_3OD), deuterium oxide (D_2O) and DMSO-d_6 were used for NMR experiments and chemical shift (δ) were referenced to signals of residual solvents at δ 7.26 (^{1}H) and 77.0 (^{13}C) for $CDCl_3$, at 4.87 (^{1}H) for CD_3OD, at 4.79 (^{1}H) for D_2O and at 2.50 (^{1}H) for DMSO-d_6.

3.1.2 Rotary Vacuum Evaporator, Buchi, Switzerland

3.1.3 Microplate Reader Spectrophotometer, software SUNRISE Magellan™ v.4.0 TECA, Austria

3.1.4 Autoclave, TOMY ss-325, Tomy Seiko Co., Ltd., Tokyo, Japan

3.1.5 Incubator, Memmert BE600, Jebsen and Jebsen

3.1.6 Larminar flow, CLEAN H1, Lab Service Ltd, Part.

3.1.7 High pressure liquid chromatography (HPLC), Shimadzu model, Japan**.** C18 (ODS) reversed-phase column, water 486UV detector, with mobile phase water/methanol (HPLC grad)

3.2 Chemicals

3.2.1 Solvents

The commercial grade of hexane, dichloromethane, ethyl acetate and methanol were distilled prior to use.

3.2.2 Other chemicals

- Sephadex™ LH-20, Amersham Pharmacia Biotech AB, Sweden
- Silica 60 Art.1.09385.9025 (230-400 mesh ASTM), Merck, Germany

- Silica gel containing gypsum 60 PF_{254}, Merck, Germany used for preperative TLC

A slurry of silica gel 60 PF_{254} (100 g) in distilled water (250 ml) was used for 6 glasses of preparative TLC (20x20 cm).

- TLC aluminum sheet which stationary phase was silica gel 60 F_{254}, Merck, Germany size 20x20 cm, thick 0.2 mm for TLC technique.

- INT (*p*-iodonitrotetrazolium violet, or 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride), Fluka, Austria was used as staining agent for antibacterial activity and Minimal Inhibitory Concentration (MIC) testing by microtiter plate broth dilution technique.

- Dimethyl sulfoxide, Riedel-deHaën®, Germany

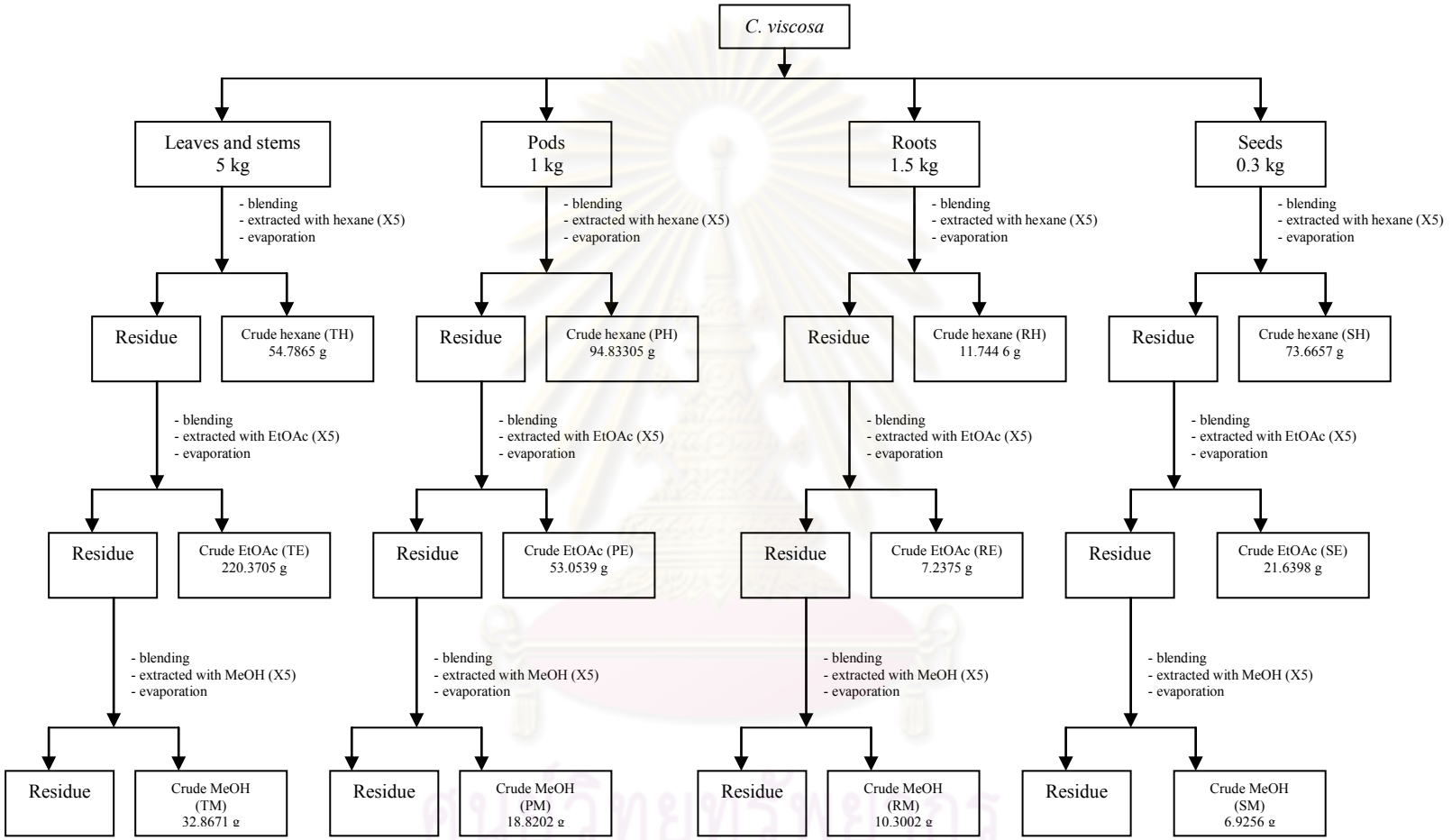
- Tween 80

- Anaerogen™, Oxoid, Mitsubishi Gas Chemical Company Inc., England

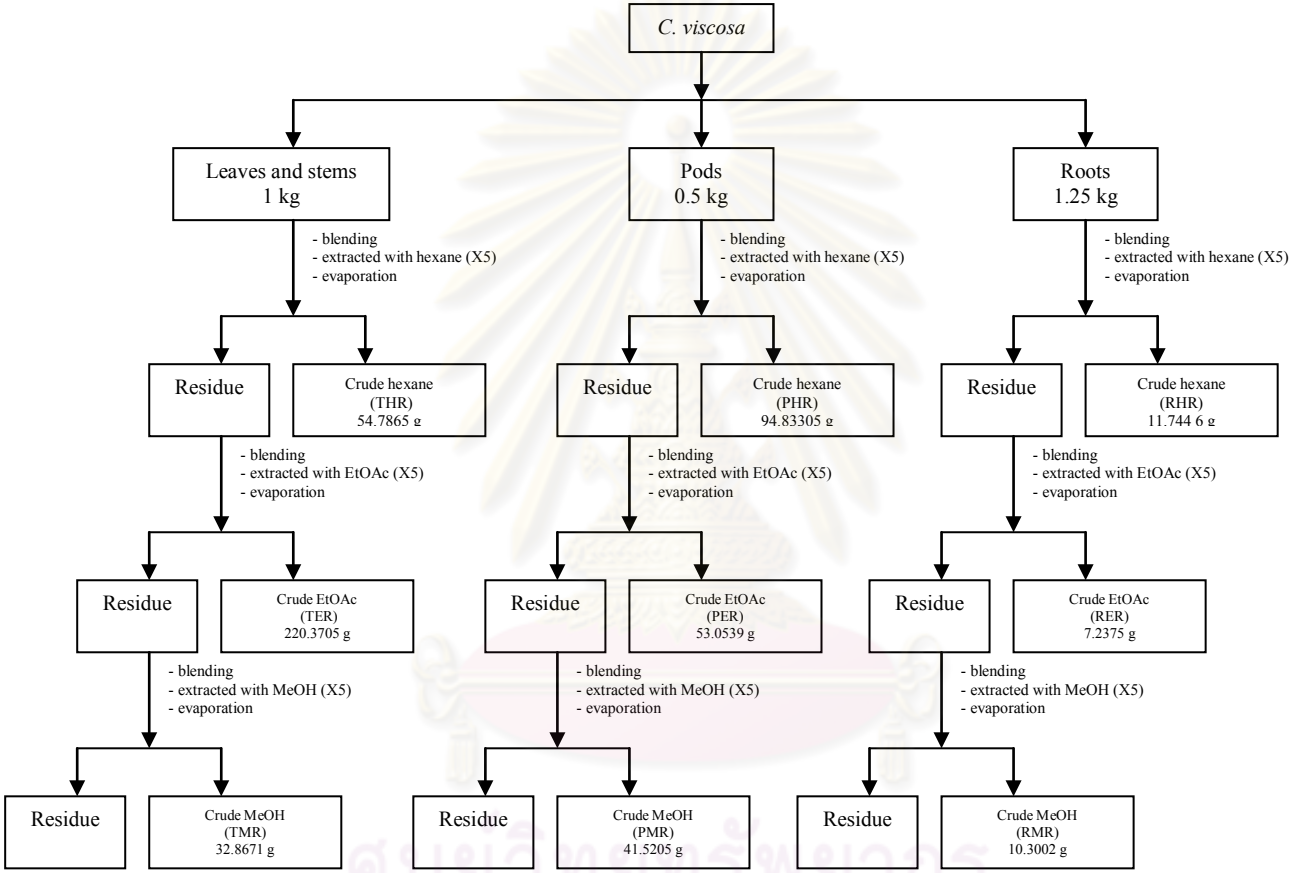
3.3 Isolation of antimicrobial agents from *C. viscosa* and *G. griffithii*

3.3.1 Extraction of *C. viscosa*

Cleome viscosa Linn. was collected from Nakhonsawan in November and from Ratchaburi in December. The whole plant were air-dried in shade and separated into four parts: leaves and stems, pods, seeds and roots. The leaves and stems, pods, seeds and roots of *C. viscosa* were ground separately and then extracted with hexane, ethyl acetate and methanol, respectively. The solvents of the extracts were removed by vacuum rotary evaporator to give hexane extracts, ethyl acetate extracts and methanol extracts as shown in Scheme 3.1 and 3.2. All crude extracts were subjected to antimicrobial assay.



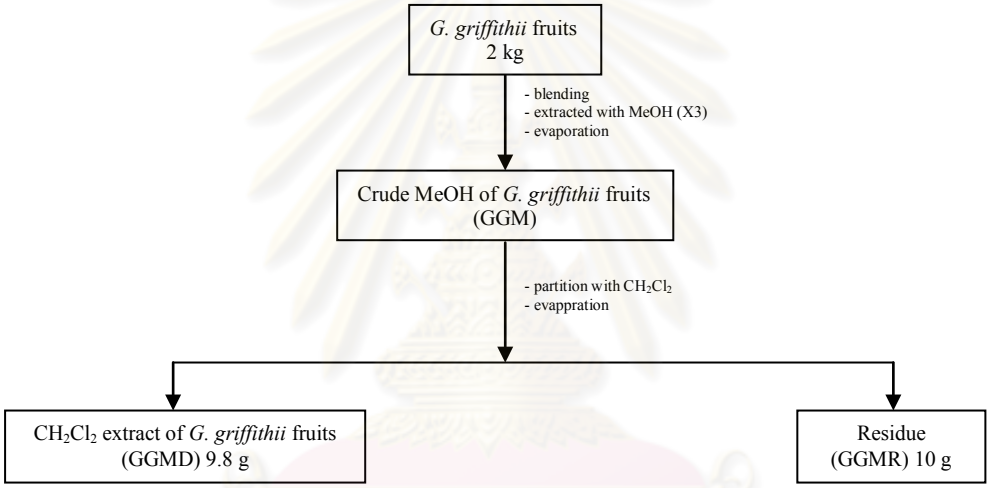
Scheme 3.1 Extraction leaves and stems of *C. viscosa* from Nakhonsawan



Scheme 3.2 Extraction leaves and stems of *C. viscosa* from Ratchaburi

3.3.2 Extraction of *G. griffithii*

Fresh fruits of *G. griffithii* (2 kg) were collected from Petchaburi in October 2010. *G. griffithii* fresh fruits were cut to small pieces, soaked in methanol (2000 ml x3) and then filtered through filter paper (Whatman No.1). The filtrate was concentrated by a rotary evaporator under reduced pressure at 40°C to give a brown liquid (2500 ml) and then partitioned with CH_2Cl_2 (2000 ml x3). The CH_2Cl_2 extract was evaporated under the reduced pressure at 40°C to give a dark green extract (9.8 g).



Scheme 3.3 Extraction of *G. griffithii* fruits from Petchaburi

3.4 Antimicrobial assay

3.4.1 Test microorganisms

- Gram positive bacterium

Staphylococcus aureus ATCC 25923 from Thailand Institute of Scientific and Technology Research (TISTR), Chatuchak, Bangkok

Propionibacterium acnes DMST 14916 from Department medical science, Muang, Nonthaburi

- Gram negative bacterium

Escherichia coli ATTC 25922 from Thailand Institute of Scientific and Technology Research (TISTR), Chatuchak, Bangkok

Pseudomonas ae ruginosa ATCC 27853 from Thailand Institute o f Scientific and Technology Research (TISTR), Chatuchak, Bangkok

- Yeast

Candida al bicans ATCC 10231 from Thailand Institute o f S cientific and Technology Research (TISTR), Chatuchak, Bangkok

3.4.2 Preparation of test microorganism inoculums

Test microorganisms, *S. aureus*, *E. coli*, *P. aeruginosa* and *C. albicans* were grown on nut rient a gar (NA) a t 37 °C for 24 h. S elected s ingle c olonies w ere inoculated into NB (5 ml) and inoculated at 37°C for 4-8 h, depending on the growth rate. T he turbidity of the ba cterial s uspension was a djusted w ith NB t o m atch t he turbidity of 0.5 McFarland (OD 0.08-0.1 at 600 nm).

P. ac nes was grown on br ain heart i nfusion a gar (BHI a gar) a t 37°C under anaerobic condition for 48 h. S elected single colonies were inoculated into BHI broth (5 ml) and incubated at 37°C for 8 h. T he turbidity of the *P. ac nes* suspension was adjusted with BHI broth to match the turbidity of 0.5 McFarland (OD 0.08-0.1 at 600 nm).

3.4.3 Antibacterial screening of crude extracts

Crude ex tracts w ere di ssolved by 10% D MSO i n s terile water w hich containing 5% Tween 80.

3.4.3A Agar well techniques (Cole, 1997)

Agar plates containing a fixed volume of agar were poured and the plates were left a t room te mperature f or 24 h . Bacterial s uspension (10^6 CFU/ml) was swabbed and the p lates were left a t room te mperature for 10 min to a llow the ag ar surface t o dry. A sterile cork bor er was u sed to cut th e c ircular s mall w ells in th e agar. 100 µl of crude extracts (serial dilution) was loaded into the wells. Streptomycin

was used as positive control and 10% DMSO solution containing 5% Tween 80 was used as negative control. *S. aureus*, *E. coli*, *P. aeruginosa* and *C. albicans* were incubated at 37 ^{o}C for 24 h and *P. acnes* was incubated at 37^{o}C for 48 h under anaerobic condition. After incubation, the inhibition zones were measured.

3.4.3B Microdilution assay (Athikomkulchai et al., 2008)

The inocula of microorganisms were prepared by inoculation of 24 h single colonies of *S. aureus*, *C. albicans*, *E. coli* and *P. aeruginosa* which grew on NA to NB while inocula of *P. acnes* was prepared by inoculation of 48 h single colonies of *P. acnes* which grew on BHI agar to BHI broth. The suspensions were adjusted to McFarland standard turbidity number 0.5. The stock solutions of each extract were diluted in serial 2-fold dilutions. The 96-well plates were prepared by dispensing into each well 90 µl of broth, and 5 µl of the inoculum. A 100 µl of highest concentrations were added into the first wells. Then, 100 µl from their serial dilutions was transferred into 3 consecutive wells. The final volume in each well was 195 µl. The broth and inocula were used as negative controls. Streptomycin was used as positive controls to determine the sensitivity of tested solutions. The plate was covered with a sterile plate sealer. The plates were incubated at appropriate condition for 18-24 hours. After incubation time, 5 µl INT solutions (4 mg/ml of distilled water) were added into each well. The first concentration which not showed the red color was the MIC value. The 5 µl of solution from clear wells were spread on NA plates for *S. aureus*, *E. coli*, *P. aeruginosa* and *C. albicans* while BHI agar plate for *P. acnes*. The Minimum Bactericidal Concentration (MBC) was defined as the concentration of the compounds to kill the microorganisms. So that, the sample concentration was not exhibited the growth of microorganisms as the MBC value.

3.4.3C TLC bioautography method (Cole, 1997)

A stock solution (1 mg/ml) of those extracts was applied to a TLC plate and TLC was developed in an appropriate mobile phase. After evaporation of the solvents, a suspension of microorganism which inoculum density was known was sprayed on the TLC plate, incubated for 12-18 hour. After incubation, a solution of INT (4 mg/ml of distilled water) was sprayed on the TLC plate. Under incident white light, zones of inhibition of microorganism growth appeared as light spots on a colored background.

3.5 Chemical investigation of *C. viscosa*

The crude extracts of *C. viscosa* which exhibited the antimicrobial activity were subjected to column chromatography.

3.5.1 Chemical investigation of methanol crude extract of leaves and stems of *C. viscosa*

16.43 g of methanol extract of leaves and stems of *C. viscosa* part 1 (TMsep) and 16.45 g of methanol extract of leaves and stems of *C. viscosa* part 2 (TM2sep), were isolated by sephadex LH-20 column chromatography eluted with methanol. The similar fractions were combined on the basis of TLC profile to give 13 and 15 combined fractions of TMsep and TM2sep respectively as shown in Table 3.1 and 3.2. The combined fractions were tested for antimicrobial activity.

Table 3.1 The combined fractions obtained from isolation of the methanol extract of leaves and stems of *C. viscosa* (TMsep) using Sephadex LH-20 column chromatography.

Fraction code	Fractions	Appearance	Weight (g)
TMsep1	1-7	Brown viscous liquid	0.6388
TMsep2	8-11	Yellow viscous liquid	1.136
TMsep3	12-13	Yellow viscous liquid	1.0437
TMsep4	14-17	Yellow viscous liquid	4.6383
TMsep5	18-22	Yellow viscous liquid	0.2826
TMsep6	23-28	Brown viscous liquid	0.1591
TMsep7	29-31	Brown viscous liquid	0.1156
TMsep8	32-38	Brown viscous liquid	0.0258
TMsep9	39-44	Yellow viscous liquid	0.1779
TMsep10	45-56	Yellow viscous liquid	0.30751
TMsep11	57-66	Brown viscous liquid	0.0789
TMsep12	67-82	Yellow viscous liquid	0.2891
TMsep13	83-100	Yellow viscous liquid	0.10669

Table 3.2 The combined fractions obtained from isolation of the methanol extract of leaves and stems of *C. viscosa* (TM2sep) using Sephadex LH-20 column chromatography.

