สารออกฤทธิ์ทางชีวภาพจากราเอนโดไฟต์จากต้นขันทองพยาบาท *Suregada multiflora* (A. Juss.) Baill.

นายเกียรติวุฒิ ดิษฐป้าน

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BIOACTIVE COMPOUNDS FROM ENDOPHYTIC FUNGI FROM *Suregada multiflora* (A. Juss.) Baill.

Mr. Keattivoot Ditpan

A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	BIOACTIVE COMPOUNDS FROM ENDOPHYTIC FUNGI FROM *Suregada multiflora* (A. Juss.) Baill.
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เกียรติวุฒิ ดิษฐป้าน: สารออกฤทธิ์ทางชีวภาพจากราเอนโดไฟต์จากต้นขันทองพยาบาท *Suregada multiflora* (A. Juss.) Baill. (BIOACTIVE COMPOUNDS FROM ENDOPHYTIC FUNGI FROM *Suregada multiflora* (A. Juss.) Baill.) อ.ที่ปรึกษา: รศ. ดร. นาตยา งามโรจนวณิชย์, อ.ที่ปรึกษาร่วม: ดร. ประสาท กิตตะคุปต์, 69 หน้า

ในการศึกษาเพื่อแยกสารออกฤทธิ์ทางชีวภาพจากราเอนโดไฟต์ไอโซเลต SM1 ที่แยกได้จากกิ่งขันทองพยาบาท พบว่าเมื่อนำสารสกัดหยาบที่ได้จากน้ำหมักเชื้อของราเอนโดไฟต์ไอโซเลต SM1 มาทำการแยกสารบริสุทธิ์โดยเทคนิคโครมาโทรกราฟี ได้สารบริสุทธิ์ 2 ชนิด คือ 2-phenylethanol และ 4-methyl-5-oxo-2-propyl-tetrahydro-furan-3-carboxylic acid ซึ่งได้พิสูจน์ทราบโครงสร้างทางเคมีของสารเหล่านี้ด้วยเทคนิคทางสเปคโทรสโคปี ร่วมกับการเปรียบเทียบข้อมูลที่มีรายงานมาแล้ว เมื่อนำสารบริสุทธิ์ที่แยกได้ไปทดสอบฤทธิ์ต้านจุลชีพ, ฤทธิ์ยับยั้งเชื้อมาลาเรีย และฤทธิ์ยับยั้งเซลล์มะเร็ง พบว่าสารบริสุทธิ์ทั้งสองชนิดไม่แสดงฤทธิ์ทางชีวภาพ การศึกษาทางสัญฐานวิทยาและการวิเคราะห์ลำดับนิวคลีโอไทด์ในส่วน ITS1-5.8S-ITS2 ของ rDNA สามารถจำแนกประเภทราเอนโดไฟต์ไอโซเลต SM1 คือ *Lasiodiplodia* sp. ซึ่งคาดว่าเป็น anamorph ของ *Botryosphaeria rhodina* (ลำดับนิวคลีโอไทด์เหมือนกัน 100 %)

สาขาวิชา.....เทคโนโลยีชีวภาพ..... ลายมือชื่อนิสิต.....เกียรติวุฒิ ดิษฐป้าน.....

ปีการศึกษา....2550..... ลายมือชื่ออาจารย์ที่ปรึกษา.....นาตยา งามโรจนวณิชย์.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

KEATTIVOOT DITPAN: BIOACTIVE COMPOUNDS FROM ENDOPHYTIC FUNGI FROM *Suregada multiflora* (A. Juss.) Baill. THE THESIS ADVISOR: ASSOC. PROF. NATTAYA NGAMROJANAVANICH, Ph.D., THESIS COADVISOR: PRASAT KITTAKOOP, Ph.D., 69 pp.

In this study we isolated bioactive compounds from the endophytic fungus isolate SM1 from a bark of *Suregada multiflora* (A. Juss.) Baill. Crude extract from the YES culture broth of endophytic fungus isolate SM1 was purified by chromatographic techniques to afford two compounds, which were identified as 2-phenylethanol, and 4-methyl-5-oxo-2-propyl-tetrahydro-furan-3-carboxylic acid. The chemical structures of isolated compounds were elucidated through extensive analyses of UV, IR, MS, and NMR spectroscopic data and by comparison with literature. The isolated compounds showed no antimicrobial activity, antimalarial activity, and anticancer activity. Based on morphology and nucleotide sequence of ITS1-5.8S-ITS2 regions of rDNA, the endophytic fungus isolate SM1 was identified as *Lasiodiplodia* sp., possibly an anamorph of *Botryosphaeria rhodina* (100 % sequence homology).

Field of Study.....Biotechnology..... Student's Signature.....เกียรติวุฒิ ดิษฐปาน.....

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Co-advisor's Signature.....

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LIST OF ABBREVIATION

δ	=	Chemical shift
ε	=	molar absorptivity
λ_{max}	=	wavelength at maximum absorption
V_{max}	=	wave number at maximum absorption
et al.	=	and other
IC_{50}	=	inhibitory concentration required for 50% inhibition of growth
IR	=	infrared
ITS	=	internally transcribed spacers
J	=	coupling constant
L	=	liter
µl	=	microliter
M	=	molar
m	=	multiplet (for NMR spectral data)
mg	=	milligram
min	=	minute
ml	=	milliliter
mm	=	millimeter
mM	=	millimolar
MHz	=	megahertz
m/z	=	mass to charge ratio
nm	=	nanometer
ppm	=	part per million
sp.	=	species
t	=	triplet (for NMR spectral data)

CHAPTER I

INTRODUCTION

Environmental pollution, increasing in human populations and their lifestyle changes such as dietary habits, obesity and stress are factors of new health problems in several developed countries. Cancer, a major global health problem, is a serious problem which the exact cause of it is under investigation. In addition, drug-resistant bacteria, parasitic protozoans and fungi are also cause of this problem, as shown in Table 1 (Lee Yuan Kun, 2006). Finding the new drugs is needed. Long time ago natural products are used in the medicinal source especially plants. There are several bioactive substances such as alkaloid, flavonoid, anthraquinone and phenolic from medicinal plants. These are found and developed to new drugs. But how to obtain them needs to lose the natural resources.

Since penicillin was discovered as antibacterial agent, microbes have been the interesting source for screening of new bioactive compounds. Especially fungi, because they are important components of biological communities. Approximately 10% of all have been described., Fungi are found in soil, water, marine, air, plants, plant remains, and carcass. In living plants, the fungi can live in all parts of plant (inside and outside) such as leave, stem, seed and root, and in diverse ecological habital. Thus, screening of new fungi that may produce novel bioactive compounds from medicinal plants is interesting and can preserve the natural resources.

Many researchs report about finding of endophytic fungi and their behaviour. Endophytic fungi are fungi that live inter- and/or intra-cellularly plant tissues for all, or at least a significant part, of their life cycle asymptomatically (Wilson, 1995). There are several species of fungal endophytes in one plant and they spread to all parts of host plant. The fungal endophytes get the living and foods from their host and some of them produce some bioactive substances to support healthy plant's growth. Several researchs find novel bioactive compounds from endophytic fungi. In addition, some researchs find that some endophytic fungi also produce same substance as their host (Stierle and Strobel 1995). Thus, it is possible to find the new strains of endophytic fungi

from the medicinal plants that produce novel bioactive compounds or same substance to their host.

Table 1. The emergence of significant microbial pathogens and clinical infectious diseases (1965-2000)

Viruses	Diseases
Ebola virus	Ebola hemorrhagic fever
Enterovirus 71	Hand, foot and mouth disease
	Encephalitis
Guanarito virus	Venezuelan hemorrhagic fever
Hantavirus	Hemorrhagic fever with renal syndrome
	Hantavirus pulmonary syndrome
Hendra virus (equine morbillivirus)	Encephalitis
Hepatitis A	Infectious hepatitis
Hepatitis B	Chronic hepatitis, cirrhosis
	Primary liver cancer
Hepatitis C	Non-A, non-B hepatitis
	Liver cirrhosis
	Primary liver cancer
Hepatitis E	Enteric-transmitted hepatitis
Human herpesvirus 6	Exanthem subitum (Roseola infantum)
Human herpesvirus 8	Kaposi's sarcoma
Human immunodeficiency viruses 1 and 2	Acquired immunodeficiency syndrome
Human papillomaviruses 16, 18 and other oncogenic types	Genital cancers
Human T-cell lymphotropic virus I	Adult T-cell leukemia and lymphoma
Human T-cell lymphotropic virus II	Hairy cell leukemia

Table 1. The emergence of significant microbial pathogens and clinical infectious diseases (1965-2000) (continued)