Fraction code	Fractions	Appearance	Weight (mg)
TM2sep1	1	Brown viscous liquid	2.238
TM2sep2	2-10	Brown viscous liquid	0.1084
TM2sep3	11-13	Brown viscous liquid	0.6738
TM2sep4	14-15	Yellow viscous liquid	0.5274
TM2sep5	16	Yellow viscous liquid	0.4509
TM2sep6	17	Yellow viscous liquid	0.3606
TM2sep7	18-19	Yellow viscous liquid	0.7335
TM2sep8	20-21	Yellow viscous liquid	0.477
TM2sep9	22-25	Yellow viscous liquid	0.3918
TM2sep10	26-42	Brown viscous liquid	0.4755
TM2sep11	43-45	Green viscous liquid	0.216
TM2sep12	46-47	Green viscous liquid	0.0198
TM2sep13	48	Green viscous liquid	0.0062
TM2sep14	49-53	Green viscous liquid	0.0949
TM2sep15	54-70	Brown viscous liquid	0.2262

3.5.2 Chemical investigation of hexane crude extract of pods of *C. viscosa*

The crude hexane extract of pods (50 mg) was isolated by preparative TLC (Table 3.3). The plate TLC was prepared by mixing silica gel (100 g) with distilled water. A slurry of silica gel was coated on the glass. The plate was dried in oven at 110 °C overnight. After preparation of TLC plate, the solution of this extract (50 mg) in hexane (1 ml) was loaded and developed by Hex:EtOAc (6:4) as mobile phase. Each band was scraped and extracted with solvent. The extracts of bands were tested for antimicrobial activity.

Table 3.3 The combined fractions obtained from isolation of the hexane extract of pods of *C. viscosa* part 1 (PHB) using preparative TLC

Fraction code	Appearance	Weight (mg)
PHB 1	Yellow viscous liquid	30.8
PHB2	Yellow viscous liquid	0.1
PHB 3	Yellow viscous liquid	5.2
PHB 4	Yellow viscous liquid	4.0
PHB 5	Yellow viscous liquid	9.6
PHB 6	Yellow viscous liquid	5.9
PHB 7	Yellow viscous liquid	2.7
PHB 8	Yellow viscous liquid	4.0
PHB 9	Yellow viscous liquid	3.7
PHB 10	Yellow viscous liquid	3.4
PHB 11	Yellow viscous liquid	0.3
PHB 12	Yellow viscous liquid	23.7

The second part of hexane extract of *C. viscosa* pods (50 mg) was also isolated by using preparative TLC and the results were shown in Table 3.4. All fractions were tested for antimicrobial activity.

Table 3.4 The combined fractions obtained from isolation of the hexane extract of pods of *C. viscosa* part 2 (PHBrd) using preparative TLC

Fraction code	Appearance	Weight (mg)
PHB1rd	Yellow viscous liquid	9.1
PHB2rd	Yellow viscous liquid	6.4
PHB3rd	Yellow viscous liquid	3.2
PHB4rd	Yellow viscous liquid	8.0
PHB5rd	Yellow viscous liquid	7.6
PHB6rd	Yellow viscous liquid	4.4
PHB7rd	Yellow viscous liquid	2.8
PHB8rd	Yellow viscous liquid	7.8
PHB9rd	Yellow viscous liquid	6.8
PHB10rd	Yellow viscous liquid	3.4
PHB11rd	Yellow viscous liquid	8.3
PHB12rd	Yellow viscous liquid	0.3
PHB13rd	Yellow viscous liquid	0.3

3.5.3 Chemical investigation of ethyl acetate crude extract of pods

Ethyl acetate crude extract of pods 26.31 g was isolated by silica column chromatography. The similar fractions were combined on the basis of TLC profile to give 30 combined fractions of PE as shown in Table 3.5. Antimicrobial activity of the combined fractions was tested.

Table 3.5 The combined fractions obtained from isolation of the ethyl acetate extract of pods of *C. viscosa* (PE) using silica column chromatography

Fraction code	Fractions	Eluents (%)	Appearance	Weight (g)
PEF1	1	hexane (100)	Yellow viscous liquid	4.3801
PEF2	2	hexane (100)	Yellow viscous liquid	7.0043
PEF3	3	hexane (100)	Yellow viscous liquid	3.3437

Table 3.5 (con.)

Fraction code	Fractions	Eluents (%)	Appearance	Weight (g)
PEF4	4	CH_2Cl_2:Hex (10:90)	Yellow viscous liquid	1.2655
PEF5	5	CH_2Cl_2:Hex (20:80)	Yellow viscous liquid	0.5990
PEF6	6-8	CH_2Cl_2:Hex (30:70)	Yellow viscous liquid	0.4604
PEF7	9-10	CH_2Cl_2:Hex (40:60)	Yellow viscous liquid	0.4418
PEF8	11	CH_2Cl_2:Hex (50:50)	Yellow viscous liquid	7.3591
PEF9	12	CH_2Cl_2:Hex (70:30)	Yellow viscous liquid	1.7061
PEF10	13	CH_2Cl_2:Hex (80:20)	Yellow viscous liquid	0.7386
PEF11	14-15	CH_2Cl_2:Hex (90:10)	Yellow viscous liquid	0.6414
PEF12	16	CH_2Cl_2 (100)	Yellow viscous liquid	0.2834
PEF13	17-24	MeOH:CH_2Cl_2 (2:98)	Yellow viscous liquid	1.8060
PEF14	25-32	MeOH:CH_2Cl_2 (2:98)	Yellow viscous liquid	4.1322
PEF15	33-36	MeOH:CH_2Cl_2 (4:96)	Yellow viscous liquid	0.3769
PEF16	37	MeOH:CH_2Cl_2 (6:94)	Yellow viscous liquid	0.0734
PEF17	38-40	MeOH:CH_2Cl_2 (6:94)	Yellow viscous liquid	0.3372

Table 3.5 (con.)

Fraction code	Fractions	Eluents (%)	Appearance	Weight (g)
PEF18	41-42	MeOH:CH_2Cl_2 (8:92)	Yellow viscous liquid	0.2901
PEF19	43	MeOH:CH_2Cl_2 (8:92)	Yellow viscous liquid	0.0728
PEF20	44	MeOH:CH_2Cl_2 (8:92)	Yellow viscous liquid	0.0558
PEF21	45-46	MeOH:CH_2Cl_2 (10:90)	Yellow viscous liquid	1.5341
PEF22	47	MeOH:CH_2Cl_2 (10:90)	Yellow viscous liquid	0.2218
PEF23	48	MeOH:CH_2Cl_2 (10:90)	Yellow viscous liquid	0.3691
PEF24	49-54	MeOH:CH_2Cl_2 (10:90)	Yellow viscous liquid	0.6280
PEF25	55-56	MeOH:CH_2Cl_2 (20:80)	Yellow viscous liquid	0.1158
PEF26	57	MeOH:CH_2Cl_2 (20:80)	Yellow viscous liquid	0.0442
PEF27	58-60	MeOH:CH_2Cl_2 (30:70)	Yellow viscous liquid	0.0550
PEF28	61	MeOH:CH_2Cl_2 (30:70)	Yellow viscous liquid	0.0483
PEF29	62-64	MeOH:CH_2Cl_2 (50:50)	Yellow viscous liquid	0.1989
PEF30	65-74	MeOH (100)	Yellow viscous liquid	0.1607

3.5.4 Chemical investigation of hexane crude extract of seeds of *C. viscosa*

100 mg of crude hexane extract of seeds was also isolated by using preparative TLC. The TLC plate was prepared by coating the 6 glasses of TLC plates (20x20 cm) with a slurry of silica gel 60 PF_{254} (100 g) in distilled water (250 ml). The plate was dried in oven at 110 °C overnight. After preparation of TLC plate, the solution of this extract (100 mg) in hexane (2 ml) was loaded and developed by

Hex:EtOAc (6:4) as mobile phase. Each band was scraped and extracted with solvent. The extracts of bands were tested for antimicrobial activity (Table 3.6).

Table 3.6 The combined fractions obtained from isolation of the hexane extract of seeds of *C. viscosa* (SEB) using preparative TLC

Fraction code	Appearance	Weight (mg)
SHB1	Yellow viscous liquid	1.6
SHB2	Yellow viscous liquid	5.7
SHB3	Yellow viscous liquid	72.1
SHB4	Yellow viscous liquid	1.0
SHB5	Yellow viscous liquid	0.3
SHB6	Yellow viscous liquid	7.7
SHB7	Yellow viscous liquid	4.3

3.5.5 Preparation of propylene glycol crude extract of *C. viscosa*

Grounded dried whole plant of *C. viscosa* (20 g) were extracted with a solution of propylene glycol:distilled water at various ratio (1:1, 2:8 and 3:7) and the results were shown in Table 3.7. The crude extracts were filtered through Whatman paper No. 1. Then antimicrobial activity of those extracts was tested.

Table 3.7 The propylene glycol extracts of *C. viscosa*

Fraction code	Appearance
leaves and stems extracted with a solution of propylene glycol:H_2O, 1:1 (T1:1)	Brown liquid
leaves and stems extracted with a solution of propylene glycol:H_2O, 2:8 (T2:8)	Brown liquid
leaves and stems extracted with a solution of propylene glycol:H_2O, 3:7 (T3:7)	Brown liquid
pods extracted with a solution of propylene glycol:H_2O, 1:1 (P1:1)	Brown liquid

Table 3.7 (con.)

Fraction code	Appearance
pods extracted with a solution of propylene glycol:H_2O, 2:8 (P2:8)	Brown liquid
pods extracted with a solution of propylene glycol:H_2O, 3:7 (P3:7)	Brown liquid
roots extracted with a solution of propylene glycol:H_2O, 1:1 (R1:1)	Yellow liquid
roots extracted with a solution of propylene glycol:H_2O, 2:8 (R2:8)	Yellow liquid
roots extracted with a solution of propylene glycol:H_2O, 3:7 (R3:7)	Yellow liquid

3.6 Chemical investigation of *G. griffithii*

3.6.1 Chemical investigation of *G. griffithii*

The dichloromethane extract of *G. griffithii* fruits (5 g) was isolated by silica column chromatography to give 16 combined fractions as shown in Table 3.8. All fractions were tested for antimicrobial activity using microdilution method as described above. The fractions which exhibited the antimicrobial activity were further isolated by using silica gel column chromatography or preparative TLC.

Table 3.8 The combined fractions obtained from isolation of the CH_2Cl_2 extract of *G. griffithii* fruits using silica gel column chromatography

Fraction code	Fractions	Eluents (%)	Appearance	Weight (mg)
GGMDF1	1-13	CH_2Cl_2 (100)	Yellow viscous liquid	7.8
GGMDF2	14-38	CH_2Cl_2 (100)	Yellow liquid	41.92
GGMDF3	39-41	CH_2Cl_2 (100)	Yellow viscous liquid	5.7
GGMDF4	42-45	MeOH:CH_2Cl_2 (1:99)	Yellow viscous liquid	5.9
GGMDF5	46-64	MeOH:CH_2Cl_2 (1:99)	White solid and yellow viscous liquid	34.1
GGMDF6	65-94	MeOH:CH_2Cl_2 (2:98)	Green solid	30.5
GGMDF7	95-103	MeOH:CH_2Cl_2 (2:98)	Green solid	20.9
GGMDF8	104	MeOH:CH_2Cl_2 (4:96)	Green solid	3.6
GGMDF9	104-124	MeOH:CH_2Cl_2 (4:96)	Green solid	51.5
GGMDF10	125-131	MeOH:CH_2Cl_2 (6:94)	Green solid	15.7
GGMDF11	132-162	MeOH:CH_2Cl_2 (8:92)	Green solid	1374.1
GGMDF12	163-181	MeOH:CH_2Cl_2 (10:90)	Green solid	1980.0
GGMDF13	182-187	MeOH:CH_2Cl_2 (12:88)	Green solid	106.57
GGMDF14	188-196	MeOH:CH_2Cl_2 (14:86)	Green solid	1188.6
GGMDF15	197-200	MeOH:CH_2Cl_2 (14:86)	Green solid	100.7
GGMDF16	201-212	MeOH:CH_2Cl_2 (15:85)	Green solid	136.7

The rest of dichloromethane extract of *G. griffithii* fruits (4.8 g) was also isolated by silica column chromatography to give 10 combined fractions as shown in Table 3.9. All fractions were tested for antimicrobial activity using microdilution method. The fractions which exhibited the antimicrobial activity were further isolated by using silica gel column chromatography or preparative TLC.

Table 3.9 The combined fractions obtained from isolation of the second part of CH_2Cl_2 extract of *G. griffithii* fruits using silica gel column chromatography

Fraction code	Fractions	Eluents(%)	Appearance	Weight (mg)
GGMDF1rd	1-3	CH_2Cl_2 (100)	Yellow viscous liquid	19.0
GGMDF2rd	4	CH_2Cl_2 (100)	Yellow liquid	7.4
GGMDF3rd	5-9	CH_2Cl_2 (100)	Yellow viscous liquid	194.9
GGMDF4rd	10-25	CH_2Cl_2 (100)	White solid and yellow viscous liquid	48.1
GGMDF5rd	26-35	CH_2Cl_2 (100)	Green solid	16.1
GGMDF6rd	36-40	CH_2Cl_2 (100)	Green solid	169.6
GGMDF7rd	41-75	CH_2Cl_2:MeOH (98:2)	Green solid	191.55
GGMDF8rd	76-95	CH_2Cl_2:MeOH (92:8)	Green solid	1003.8
GGMDF9rd	96-104	CH_2Cl_2:MeOH (90:10)	Green solid	785.33
GGMDF10rd	105-141	MeOH (100)	Green solid	500.4

3.6.2 Chemical investigation of GGMDF1

GGMDF1 (7.8 mg) (see Table 3.8) was isolated by silica gel column chromatography. All fractions were yellow viscous liquid as shown in Table 3.10.

Table 3.10 The combined fractions obtained from isolation of the GGMDF1 using silica gel column chromatography

Fraction code	Fractions	Eluents (%)	Appearance	Weight (mg)
GGMDF1-1	1-50	hexane (100)	Yellow viscous liquid	1.8
GGMDF1-2	51-90	hexane:CH_2Cl_2 (99:1)	Yellow viscous liquid	1.0
GGMDF1-3	91-159	hexane:CH_2Cl_2 (98:2)	Yellow viscous liquid	0.6
GGMDF1-4	160-179	hexane:CH_2Cl_2 (97:3)	Yellow viscous liquid	4.2
GGMDF1-5	180-250	hexane:CH_2Cl_2 (95:5)	Yellow viscous liquid	2.9

3.6.3 Chemical investigation of GGMDF2

GGMDF2 was isolated by silica gel column chromatography. The results were shown on Table 3.11.

Table 3.11 The combined fractions obtained from isolation of the GGMDF2 using silica gel column chromatography

Fraction code	Fractions	Eluents (%)	Appearance	Weight (mg)
GGMDF2-1	1-8	hexane:CH_2Cl_2 (60:40)	Yellow viscous liquid	0
GGMDF2-2	9-14	hexane:CH_2Cl_2 (50)	Yellow viscous liquid	1.8
GGMDF2-3	15-19	hexane:CH_2Cl_2 (50:50)	White viscous liquid	1.9
GGMDF2-4	20-24	hexane:CH_2Cl_2 (40:60)	Yellow viscous liquid	1.1
GGMDF2-5	25-35	hexane:CH_2Cl_2 (40:60)	Yellow viscous liquid	13.7
GGMDF2-6	36-50	hexane:CH_2Cl_2 (30:70)	Yellow viscous liquid	205.1
GGMDF2-7	51-75	hexane:CH_2Cl_2 (30:70)	Yellow viscous liquid	117.9
GGMDF2-8	76-115	hexane:CH_2Cl_2 (80)	Yellow viscous liquid	9
GGMDF2-9	116-189	hexane:CH_2Cl_2 (20:80)	Yellow viscous liquid	5.5
GGMDF2-10	190-230	hexane:CH_2Cl_2 (100)	Yellow viscous liquid	1.4

3.6.4 Chemical investigation of GGMDF3

GGMDF3 (5.7 mg) was isolated by HPLC. GGMDF3 was dissolved in chloroform (1 ml) and injected into an HPLC column (100 µl) with mobile phase MeOH:water (80, HPLC grade:20), flow rate: 3 ml/min, read at 220, 254 and 365 nm, on UV detector. Separation by HPLC gave GGMDF3H13 at retention time 13 min (Table 3.12, Figure 3.1)

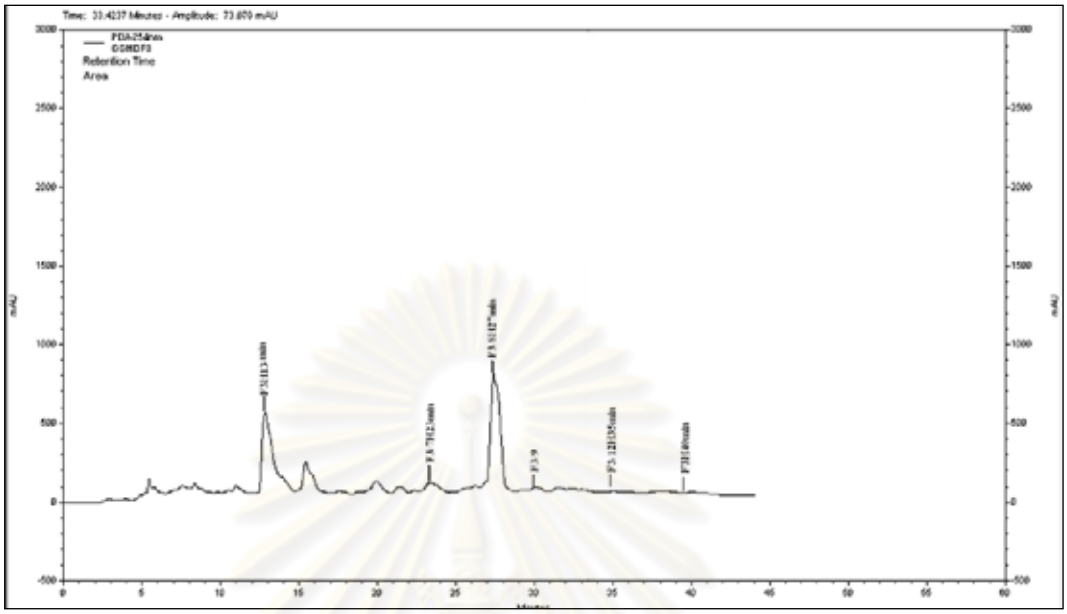


Figure 3.1 HPLC chromatogram of GGMDF3

Table 3.12 Retention time of GGMDF3 isolated from HPLC

Fractions	Retention time (min)
F3H13min	13
F3/7H23min	23
F3/8H27min	27
F3/9	30
F3H32min	35
F3/12H35min	39
F3H40min	39.9

3.6.5 Chemical investigation of GGMDF4

GGMDF4 (48.1 mg) was also isolated by HPLC. GGMDF4 was dissolved in chloroform (1 ml) and injected into an HPLC column (100 µl) with mobile phase MeOH:water (80, HPLC grade:20), flow rate: 3 ml/min, read at 220, 254 and 365 nm, on UV detector (Figure 3.2, Table 3.13).

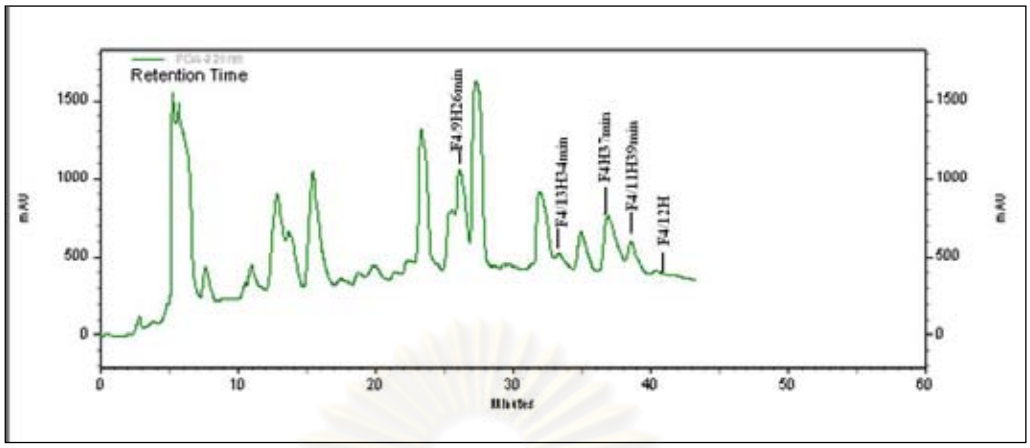


Figure 3.2 HPLC chromatogram of GGMDF4

Table 3.13 Retention time of GGMDF4 isolated from HPLC

Fractions	Retention time (min)
F4/9H26min	26
F4/13H34min	34
F4/11H39min	39
F4/12	42

3.6.6 Chemical investigation of GGMDF5

GGMDF5 w as is olated b y silica gel column c hromatography. The results were shown on Table 3.14.

Table 3.14 The combined fractions obtained from isolation of the GGMDF5 using silica gel column chromatography

Fraction code	Fractions	Eluents (%)	Appearance	Weight (mg)
GGMDF5-1	1-2	hexane:CH_2Cl_2 (60:40)	Green solid	31.0
GGMDF5-2	3-5	hexane:CH_2Cl_2 (50:50)	Green solid	1.5
GGMDF5-3	6-12	hexane:CH_2Cl_2 (40:60)	Green solid	1.8
GGMDF5-4	13-79	hexane:CH_2Cl_2 (30:70)	White solid	6.1
GGMDF5-5	80-102	hexane:CH_2Cl_2 (20:80)	Green solid	4.0
GGMDF5-6	103-200	hexane:CH_2Cl_2 (100)	Green solid	8.3

GGMDF5-4 was isolated by HPLC (Figure 3.3). The compound **1** (F5-4/8H34.49min) was obtained as white solid at retention time 34.49 min. It was subjected to spectroscopic analysis including NMR and optical rotation.

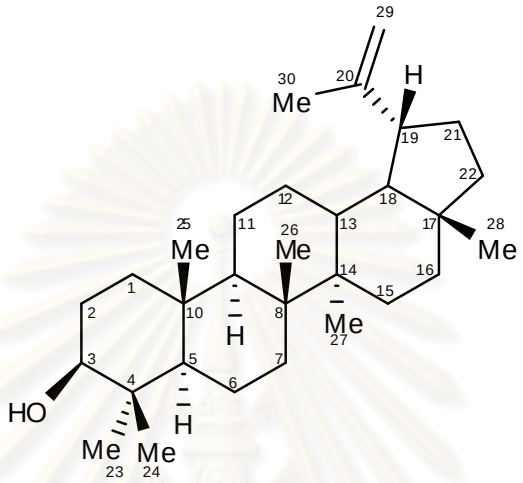


Figure 3.3 Structure of lupeol

R_f : 0.4 (CH_2Cl_2, silica gel 60 F_{254} TLC aluminum sheet)

mp. 208-210 ^oC

Optical Rotation: $[\alpha]_D^{20} = +41^o$ (c = 0.04, $CHCl_3$)

^{1}H NMR (400 MHz, $CDCl_3$): δ 0.68 (1H, d, J=9.2 Hz, H-5), 0.76 (3H, s, H-24), 0.79 (3H, s, H-28), 0.83 (3H, s, H-25), 0.92 (1H, m, H-1a), 0.94 (3H, s, H-27), 0.97 (3H, s, H-23), 0.99 (1H, m, H-2b), 1.02 (1H, m, H-15b), 1.03 (3H, s, H-26), 1.06 (1H, m, H-12a), 1.19 (1H, m, H-22a), 1.23 (1H, m, H-11a), 1.27 (1H, m, H-9), 1.32 (1H, ddd, J=10.8, 14.0, 19.6 Hz, H-21a), 1.37 (1H,m, H-18), 1.38 (1H, m, H-16a), 1.38 (1H, m, H-22b), 1.39 (1H, m, H-6a), 1.39 (2H, m, H-7), 1.41 (1H, m, H11b), 1.48 (1H, m, H-16b), 1.52 (1H, m, H-6b), 1.59 (1H, m, H2b), 1.66 (1H, m, H-13), 1.67 (1H, m, H-1b), 1.67 (1H, m, 12b), 1.68 (1H, m, H-15b), 1.68 (3H, s, H-30), 1.92 (1H, m, H-21b), 2.38 (1H, ddd, J=5.6, 10.8, 11.2 Hz, H-19), 3.19 (1, m, H-3), 4.57 (1H, br s, H-29a), 4.68 (1H, br s, H-29b)

^{13}C NMR (100 MHz, $CDCl_3$): δ 14.6 (C-27), 15.4 (C-24), 16.0 (C-26), 16.1 (C-25), 18.0 (C-28), 18.3 (C-6), 19.3 (C-30), 20.9 (C-11), 25.2 (C-12), 27.5 (C-15), 27.5 (C-2), 28.0 (C-23), 29.9 (C-21), 34.3 (C-7), 35.6 (C-16), 37.2 (C-10), 38.1 (C-13), 38.7 (C-1), 38.8 (C-4), 40.0 (C-22), 40.9 (C-14), 42.9 (C-8), 43.0 (C-17), 48.0 (C-19), 48.3 (C-18), 50.5 (C-9), 55.3 (C-5), 79.0 (C-3), 109.3 (C-29), 150.9 (C-20)

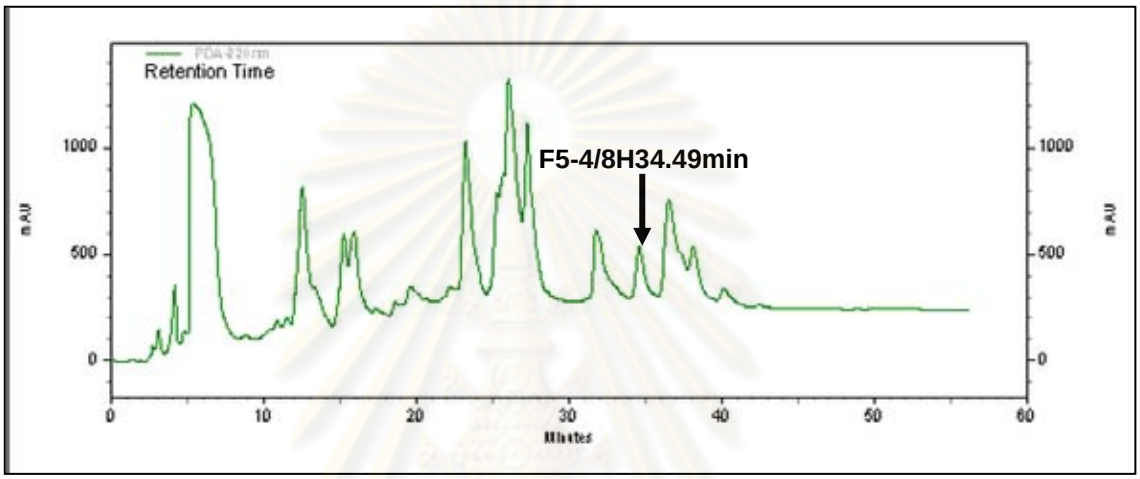


Figure 3.4 HPLC chromatogram of GGMDF5-4

3.6.7 Chemical investigation of GGMDF6

GGMDF6 (30.5 mg) was isolated by using silica gel column chromatography (Table 3.15). The green solids in yellow liquid oil obtained in the combined fractions GGMDF6-4 were washed by 90% CH_2Cl_2 in MeOH followed by crystallization from CH_2Cl_2 and MeOH to give the white solid compound (7.2 mg) as GGMDF6-4/1.

Table 3.15 The combined fractions obtained from isolation of the GGMDF6 using silica gel column chromatography

Fraction code	Fractions	Eluents (%)	Appearance	Weight (mg)
GGMDF6-1	1-35	hexane:CH_2Cl_2 (60:40)	White solid	0.1
GGMDF6-2	36-75	hexane:CH_2Cl_2 (50:50)	White solid	5.0
GGMDF6-3	76-110	hexane:CH_2Cl_2 (40:60)	White solid	21.7
GGMDF6-4	111-150	hexane:CH_2Cl_2 (30:70)	White solid and yellow viscous liquid	8.7

GGMDF6-2, GGMDF6-3 and 6-4/1 were isolated by HPLC, the retention time showed in Table 3.16 and 3.17. All fractions were dissolved in 1 ml chloroform:MeOH, (1:1) and injected into an HPLC column (100 µl) with mobile phase MeOH:water (80, HPLC grade: 20), flow rate: 3 ml/min, read at 220, 254 and 365 nm, on UV detector (Figure 3.4-3.6).

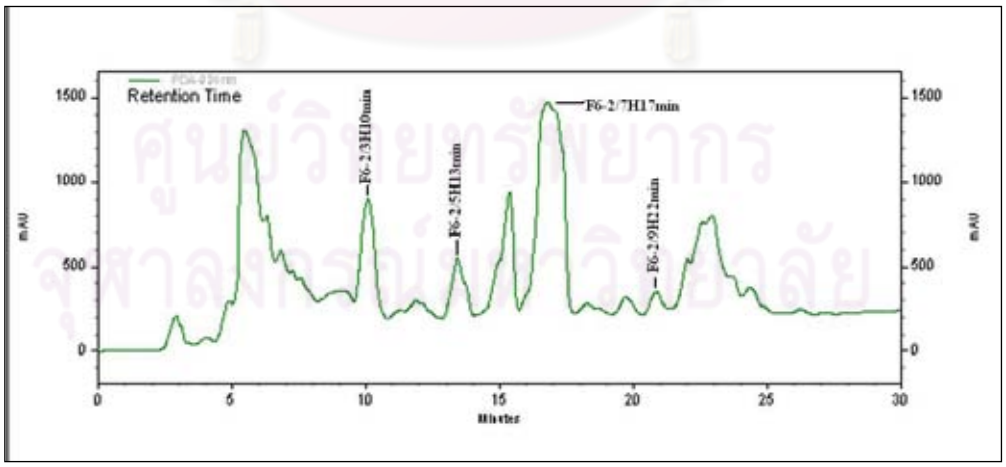


Figure 3.5 HPLC chromatogram of GGMDF6-2

Table 3.16 Retention time of GGMDF6-2 isolated from HPLC

Fractions	Retention time (min)
F6-2/3H10min	10
F6-2/5H13min	13
F6-2/7H17min	17
F6-2/9H22min	22

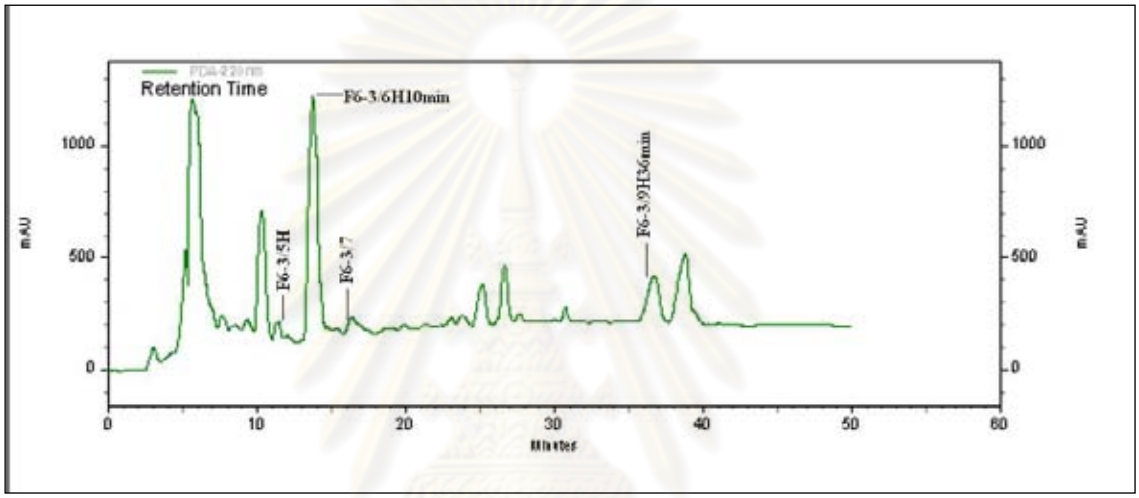


Figure 3.6 HPLC chromatogram of GGMDF6-3

Table 3.17 Retention time of GGMDF6-3 isolated from HPLC

Fractions	Retention time (min)
F6-3/5	11
F6-3/6H10min	14.9
F6-3/7	17
F6-3/8	28
F6-3/9	37

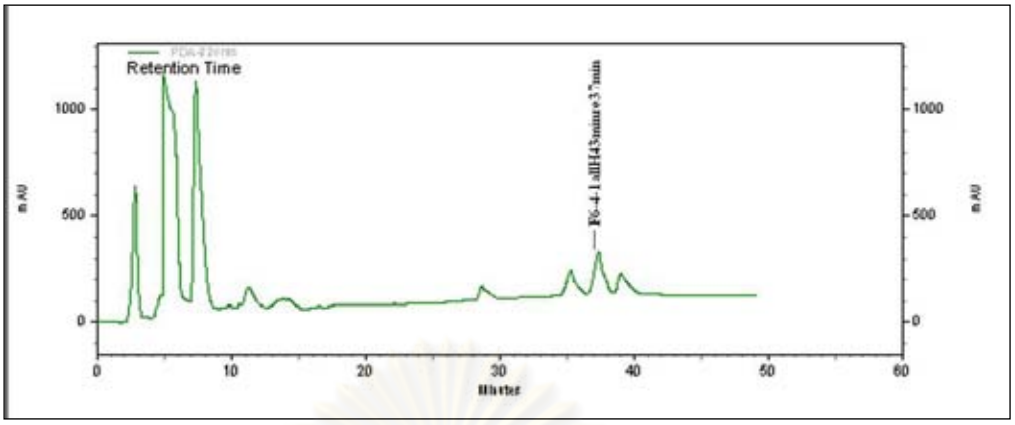


Figure 3.7 HPLC chromatogram of GGMDF6-4-1allH43min

3.6.8 Chemical investigation of GGMDF7

GGMDF7 was isolated by silica gel column chromatography to give 5 combined fractions (Table 3.19). GGMDF7-2, green solid, was isolated by HPLC as showed in Figure 3.7.

Table 3.18 The combined fractions obtained from isolation of the GGMDF7 using silica gel column chromatography

Fraction code	Fractions	Eluents (%)	Appearance	Weight (mg)
GGMDF7-1	1-5	CH_2Cl_2 (100)	Green solid	0.3
GGMDF7-2	6-20	CH_2Cl_2:MeOH(98:2)	Green solid	0.2
GGMDF7-3	21-65	CH_2Cl_2:MeOH (97:3)	Green solid	4.7
GGMDF7-4	66-200	CH_2Cl_2:MeOH (96:4)	Green solid	5.1
GGMDF7-5	201-268	CH_2Cl_2:MeOH (95:5)	Green solid	4.2

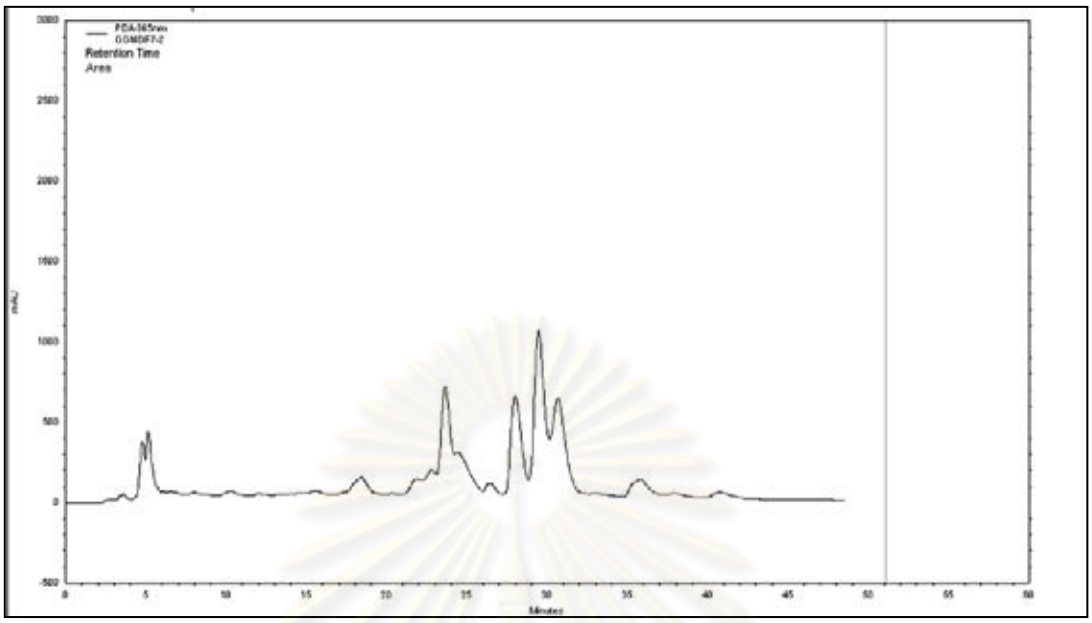


Figure 3.8 HPLC chromatogram of GGMDF7-2

3.6.9 Chemical investigation of GGMDF8

GGMDF8 (3.6 mg) was isolated by preparative TLC and developed by MeOH:CH_2Cl_2 (1:9) as mobile phase (Table 3.19).

Table 3.19 The combined fractions obtained from isolation of the GGMDF8 by preparative TLC

Fraction code	R_f	Appearance	Weight (mg)
GGMDF8B1	0.76	Green solid	1.3
GGMDF8B2	0.70	White solid	0.7
GGMDF8B3	0.62	Green solid	0.5
GGMDF8B4	0	White solid	0.6

3.6.10 Chemical investigation of GGMDF9

GGMDF9 (51.5 mg) was isolated by preparative TLC and developed by MeOH:CH_2Cl_2 (1:9) as mobile phase (Table 3.20).

Table 3.20 The combined fractions obtained from isolation of the GGMDF9 by preparative TLC

Fraction code	R_f	Appearance	Weight (mg)
GGMDF9B1	0.90	Green solid	17.5
GGMDF9B2	0.80	Green solid	6.8
GGMDF9B3	0.70	Green solid	8.3
GGMDF9B4	0.55	Green solid	3.0
GGMDF9B5	0.40	Green solid	2.0
GGMDF9B6	0	Green solid	4.0

3.6.11 Chemical investigation of GGMDF10

GGMDF10 (15.7 mg) was isolated by preparative TLC and developed by MeOH:CH_2Cl_2 (1:9) as mobile phase (Table 3.21).

Table 3.21 The combined fractions obtained from isolation of the GGMDF10 by preparative TLC

Fraction code	R_f	Appearance
GGMDF10B1	0.6	Green solid
GGMDF109B2	0.54	Green solid
GGMDF10B3	0.4	Green solid
GGMDF10B4	0.3	Green solid

3.6.12 Chemical investigation of GGMDF1rd

GGMDF1rd (19 mg) was isolated by preparative TLC and developed by Hexane:CH_2Cl_2 (9:1) as mobile phase (Table 3.22).

Table 3.22 The combined fractions obtained from isolation of the GGMDF1rd by preparative TLC

Fraction code	R_f	Appearance	Weight (mg)
GGMDF1rdB1	0.22	White solid	0.1
GGMDF1rdB2	0.67	White solid	1.4
GGMDF1rdB3	0	White solid	1

3.6.13 Chemical investigation of GGMDF2rd

GGMDF2rd (7.4 m g) w as i solated by pr eparative T LC a nd de veloped by hexane:CH_2Cl_2 (9:1) as mobile phase (Table 3.23).

Table 3.23 The combined fractions obtained from isolation of the GGMDF2rd by preparative TLC

Fraction code	Appearance	Weight (mg)
GGMDF2rdB1	White solid	0.6
GGMDF2rdB2	White solid	3.7
GGMDF2rdB3	White solid	1.2
GGMDF2rdB4	White solid	0.6

3.6.14 Chemical investigation of GGMDF3rd

GGMDF3rd (194.9 mg) was also i solated b y preparative T LC an d developed by MeOH: CH_2Cl_2 (1:9) as mobile phase (Table 3.24).

Table 3.24 The combined fractions obtained from isolation of the GGMDF3rd by preparative TLC

Fraction code	R_f	Appearance
GGMDF3rdB1	0.8	White solid
GGMDF3rdB2	0.6	Yellow viscous liquid
GGMDF3rdB3	0.3	White solid

3.6.15 Chemical investigation of GGMDF4rd

GGMDF4rd (48.1 mg) was isolated by silica gel column chromatography. The results were showed in Table 3.25.

Table 3.25 The combined fractions obtained from isolation of the GGMDF4rd using silica gel column chromatography

Fraction code	Fractions	Eluents (%)	Appearance	Weight (mg)
GGMDF4rd-1	1-5	hexane:CH_2Cl_2 (60:40)	White solid	2
GGMDF4rd-2	6-10	hexane:CH_2Cl_2 (50:50)	White solid	3.2
GGMDF4rd-3	11-35	hexane:CH_2Cl_2 (40:60)	White solid	6.7
GGMDF4rd-4	36-120	hexane:CH_2Cl_2 (30:70)	White solid	9.7
GGMDF4rd-5	121-201	hexane:CH_2Cl_2 (20:80)	not appearence	0
GGMDF4rd-6	202-226	CH_2Cl_2 (100)	Brown solid	3.1

GGMDF4rd-3 and GGMDF4rd-4 were isolated by using HPLC, showed in Figure 3.8 -3.9 and Table 3.26-3.27. All fractions were tested for antimicrobial activity.

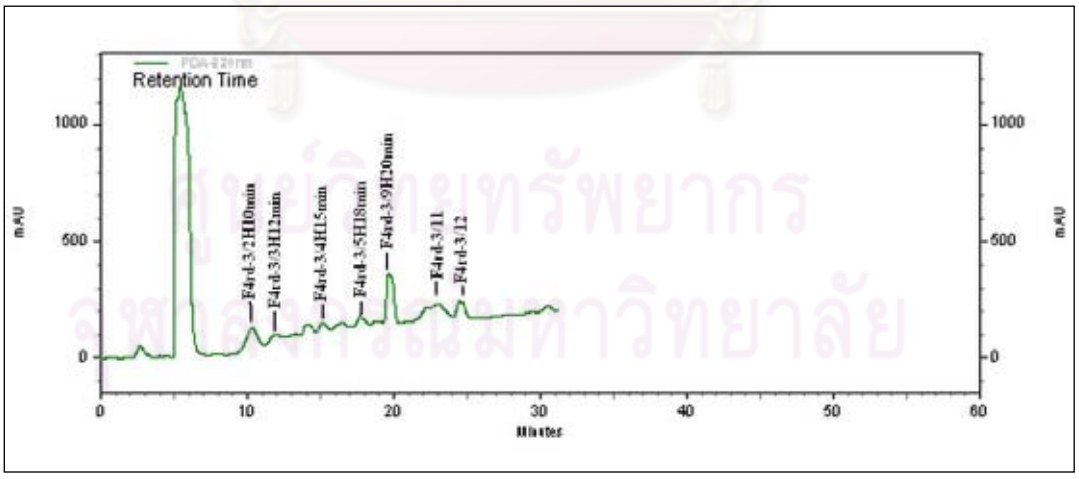


Figure 3.9 HPLC chromatogram of GGMDF4rd-3

Table 3.26 Retention time of GGMDF4rd-3 isolated from HPLC

Fractions	Retention time (min)
F4rd-3/2H10min	10
F4rd-3/3H12min	12
F4rd-3H15min	15
F4rd-3H18min	18
F4rd-3H22min	22
F4rd-3/11	23
F4rd-3/12	25

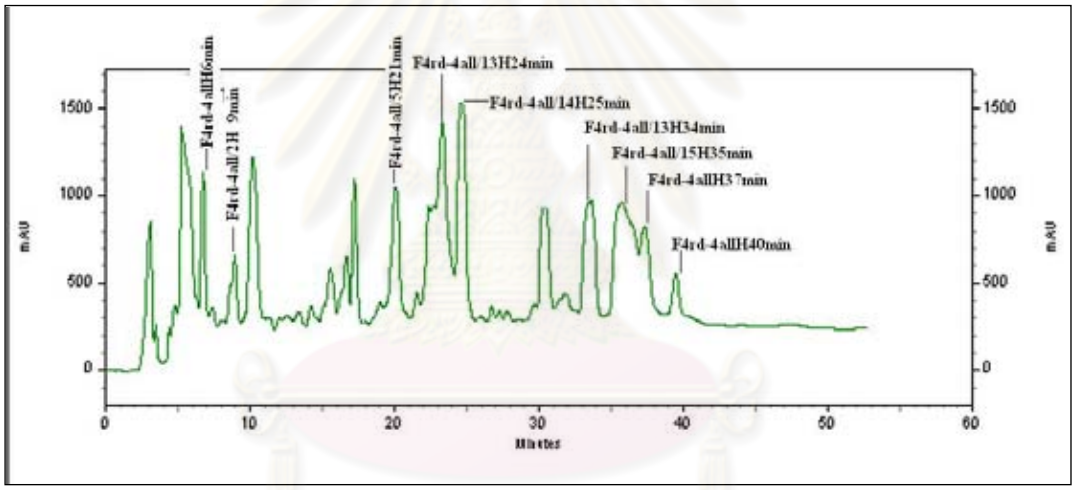


Figure 3.10 HPLC chromatogram of GGMDF4rd-4

Table 3.27 Retention time of GGMDF4rd-4 isolated from HPLC

Fractions	Retention time (min)
F4rd-4allH6min	6
F4rd-4all/2H9min	9
F4 rd -4all/5H21min	21
F4rd -4all/3H24min	24
F4rd-4all/5H25min	25
F4rd-4all/13H34min	34
F4rd-4all/12H35min	35
F4rd-4allH37min	37
F4rd-4allH40min	40

3.6.16 Chemical investigation of GGMDF5rd

GGMDF5rd (16.1 m g) was isolated b y H PLC. GGMDF5rd was dissolved in MeOH (1 ml) and injected into an HPLC column (100 µl) with mobile phase MeOH : water (80, HPLC grade : 20), flow rate: 3 ml/min, read at 220, 254 and 365 nm, on UV detector.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Antimicrobial activity screening of crude extracts of *C. viscosa*

4.1.1 Agar well diffusion and bioautography method

The healthy plants were collected from 2 provinces of Thailand. The plants from Nakhonsawan were divided to 4 parts, leaves and stems, pods, roots and seeds. *C. viscosa* from Ratchaburi were divided to 4 parts, there were leaves and stems, pods and roots. All parts of plants were extracted with distilled commercial solvents, hexane, EtOAc and MeOH. After evaporated under reduced pressure, all crude were tested for antimicrobial activity using agar well diffusion and bioautography method. The results showed that all crude extracts of *C. viscosa* could not inhibit *E. coli* and *P. aeruginosa* while all crude extracts of *C. viscosa* showed antimicrobial activity against *S. aureus* and *P. acnes* except crude extracts of root from Nakhonsawan (RH, RE and RM), crude extracts of leaves and stems from Rajchaburi (THR, TER and TMR) and MeOH extract of pods and roots from Rajchaburi (Table 4.1).

Hexane extract of pods (PH), hexane extract of seeds (SH) and EtOAc extract of seeds (SE) of *C. viscosa* from Nakhonsawan showed the lowest concentration for inhibition of *S. aureus* and *P. acnes* at 50 µg/ml (Figure 4.1) and MeOH extract of seeds (SM) at 50 µg/ml inhibited only *P. acnes*.

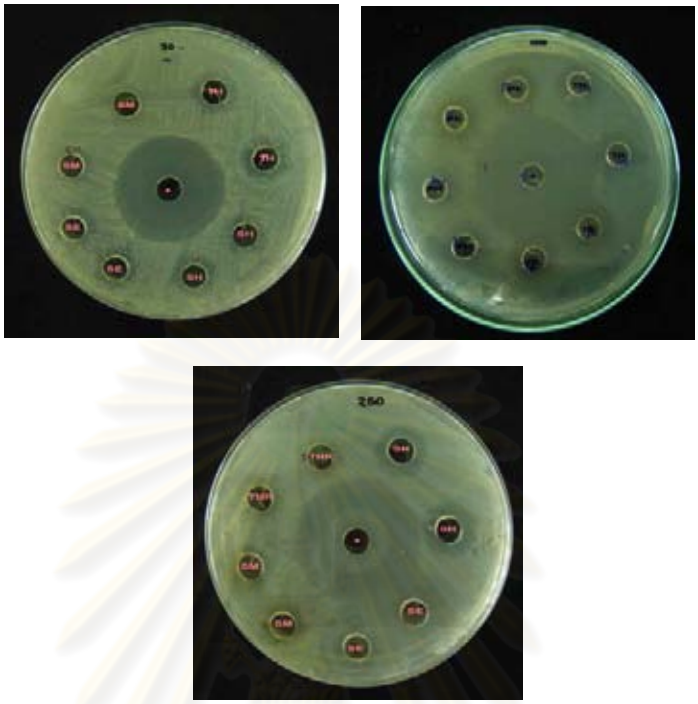
Hexane extract of *C. viscosa* pods collected from Ratchaburi showed the lower antimicrobial activity against *S. aureus* than hexane extract of pods from Nakhonsawan. Hexane extract of pods (PHR), EtOAc extract of pods (PER) and EtOAc extract of roots (RER) collected from Ratchaburi inhibited *S. aureus*. at 250 µg/ml and also inhibited *P. acnes* at 50 µg/ml.

All crudes were also investigated for antimicrobial activity by using TLC bioautography technique. The activity was determined by the white zones which appeared on TLC after spraying INT (Figure 4.2-4.5).

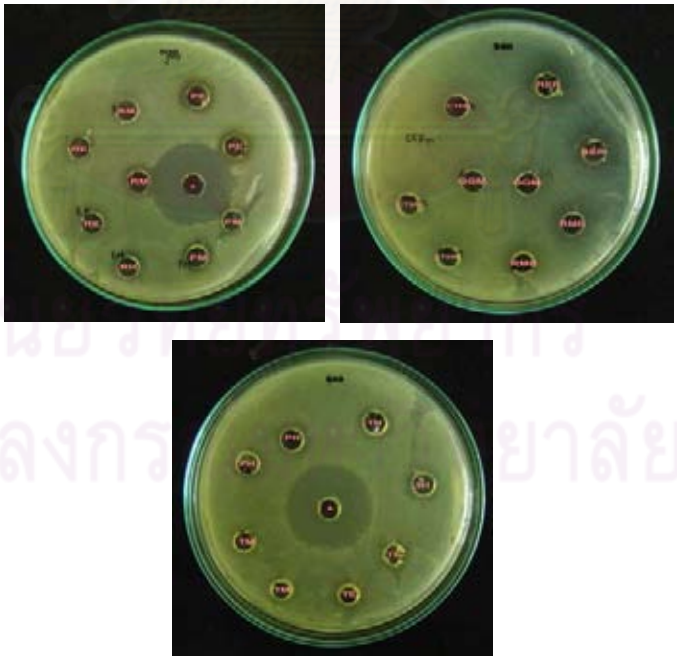
Table 4.1 Inhibition zone diameter in mm from agar well diffusion assay on antimicrobial activity of crude *C. viscosa*

Test microorganisms	***S. aureus***				***P. acnes***			
Conc. (µg/ml)* / **Crude****	**500**	**250**	**100**	**50**	**500**	**250**	**100**	**50**
TH	12	-	-	-	11	12	12	-
TE	13	-	-	-	9	11	13	-
TM	11	-	-	-	13	12	11	-
PH	18	15	14	13	18	15	13	10
PE	13.5	-	-	-	12	13	13.5	-
PM	-	-	-	-	10	-	-	-
RH	-	-	-	-	-	-	-	-
RE	-	-	-	-	-	-	-	-
RM	-	-	-	-	-	-	-	-
SH	15	13.5	12	11	12	14	15	13.5
SE	18	16	11	9	18	16	15	16
SM	11	9	-	-	13	12	11	9
THR	-	-	-	-	-	-	-	-
TER	-	-	-	-	-	-	-	-
TMR	-	-	-	-	-	-	-	-
PHR	15.5	13.5	-	-	16	15	15.5	13.5
PER	14.5	11.5	-	-	11	13	14.5	11.5
PMR	-	-	-	-	-	-		-
RHR	9.5	-	-	-	14	12	9.5	-
RER	13	11.5	-	-	12	13	13	11.5
RMR	-	-	-	-	-	-	-	-

* The inhibition diameter was an average of diameter (triplicate). The diameter of each well was 6 mm; symbol "-" means no inhibition zone;
** The code of crude *C. viscosa* were shown on scheme 3.1 and 3.2



(A) Crude extracts of *C. viscosa* against *S. aureus*



(B) Crude extracts of *C. viscosa* against *P. acnes*

Figure 4.1 Agar well diffusion assay of crude extracts of *C. viscosa* exhibiting antimicrobial activity against test microorganisms

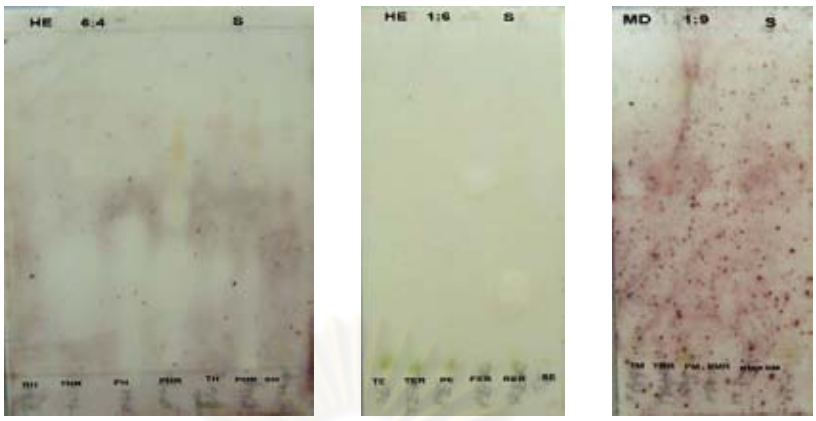


Figure 4.2 Bioautography of *C. viscosa* crude extract against *S. aureus*

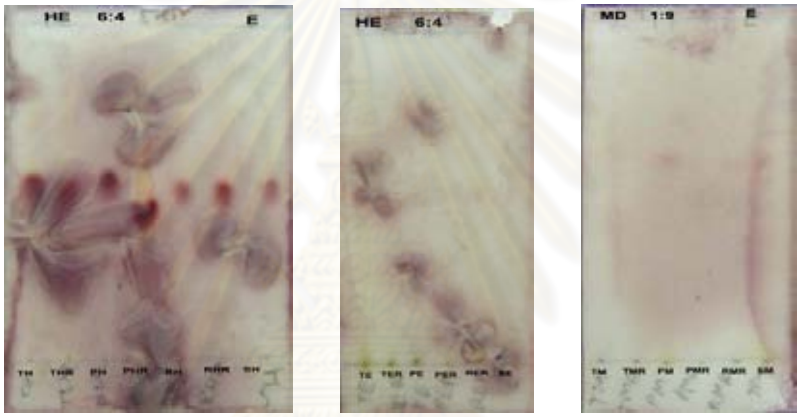


Figure 4.3 Bioautography of *C. viscosa* crude extract against *E. coli*

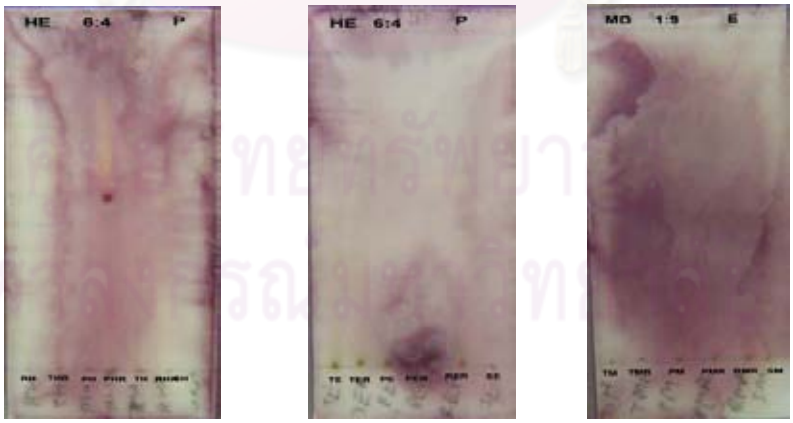


Figure 4.4 Bioautography of *C. viscosa* crude extract against *P. aeruginosa*

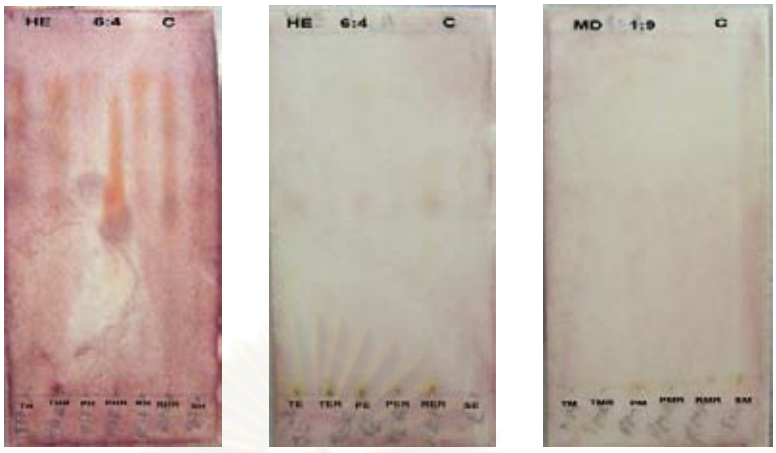


Figure 4.5 Bioautography of *C. viscosa* crude extract against *C. albicans*

According to this study, crude extracts of *C. viscosa* showed the similar antimicrobial activity as reported by Sudhakar. From Sudhakar et al., (2006) studied antimicrobial activity of *C. viscosa* agar well method. They found that ethanol extract of *C. viscosa* leaves and flowers could inhibit *S. aureus* at 40 µg/ml with clear zones 22 and 14 mm respectively. The ethanol extract of *C. viscosa* leaves and flowers also had the activity at the same concentration (40 µg/ml) against bacteria *P. aeruginosa*, *E. coli*, *P. vulgaris*, *B. subtilis*, *B. pumilus*, *Streptococcus faecalis,* fungi: *Aspergillus niger* and *Rhizopus oligosporus* and yeast *C. albicans*.

Williams et al. (2003) reported that ethanol extract of leaves and stems of *C. viscosa* had strong activity against *Bacillus subtilis* and *P. fluorescens* by using bioautography method and the ethanol extract of leaves and stems could inhibit fungus *Cladosporium cucumerinum.*

4.1.2 Minimum inhibition concentration assay of *C. viscosa*

Minimum inhibition concentration (MIC) of all crudes of *C. viscosa* were determined against five microorganisms using microdilution method (Athikomkulchai et al., 2008). The concentration of all crude was investigated as 500 µg/ml and then dilution two-fold to 6.25 µg/ml (last concentration). MIC results were exhibited the lowest concentration of crude extracts showing inhibition of growth (Table 4.2).

Table 4.2 Minimum inhibition concentration (MIC) of crude extracts of *C. viscosa*

Crude extracts	MIC (µg/ml)*				
	S. aureus	***P. acnes***	***C. albicans***	***E. coli***	***P. aeruginosa***
TH	500	500	50	-	-
TE	500	500	50	-	-
TM	500	-	50	-	-
PH	100	100	-	-	-
PE	200	100	50	-	-
PM	-	100	50	-	-
RH	-	500	50	-	-
RE	-	200	50	-	-
RM	-	200	100	-	-
SH	100	200	50	100	-
SE	100	-	50	100	-
SM	200	-	50	100	-
THR	-	-	100	-	-
TER	-	-	100	-	-
TMR	-	-	50	-	-
PHR	-	-	50	-	-
PER	500	500	50	-	-
PMR	-	-	50	-	-
RHR	500	-	50	-	-
RER	-	-	-	100	-
RMR	-	-	-	100	-

* - means no inhibition

Most of crude extracts of *C. viscosa* showed the efficiency effect against *C. albicans* at 50 µg/ml. All leaves and stems crude extract from Nakhonsawan inhibited *S. aureus* at 500 µg/ml. Crude extracts of seed also inhibited *S. aureus*. Leaves and stems crude extract from Ratchaburi had no antimicrobial activity against *S. aureus*. Most of crude extracts from Nakhonsawan inhibited *P.*

acnes, except MeOH extract of leaves and stems, EtOAc and MeOH extract of seeds. Only EtOAc extract of pods from Ratchaburi inhibited *P. acnes.* Crude extracts from Nakhonsawan and Rajchaburi were different.

Sudhakar et al. (2006) reported that ethanol extract of leaves of *C. viscosa* had the MIC value 0.1 mg/ml against *E. coli* while ethanol extract of flowers had the MIC value 0.2 mg/ml. The ethanol extract of leaves and flowers had the efficiency against microorganism such as *C. albicans*, *P. vulgaris*, *B. subtilis*, *B. pumilus*, *S. faecalis, A. niger* and *R. oligosporus*. Moreover, the ethanol extract of leaves had the MIC value 0.15 mg/ml against *S. aureus* and 0.2 mg/ml against *P. aeruginosa*. But the ethanol extract of flowers showed more MIC value than ethanol extract of leaves.

Williams et al. (2003) were studied antimicrobial activity by using TLC bioautography method. The results showed that the hexane extract of leaves and stems of *C. viscosa* could inhibit *B. subtilis*, *P. fluorescens* and *Cladosporium cucumerinum.* The MIC values obtained from *B. subtilis* and *P. fluorescens* were 5.0 and 1.0 µg/spot, respectively.

Source of plant is one of the factors that effect to production of secondary metabolites. Same species of plant from different sources can produce different bioactive compound depend on climate and geography.

4.2 Antimicrobial activity test of fraction from isolation of methanol extract of leaves and stems

The fraction from isolation of MeOH extract of leaves and stems by Sephadex LH-20 column chromatography were analyzed by MIC method. The concentration of all crude extracts were investigated as 250 µg/ml and then diluted two-fold to 6.25 µg/ml (last concentration). As a consequence of sufficient bacterial metabolism and growth, the oxidation-reduction indicator INT changes color from yellow to red. MIC value was determined as the lowest concentration which INT did not change to the red color. The results were shown in Table 4.3.

Table 4.3 The minimum inhibition concentration (MIC) using microdilution method of fraction from methanol extract of leaves and stems

Fractions	MIC (µg/ml)*				
	S. aureus	***P. acnes***	***C. albicans***	***E. coli***	***P. aeruginosa***
TMsep1	50	100	100	-	-
TMsep2	50	-	100	-	-
TMsep3	100	-	100	-	-
TMsep4	250	-	-	-	-
TMsep5	100	-	100	-	-
TMsep6	100	-	50	-	-
TMsep7	-	-	-	-	-
TMsep8	50	-	100	-	-
TMsep9	50	-	50	-	-
TMsep10	50	-	-	-	-
TMsep11	500	-	-	-	-
TMsep12	100	-	-	-	-
TMsep13	250	100	50	-	-
TM2sep1	250	-	-	-	-
TM2sep2	-	-	-	-	-
TM2sep3	-	-	-	-	-
TM2sep4	500	-	-	-	-
TM2sep5	500	-	-	-	-
TM2sep6	500	-	-	-	-
TM2sep7	250	-	-	-	-
TM2sep8	500	-	-	-	-
TM2sep9	500	-	-	-	-
TM2sep10	250	-	-	-	-
TM2sep11	250	-	-	-	-
TM2sep12	250	-	-	-	-
TM2sep13	250	-	-	-	-
TM2sep14	250	-	-	-	-

All fractions showed the efficiency against *S. aureus* except TMsep7, TM2sep2 and TM2sep3. Only TMsep1 and TMsep13 inhibited *P. acnes* at 100 µg/ml. Moreover, fractions TMsep6, TMsep9 and TMsep13 also had the antimicrobial activity against *C. albicans* with MIC value of 50 µg/ml. ^{1}H NMR spectra of the active fractions revealed that those fraction mainly comprised of oil and fatty acids, so they were complicating to be isolated to give any pure compounds.

4.3 Antimicrobial activity test of fraction from isolation of hexane extract of pods

Hexane extract of pods (PH), (50 mg) was isolated by preparative TLC using 40% EtOAc in hexane as mobile phase to give 12 bands. All bands were isolated and tested for antimicrobial activity against *S. aureus* and *P. acnes* by using microdilution assay (Table 4.4).

The results showed that most of them inhibited *S. aureus* at 500 µg/ml except PHB 2, PHB 4 and PHB 12 which all of them had no activity against *P. acnes*. Attempts to isolate pure antimicrobial agent by silica gel preparative TLC were unsuccessful due to < 1 mg of the active fraction.

The rest of hexane extract of pods (PHrd), 50 mg was also isolated by preparative TLC using 40% EtOAc in hexane as mobile phase to give 13 bands. All bands were isolated and tested for antimicrobial activity. The results showed that most of band inhibited *S. aureus* with MIC value of 500 µg/ml. Moreover, only PH 3rd exhibited antimicrobial activity against *P. acnes* with MIC value of 500 µg/ml.

Table 4.4 The minimum inhibition concentration (MIC) using microdilution method of fraction from hexane extract of pods

Fraction code	MIC (µg/ml)*	
	S. aureus	***P. acnes***
PHB 1	500	-
PHB 2	-	-
PHB 3	500	-
PHB 4	-	-
PHB 5	500	-
PHB 6	500	-
PHB 7	500	-
PHB 8	500	-
PHB 9	500	-
PHB 10	500	-
PHB 11	500	-
PHB 12	-	-

* - means no inhibition

Table 4.5 The minimum inhibition concentration (MIC) using microdilution method of fraction from the second part of hexane extract of pods

Fraction code	MIC (µg/ml)*	
	S. aureus	***P. acnes***
PHB 1rd	500	-
PHB 2rd	-	-
PHB 3rd	-	500
PHB 4rd	500	-
PHB 5rd	-	-
PHB 6rd	500	-
PHB 7rd	500	-
PHB 8rd	500	-
PHB 9rd	500	-
PHB 10rd	-	-
PHB 11rd	500	-
PHB 12rd	500	-
PHB 13rd	-	-

* - means no inhibition

4.4 Antimicrobial activity test of fraction from isolation of ethyl acetate extract of pods

The combined fractions from isolation of EtOAc extract of pods collected from Nakhonsawan (PE) by silica gel column chromatography were tested for antibacterial activity by using bioautography method (Cole, 1994). The TLC was developed in 1% MeOH in CH_2Cl_2 and the results were presented in Figures 4.6-4.7. White zones in colored background on TLC indicated inhibition zone of antimicrobial agents. The combined fractions from isolation of EtOAc extract of pods exhibited the antimicrobial activity against both *S. aureus* and *P. acnes*.

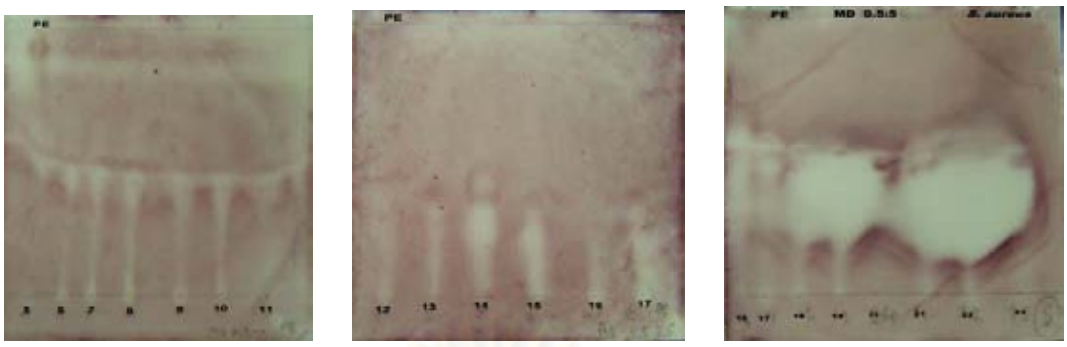


Figure 4.6 Bioautography of combined fractions from the isolation of EtOAc extract of pods against *S. aureus*

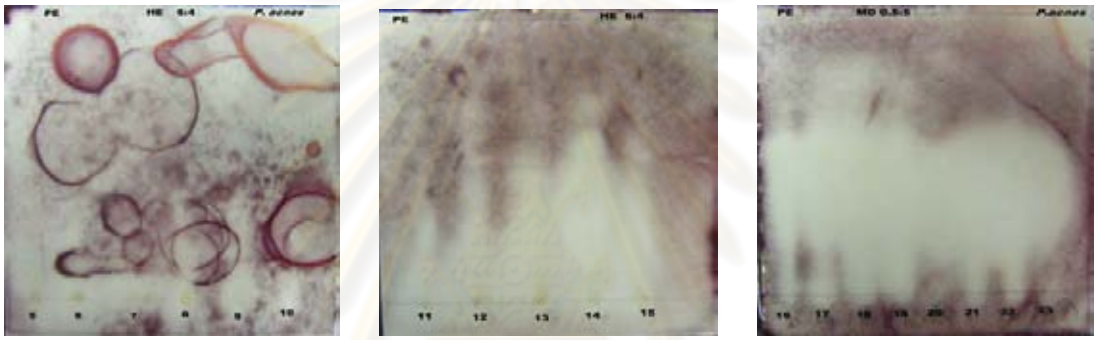


Figure 4.7 Bioautography of combined fractions from the isolation of EtOAc extract of pods against *P. acnes*

4.5 Antimicrobial activity test of fraction from isolation of hexane extract of seeds

Hexane extract of seeds (SH), 100 mg, was isolated by preparative TLC and developed in 40% EtOAc in hexane. 7 bands were isolated and tested for antimicrobial activity against *S. aureus* and *P. acnes* by using microdilution assay. The fractions from isolation of hexane extract of seeds showed the antimicrobial activity against both *S. aureus* and *P. acnes* at 500 µg/ml (Table 4.6). Four factions fractions from isolation of hexane extract of seeds could inhibit *S. aureus*. But only SHB 3 which could inhibit *P. acnes*. However, the active fractions had the low weight (less than 1 mg), so they were complicating to be isolated to give any pure compounds.

Table 4.6 The minimum inhibition concentration (MIC) using microdilution method of fraction from hexane extract of seeds

Fraction code	MIC (µg/ml) *	
	S. aureus	***P. acnes***
SHB 1	500	-
SHB 2	-	-
SHB 3	-	500
SHB 4	500	-
SHB 5	-	-
SHB 6	500	-
SHB 7	500	-

* - means no inhibition

4.6 Antimicrobial activity test of propylene glycol of *C. viscosa*

C. viscosa was extracted by propylene glycol which widely used in cosmetic production. The ratios of propylene glycol and distilled water were varying to 1:1, 2:8 and 3:7. After extraction, the extracts were filtered through Whatman no.1 and tested for antimicrobial activity by using microdilution method. The results showed that only propylene glycol extract with ratio 1:1 inhibited *S. aureus* at 250 µg/ml. All of the extracts had no activity against *P. acnes.* It could be a guide line for producing anti acnes from *C. viscosa*.

4.7 Antimicrobial activity screening of crude extracts of *G. griffithii*

After MeOH extract of *G. griffithii* was partitioned with CH_2Cl_2, the CH_2Cl_2 extract and a residue were tested for antimicrobial activity against 5 microorganisms (*S. aureus*, *P. acnes*, *E. coli*, *P. aeruginosa* and *C. albicans*) by using microdilution assay. Both CH_2Cl_2 extract and residue showed the antimicrobial activity against *P. acnes* and *E. coli.* The residue after partitioning inhibited *P. acnes* and *E. coli* at 100 µg/ml. While the CH_2Cl_2 extract inhibited *P. acnes* at 50 µg/ml and *E. coli* at 100 µg/ml.

4.7.1 Antimicrobial activity test of fractions from isolation of CH_2Cl_2 extract of *G. griffithii* fruits

Antimicrobial activity of the fractions from isolation of CH_2Cl_2 extract of *G. griffithii* fruits by silica gel column chromatography was examined by microdilution and bioautography method. For microdilution method, the fractions (GGMDF1, GGMDF2, GGMDF3, GGMDF4, GGMDF5, GGMDF6, GGMDF7, GGMDF9 and GGMDF10) were active against *E. coli* and *P. acnes* with MIC 12.5 to 50 µg/ml. Fraction GGMDF2 showed the lowest MIC value of 12.5 µg/ml against *E. coli* and *P. acnes*. Fractions GGMDF5 and GGMDF10 exhibited antimicrobial activity against *E. coli* at 25 µg/ml. Fraction GGMDF3 showed antimicrobial activity with the MIC value of 25 µg/ml against *P. acnes*. In contrast, GGMDF8, GGMDF11, GGMDF12, GGMDF13, GGMDF14, GGMDF15 and GGMDF16 had no activity against both bacteria (Table 4.7).

Table 4.7 The minimum inhibition concentration (MIC) using microdilution method of combine fraction from CH_2Cl_2 extract of *G. griffithii* fruits

Fraction code	MIC (µg/ml)*	
	E. coli	***P. acnes***
GGMDF1	50	50
GGMDF2	12.5	12.5
GGMDF3	50	25
GGMDF4	50	50
GGMDF5	25	50
GGMDF6	50	50
GGMDF7	50	-
GGMDF8	-	-
GGMDF9	50	-
GGMDF10	25	50
GGMDF11	-	-
GGMDF12	-	-
GGMDF13	-	-
GGMDF14	-	-
GGMDF15	-	-
GGMDF16	-	-

* - means no inhibition

By using bioautography method, CH_2Cl_2 extract of *G. griffithii* fruits was inactive against *E. coli* (Figure 4.8), while bioautography of fractions GGMDF3, GGMDF8, GGMDF9, GGMDF5, GGMDF6 and GGMDF11 showed inhibition zones against *P. acnes* (Figure 4.9).

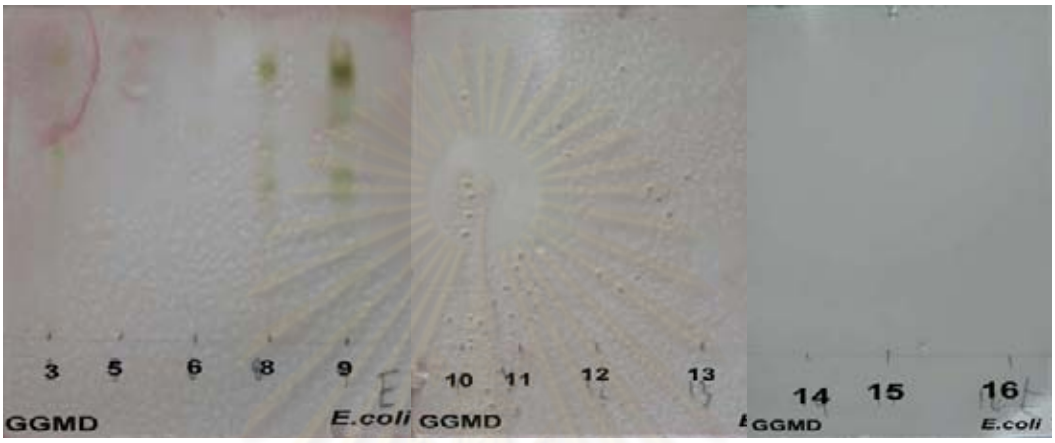


Figure 4.8 Bioautography of combined fractions from the isolation of CH_2Cl_2 extract of *G. griffithii* fruits by silica gel column chromatography against *E. coli*

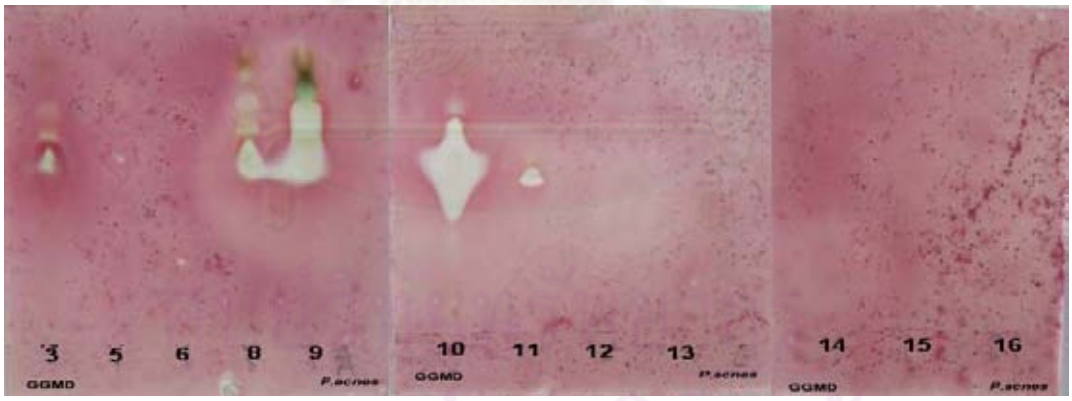


Figure 4.9 Bioautography of combined fractions from the isolation of CH_2Cl_2 extract of *G. griffithii* fruits by silica gel column chromatography against *P. acnes*

Isolation of dichloromethane extract (GGMDrd) of *G. griffithii* fruits by silica gel column chromatography gave 10 combined fractions. All fractions were tested for antimicrobial activity by using bioautography method.

The results (Figure 4.10-4.11) showed that GGMD3rd, GGMD6rd and GGMD7rd contained antimicrobial agents against *P. acnes* and all fractions were

inactive ag ainst *E. c oli*. There w ere m any c onstituents in G GMD6rd w hich could inhibit *P. acnes* (Figures 4.10-4.11).

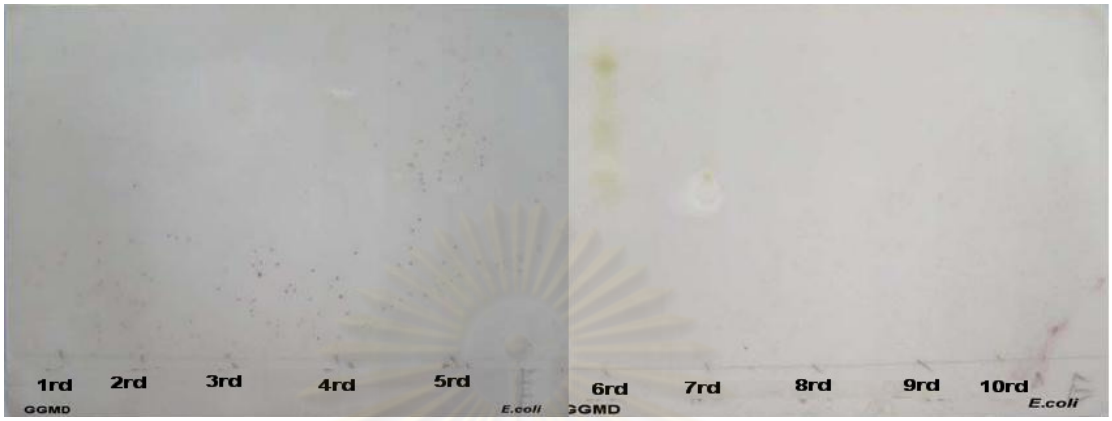


Figure 4.10 Bioautography of combined fractions from the isolation of the rest part of CH_2Cl_2 extract of *G. griffithii* fruits by silica gel column chromatography against *E. coli*

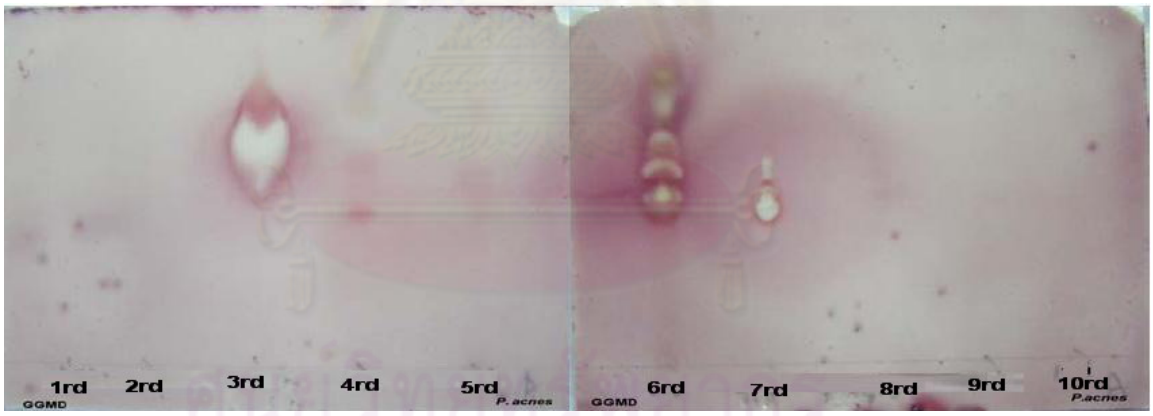


Figure 4.11 Bioautography of combined fractions from the isolation of the rest part of CH_2Cl_2 extract of *G. griffithii* fruits by silica gel column chromatography against *P. acnes*

Satdive et a l. (2003), r eported that e thanol e xtract of *G. sylvestre* leaves had the antimicrobial activity against *B. pumilis*, *B. subtilis* and *S. aureus,* gram positive bacteria, w ith the M IC value 150, 50 and 50 mg/ml by using a gar w ell diffusion m ethod. E thanol e xtract of *G. sylvestre* leaves al so s howed t he activity against gram negative bacteria, *P. aeruginosa* with the MIC value 50 mg/ml. But the ethanol extract of *G. sylvestre* could not inhibit *E. coli* and *Proteus vulgaris*.

4.7.2 Antimicrobial activity test of fractions from isolation of *G. griffithii* fruits by using HPLC

The fractions were collected by using HPLC and tested for antimicrobial activity against *E. coli* and *P. acnes*.

Table 4.8 The minimum inhibition concentration (MIC) using microdilution method of combined fractions from CH_2Cl_2 extract of *G. griffithii* fruits

Fractions	MIC (µg/ml)*	
	E. coli	***P. acnes***
F3H13min	-	-
F3/7H23min	6.25	-
F3/8H27min	-	-
F3/9	-	-
F3H32min	25	12.5
F3/12H35min	25	-
F3H40min	12.5	-
F4/9H26min	-	-
F4/13H34min	-	-
F4/11H39min	12.5	-
F4/12	12.5	-
F6-2/3H10min	-	-
F6-2/5H13min	25	-
F6-2/7H17min	6.25	-
F6-2/9H22min	12.5	-
F6-3/5	6.25	-
F6-3/6H16min	-	-
F6-3/7	-	-
F6-3/8	25	-
F6-3/9H36min	25	-

Fractions	MIC (µg/ml)*	
	E. coli	***P. acnes***
F4rd-3/2H10min	6.25	-
F4rd-3/3H12min	-	12.5
F4rd-3H15min	-	-
F4rd-3H18min	-	-
F4rd-3H22min	-	-
F4rd-3/11	-	-
F4rd-3/12	-	-
F4rd-4allH6min	12.5	-
F4rd-4all/2H9min	12.5	-
F4 rd - 4all/5H21min	25	-
F4rd-4all/5H25min	6.26	-
F4rd-4all/13H34min	12.5	-
F4rd-4all/12H35min	25	-
F4rd-4allH37min	12.5	-
F4rd-4allH40min	25	-

4.8 Characterization of compound 1

Fraction GGMDF5-4 (Table 3.14) was isolated by H PLC (using gradient system, water:methanol (HPLC grad) (15:85, 20 m in to M eOH (60 m in)) as mo bile phase) and compound **1** was obt ained at 34.49 m in of r etention t ime. After crystallization f rom MeOH, compound **1** was a fforded a s white s olid a nd i t w as soluble in chloroform and MeOH.

4.8.1 Structure elucidation of compound 1

The ^{13}C N MR an d H SQC s pectral d ata (Appendix B) showed 30 carbon signals co mprising o f s even m ethyl carbons (δ_C 14.6, 15.4, 16.0, 16.1, 18.0, 19.3, 28.0 ppm), t en sp^3 methylene car bons (δ_C 18.3, 20.9, 25.2, 27.5, 27.5, 29.9, 34.3, 35.6, 38.7, 40.0 ppm), one sp^2 methylene car bon (δ_C 109.3 ppm), s ix sp^3 methine c arbons (δ_C 50.5, 38.1, 48.0, 48.3, 55.3, 79.0 ppm), f ive sp^3 quaternary carbons (δ_C 37.2, 40.9, 42.9, 43.0, 38.9 ppm) and one sp^2 quaternary carbon (δ_C 150.9 ppm).

By 1D N MR and 2D NMR i ncluding C OSY, H SQC a nd H MBC structure of compound **1** was established.

The s ignal at δ_C 79.0 ppm i ndicated t hat t his methine car bon w as attached w ith h ydroxyl group and s ignals a t δ_C 109.3 (CH_2) a nd 150. 9 (C) w as assigned t o b e t he t erminal doubl e bond of t he i sopropenyl group. M oreover t he methyl group at δ_C 19.3 ppm and the exocyclic sp^2 methylene protons at δ_H 4.57 and 4.68 ppm , s upported t he e xistence of a n i sopropenyl group i n t he m olecule. F rom HMBC s pectral d ata w ith as sistance o f ^{1}H-^{1}H c orrelations (COSY), t he s keleton of compound **1** could be established as a lupane skeleton (Figure 4.12 and 4.13).

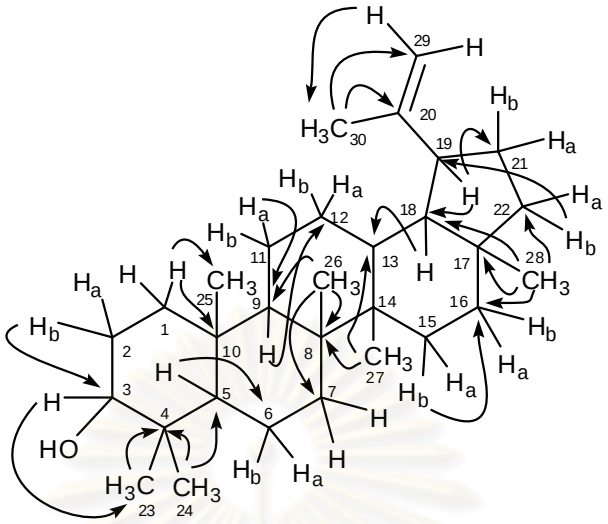


Figure 4.12 Crucial HMBC correlations of compound **1**

Melting point of compound **1** was 208-210°C (lit. m p. 211 -213°C, Mondon, 1975) . The specific optical rotation of compound **1** was $[\alpha]_D^{20}$ = +41° ($CHCl_3$, *c* 0.04) [lit. $[\alpha]_D^{25}$ = +26.5° ($CHCl_3$), Mondon et al., 1975] and the structure search in the literature revealed that compound **1** was agree with lupeol (Figure 4.14 and Table 4.9).

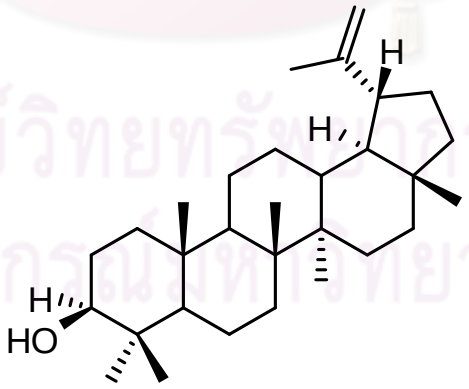


Figure 4.13 Structure of lupeol

Table 4.9 The comparison of ^{13}C NMR chemical shift of compound **1** with lupeol

Position	Chemical shift of ^{13}C NMR	
	Lupeol	**Compound 1**
1	39.1	38.7
2	27.8	27.5
3	79.3	79.0
4	39.2	38.9
5	55.6	55.3
6	18.7	18.3
7	34.6	34.3
8	41.2	40.8
9	50.7	50.5
10	37.5	37.2
11	21.3	20.9
12	25.5	25.2
13	38.7	38.1
14	43.2	40.9
15	27.8	27.5
16	35.9	35.6
17	43.4	43.0
18	48.3	48.3
19	48.6	48.0
20	151.1	150.9
21	30.2	29.9
22	40.4	40.0
23	28.4	28.0
24	15.8	15.4
25	16.5	16.1
26	16.3	16.0

Table 4.9 (con.)

Position	Chemical shift of ^{13}C NMR	
	Lupeol	**Compound 1**
27	14.9	14.6
28	18.4	18.0
29	109.6	109.3
30	19.7	19.3

(Fotie et al. 2006)

4.8.2 Antimicrobial activity of compound 1

Compound **1** was tested for antimicrobial activity by using microdilution method. The results showed that compound **1** inhibited *E. coli* and *P. acnes* with the MIC values of 25 and 50 µg/ml respectively.

Kuete et al. (2008) also found that lupeol could inhibit *E. coli* at the concentration 100 µg/disc by using paper disc diffusion technique. It could not inhibit other bacterial including *P. aeruginosa*, *S. aureus*, *B. subtilis* and *C. albicans*.

Hernhndez-PCrez et al. (1994) reported that lupeol which found in *Visnea mocanera* leaf extracts could inhibit *Bordetella bronchiseptica* and *Micrococcus luteus* with the concentration 0.025 and 0.05 mg/µl respectively

Margareth and Miranda (2009) reported that lupeol exhibited a broad spectrum of antimicrobial activity as showed in Table 4.10.

Table 4.10 Antimicrobial activity of lupeol (Margareth and Miranda, 2009)

Fugal species	MIC (µg/ml)	Bacteria species	MIC (µg/ml)
Sporothrix schenckii	12	*P. aeruginosa*	250
Microsporum canis	16	*E. coli*	250
Aspergillus fumit	93.5	*S. aureus*	250
Candida albicans	250	*Enterococcus faecalis*	63
Cryptococcus neoformans	180	*Salmonella typhi*	>200
Candida spicata	94	*Vibrio cholera*	>200

Lupeol was active against *C. albicans* on bioautography method. But in the microdilution assay, it failed to display appreciable activity against *C. albicans* with MIC value lower than 250 μg/ml (Shai et al, 2008).

CHAPTER V

CONCLUSIONS

Cleome viscosa L. collected from Nakornsawan and Ratchaburi, was extracted with hexane, ethyl acetate and methanol, respectively. All crude extracts were tested for antimicrobial activity against 5 microorganisms. Screening of antimicrobial activity by using TLC bioautography showed that the hexane extract of pods had the strong activity against *S. aureus* and it had inhibition zones of 13 and 15 mm for *S. aureus* and *P. acnes* respectively. The hexane extract of seeds also had efficiency activity similar to hexane extract of pods, it could inhibit *S. aureus* and *P. acnes* with the inhibition zones of 11 and 13.5 mm respectively. The other crude extracts also showed the antimicrobial activity but crude extracts from Ratchaburi had lower activity than crude extracts from Nakornsawan. This could suggest that plant which located from different places could produce different metabolites. The effects of geography and climate may the factors of metabolites production of plants. *C. viscosa* contained amount of oil, so that was difficult to isolate the active compound by column chromatography method.

Gymnema griffithii C. fruits were collected from Petchaburi. The fruits were extracted by methanol. The methanol crude extract was partitioned by dichloromethane. The CH_2Cl_2 extract (GGMD) and the residue were tested for antimicrobial activity. The results showed that CH_2Cl_2 extract and residue had the antimicrobial activity against *E. coli* and *P. acnes*.

Isolation of CH_2Cl_2 of *G. griffithii* by HPLC gave "lupeol" which exhibited antimicrobial activity against *E. coli* and *P. acnes* by using microdilution method with the MIC value 25 and 50 µg/ml repectivety.

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APPENDICES

Appendix A
Media

All media were added distilled water to a final volume of 1 liter and sterile in the autoclave at 121^{o}C for 15 minutes. The broth media did not add the agar in the formula.

1. Nutrient agar (NA)

Beef extract	3.0 g
Peptone	5.0 g
Agar	15.0 g

2. Brain heart infusion agar ((BHI agar)

Calf brain infusion from 200 g	7.7 g
Beef heart infusion from 250 g	9.8 g
Proteose peptone	10.0 g
Dextrose	20.0 g
Sodium chloride	5.0 g
Disodium phosphate	2.5 g

Appendix B

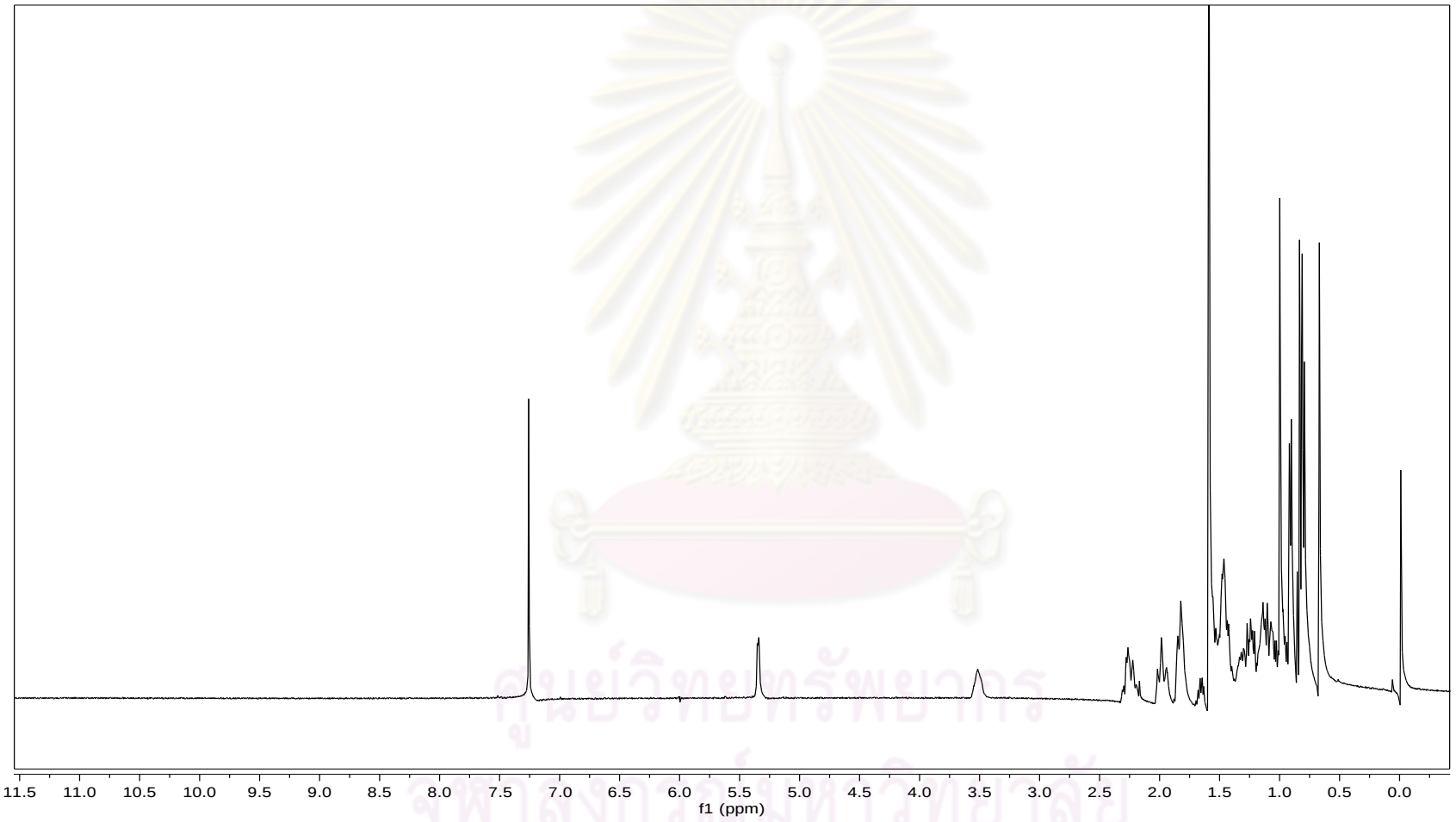

Figure B1 ^{1}H NMR of GGMDF6-4all 41 min

81

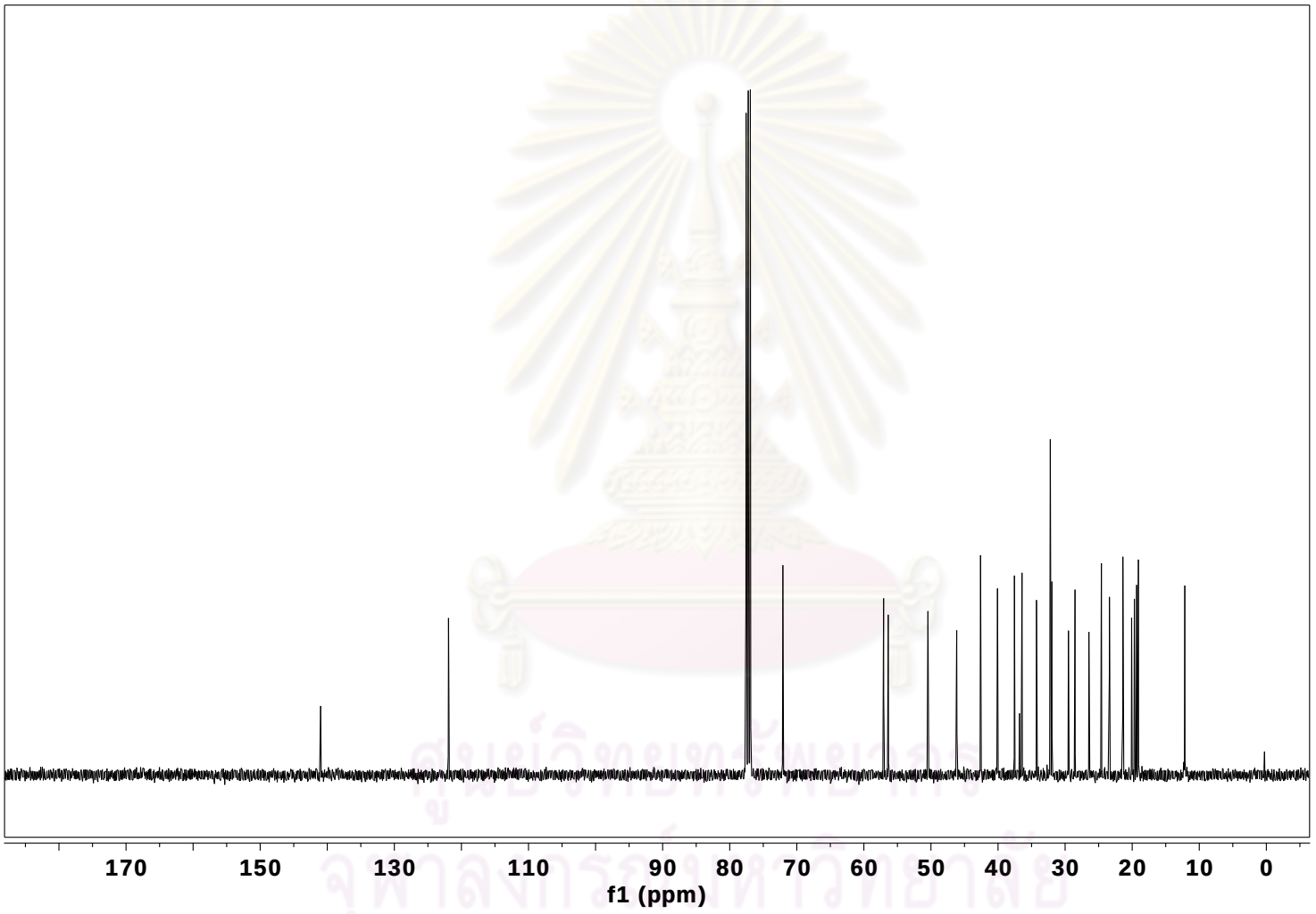

Figure B2 ^{13}H NMR of GGMDF6-4all 41 min

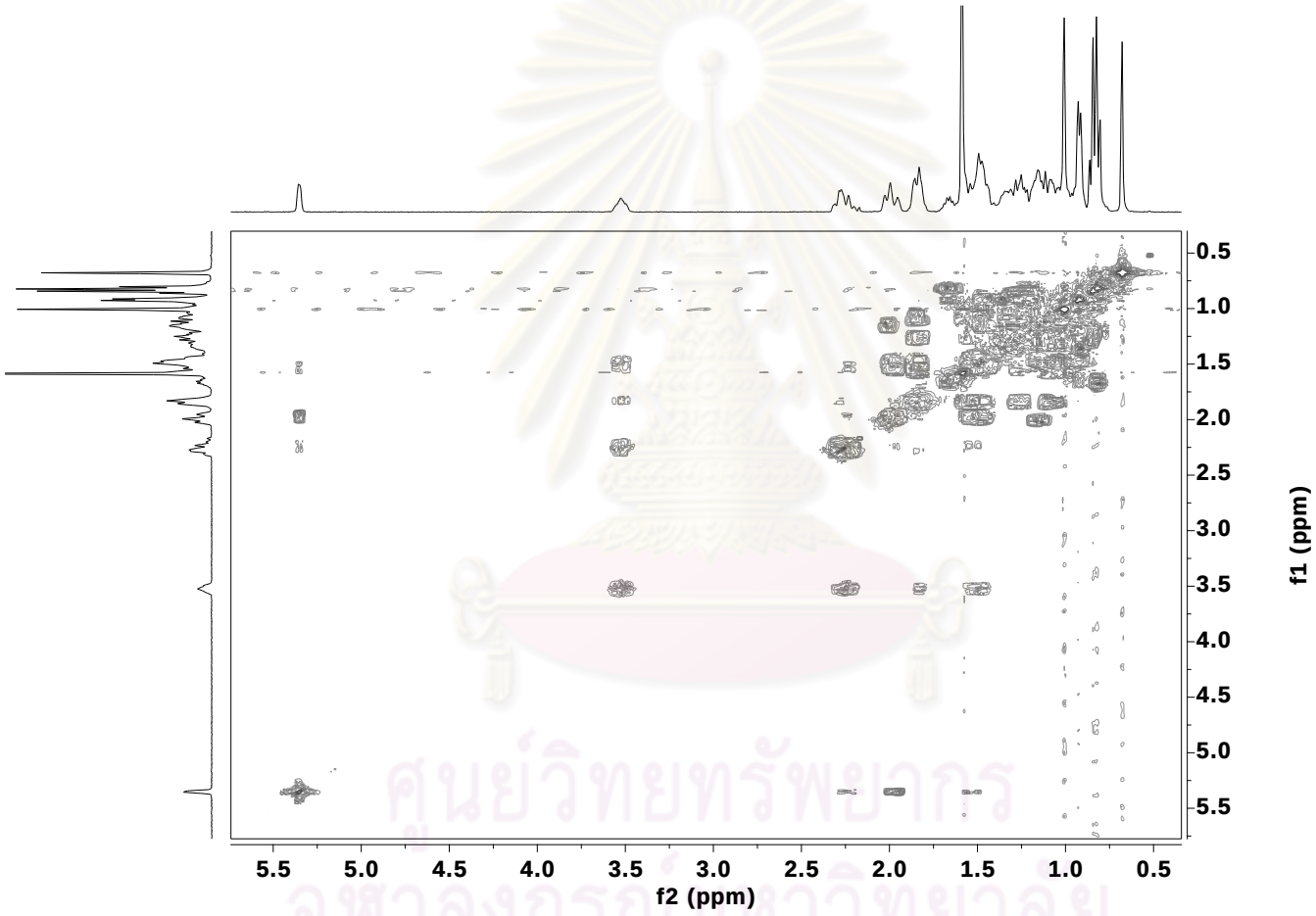

Figure B3 gCOSY of GGMDF6-4all 41 min

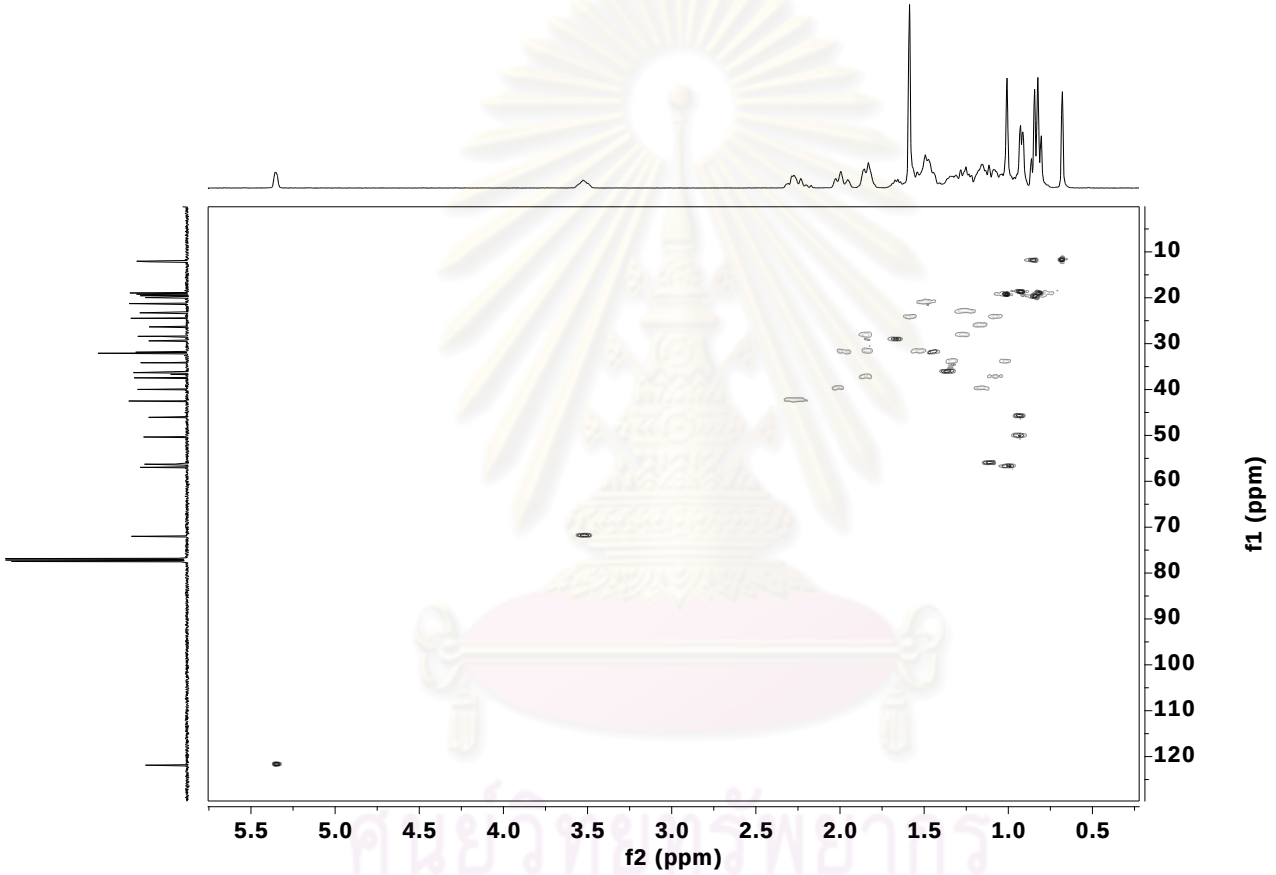

Figure B4 gHSQC of GGMDF6-4all 41 min

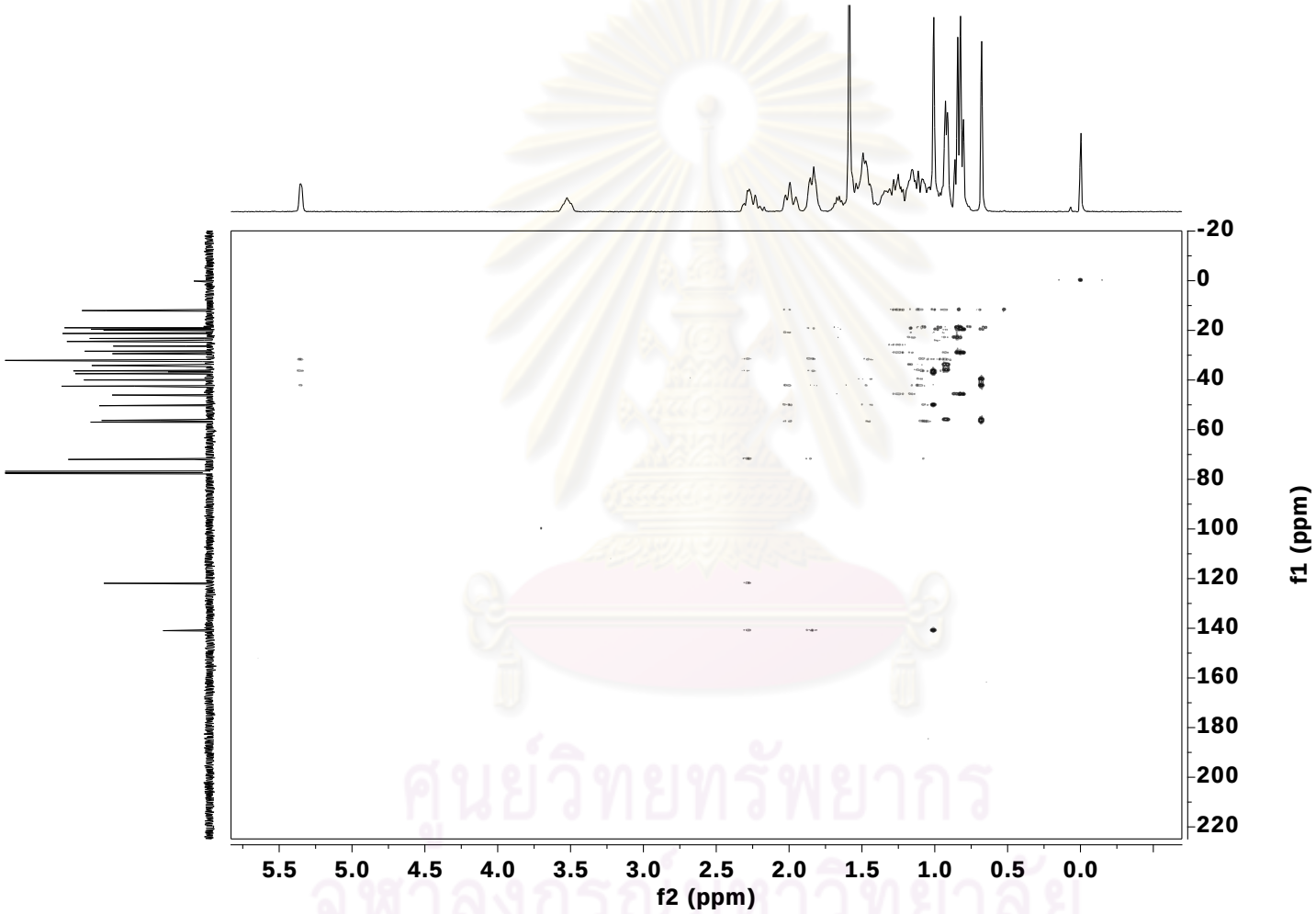

Figure B5 gHMBC of GGMDF6-4all 41 min

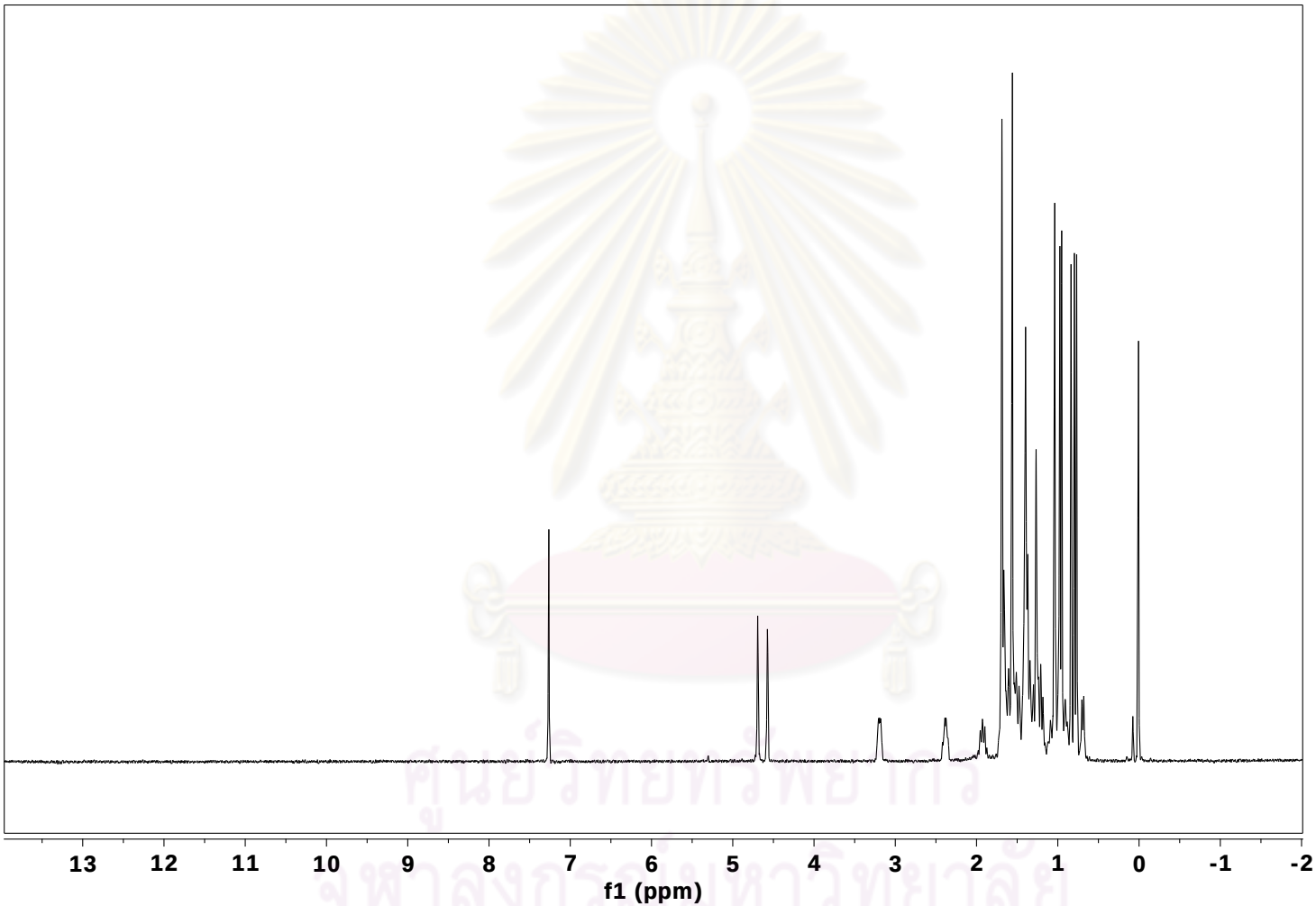

Figure B6 ^{1}H NMR of GGMDF5-4/8H34.39min

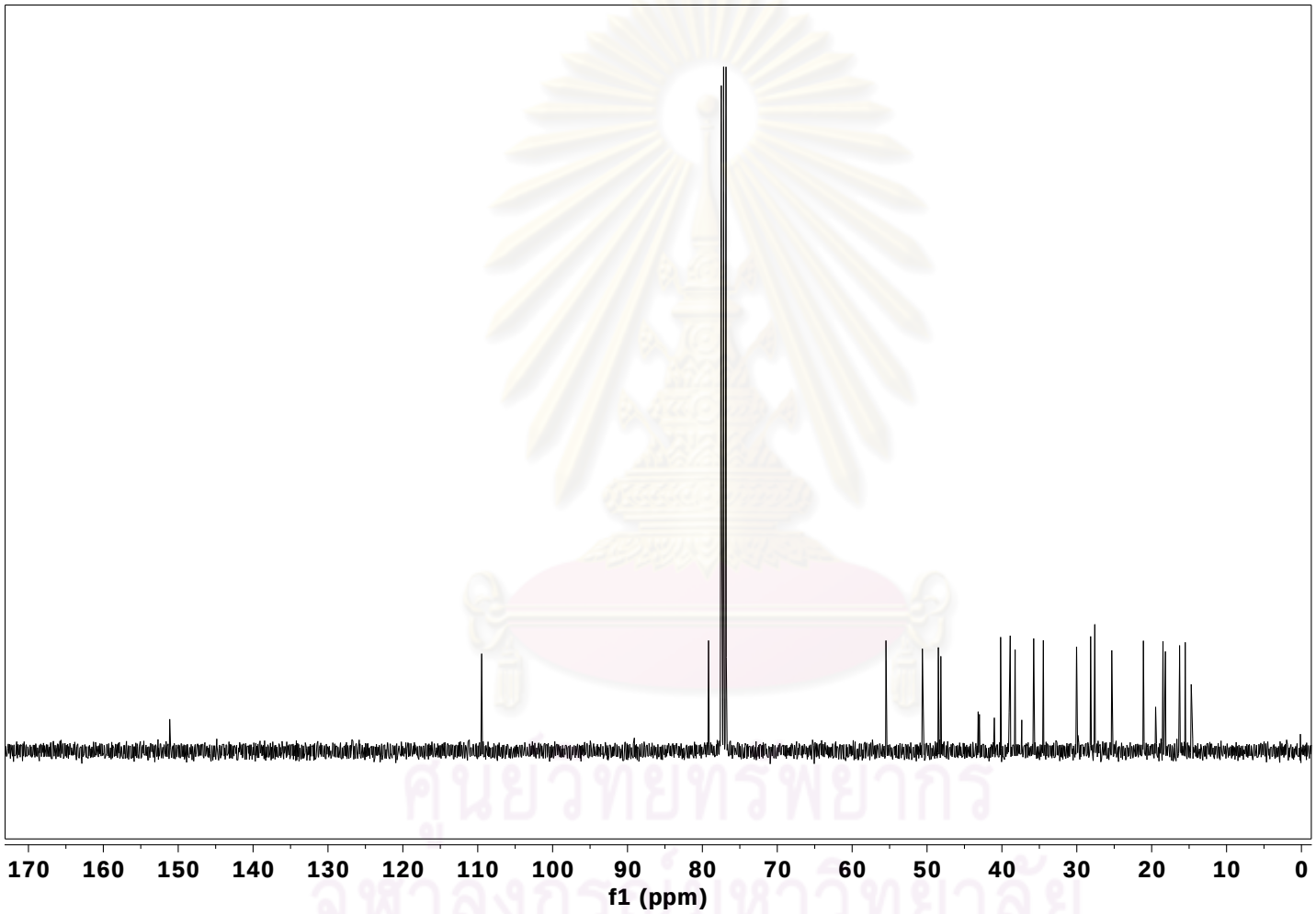

Figure B7 ^{13}H NMR of GGMDF5-4/8H34.49min

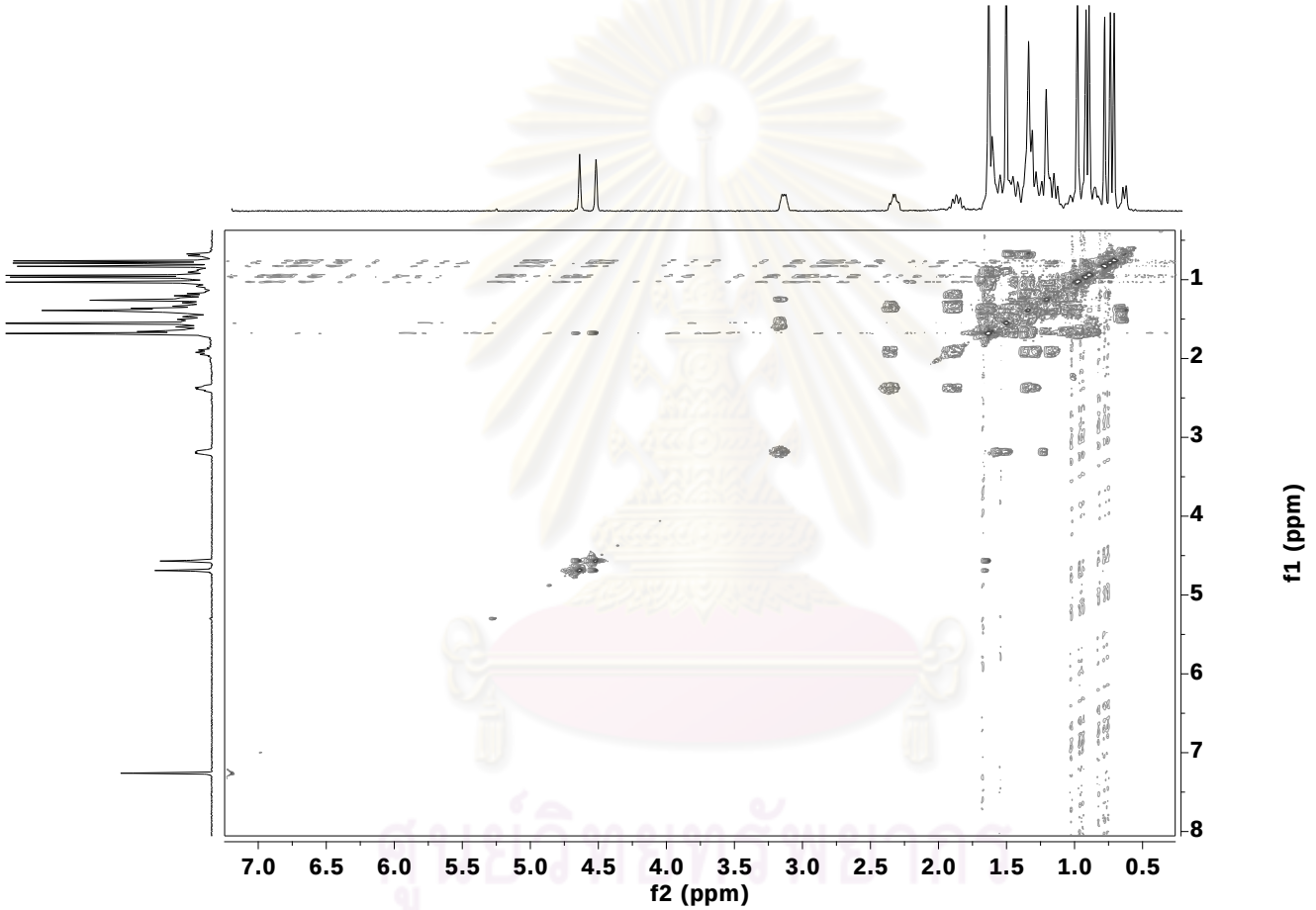

Figure B8 gCOSY of GGMDF5-4/8H34.49min

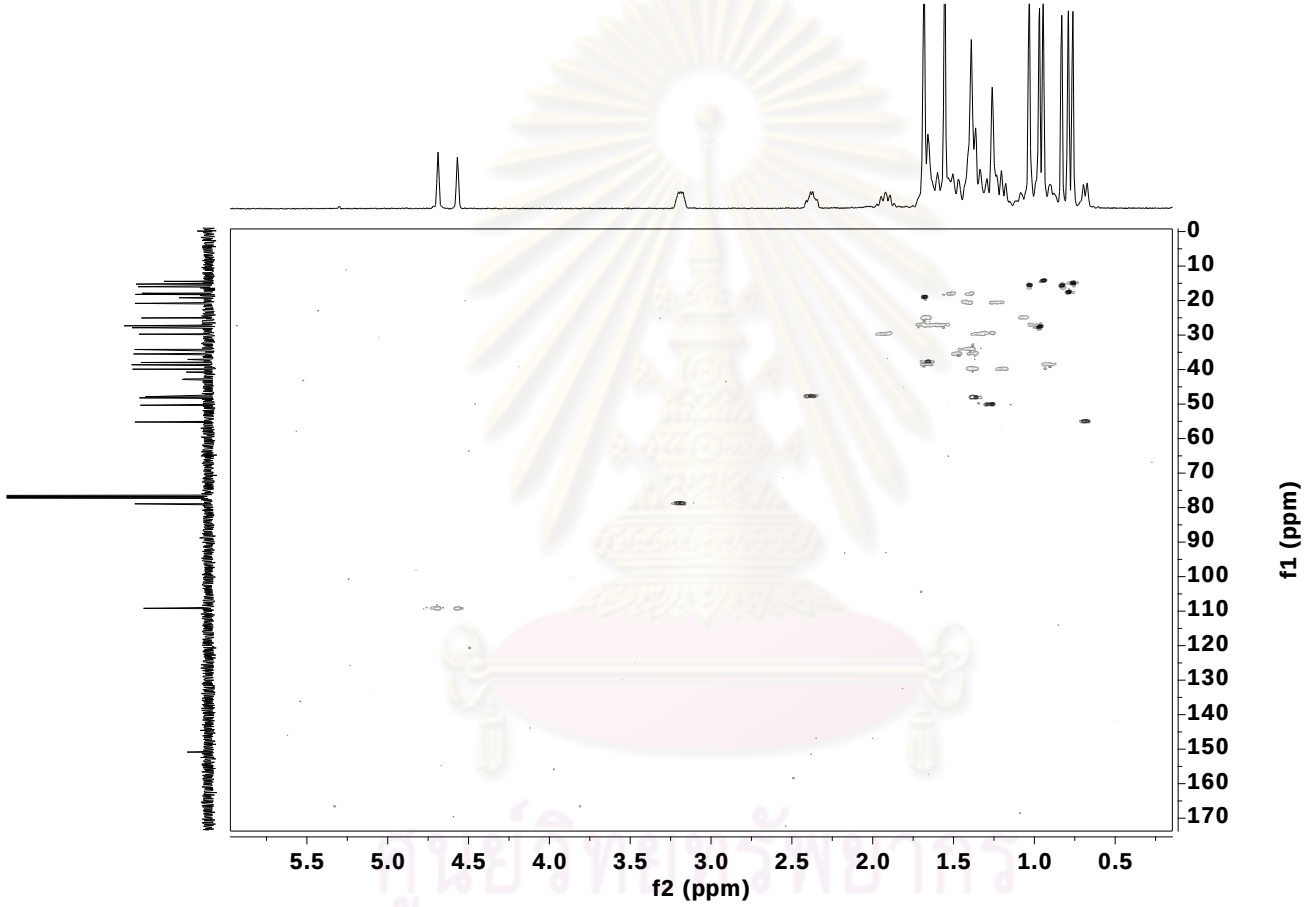

Figure B9 gHSQC of GGMDF5-4/8H34.49min

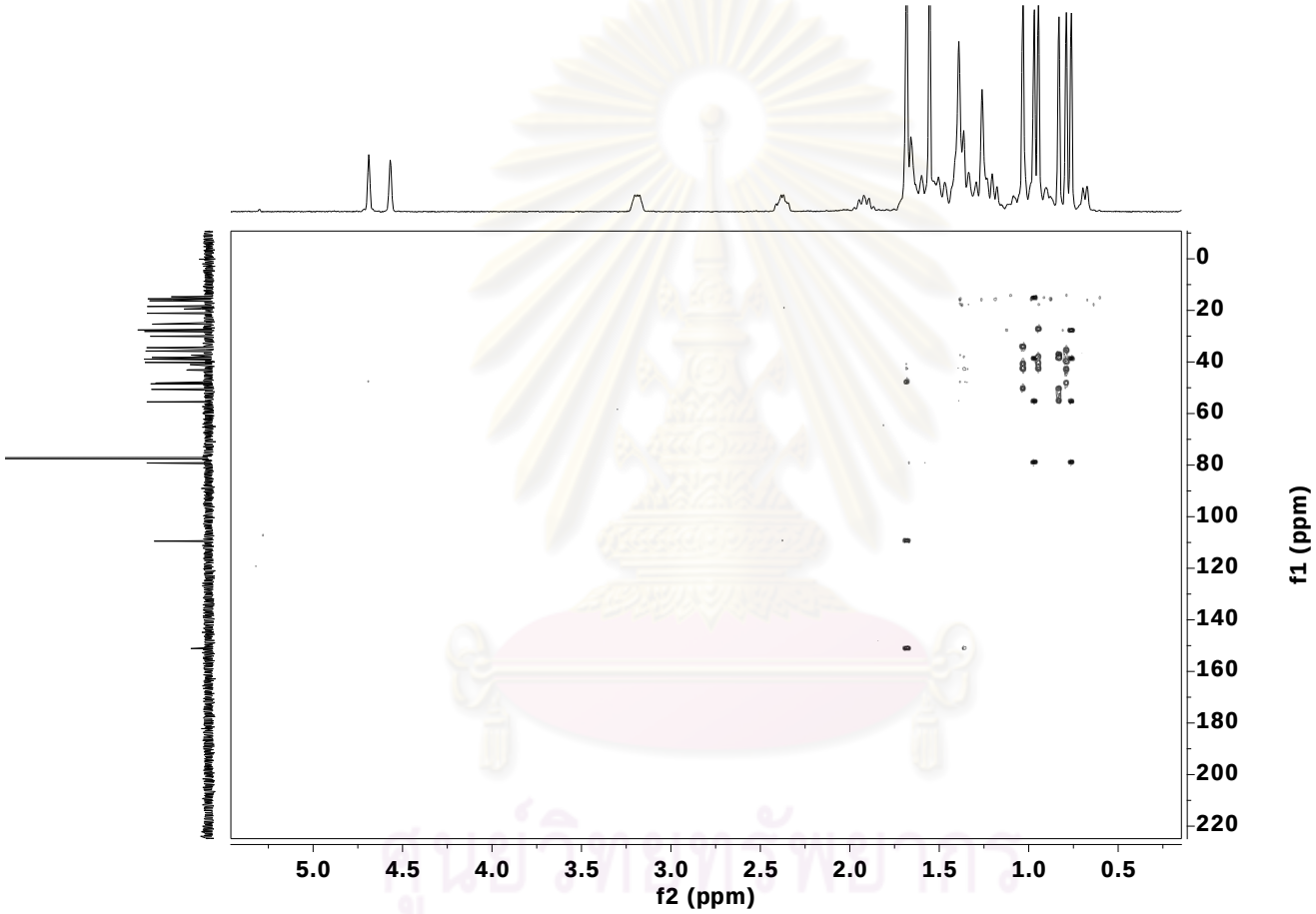

Figure B10 gHMBC of GGMDF5-4/8H34.49min

BIOGRAPHY

Miss Luntharima Prommi was born on October 9, 1985 in Sukhothai, Thailand. She obtained a Bacherlor Degree (Biotechnology) from the Department of Applied Biology, Faculty of Science, King Mongkut Institute of Technology Ladkrabang in 2007. She had been studying for a Master of Program in Biotechnology, Faculty of Science, Chulalongkorn University til 2005.