Viruses	Diseases
Lassa virus	Lassa hemorrhagic fever
Marburg virus	Marburg disease
Nipah virus	Encephalitis
Parvovirus	Fifth disease
	Fetal death
	Aplastic crisis in chronic hemolytic anemia
Prion	Variant Creutzfeldt-Jakob disease
Rotavirus	Infantile diarrhea
Sabia virus	Brazilian hemorrhagic fever
SARS coronavirus	Severe acute respiratory syndrome

Bacteria	diseases
Bartonella henselae	Cat scratch disease
	Bacillary angiomatosis
Borrelia burgdoferi	Lyme disease
Campylobacter sp.	Diarrheal disease
Clostridium difficile	Pseudomembranous colitis
	Antibiotic-associated colitis
Ehrlichia chaffeensis	Ehrlichiosis
Escherichia coli O157:H7	Hemorrhagic colitis
	Hemolytic uremic syndrome
Helicobacter pylori	Gastritis
	Peptic ulcer

Table 1. The emergence of significant microbial pathogens and clinical infectious diseases (1965-2000) (continued)

Bacteria	diseases
Legionella pneumophila	Legionnaires' disease/pneumonia
Staphylococcus aureus (toxigenic strians)	Toxic shock syndrome
Vibrio cholerae O139	Epidemic cholera

Parasites	Diseases
Cryptosporidium parvum	Enterocolitis
Cyclospora cayetanensis	Diarrheal disease
Microsporidia	Immunodeficiency-related microspoidiosis

In Thailand, there are several reports about medicinal property of many medicinal plants. Thailand is in the tropical land that has high biological diversity. The famous Thai medicinal plant is “Plaunoi” (*Croton sublyratus* Kurz.). Plaunotol from this plant are used in treatment of peptic ulcre. In addition, several Thai medicinal plants are presented the medicinal property such as *Kaempferia praviflora* Wall., *Stemona tuberosa* Lour., and *Curcuma longa* Linn. This study is interested in *Suregada multiflora* (A. Juss.) Baill. There are some reports about medicinal property of the crude extracts from this plant. “ขันทองพยาบาท”or *Suregada multiflora* (A. Juss.) Baill. is a plant that belonging to the Euphorbiaceae family. It has long been used as traditional medicine for the treatment of leprosy, urtication, veneral disease, and used as cathartic. The crude extract of some endophytic fungi from this plant had antimicrobial activities including anti-*Candida albicans* and anti- MRSA activities.

The present research aims to study bioactive metabolites that are produced by selected endophytic fungi from *Suregada multiflora* (A. Juss.) Baill.The crude extract of selected endophytic fungi that have high yields and antimicrobial activities, are purified

to pure compounds. The pure compounds are characterized and evaluated of biological activities.

The objectives of this study are as follows:

1. Isolation and characterization of bioactive compounds of the selected endophytic fungus from *Suregada multiflora* (A. Juss.) Baill.
2. Classsification of the selected endophytic fungus.
3. Evaluation of biological activities of the isolated compounds

CHAPTER II

LITERATURE REVIEWS

2.1 *Suregada multiflora* (A. Juss.) Baill.(Euphorbiaceae)

Suregada multiflora (A. Juss.) Baill. (syn. *Gelonium multiflorum* A. Juss., family Euphorbiaceae, Figure 1) grows in the tropical and the subtropical areas of Asia and Africa. The plant is used for the treatment of hepatic and gum diseases in folk medicines (Choudhary *et al*., 2004).

This plant is a small or middle-sized tree, about 3-15 m high and glabrous, with a crooked trunk but smooth bark; branches slender smooth, bark pale and gray. Leaf is simple, elliptical-oblong shape, about 12-15 cm long, 5-7 cm broad, base cuneate, margin entire, apex acute, dark green, secondary veins 5-7 pairs. Flowers unisexual, about 0.8-1.2 cm in diameter; pedicel 4-6 mm long, covered with short hair. The flower consists of five sepals, round, blunt sepals 3-4 mm long, 2.5-3.5 mm broad, the receptacle with numerous small disc glands among the filaments; pistillode minute. Pistillate flowers: sepals longer than the staminate ones, disc with papery margin, concave, sometime with tiny staminodes; ovary 3-locular, stigmas shortly bifid, spreading. Fruits are sub-globular and shallowly 3-lobed, about 1.5-2 cm in diameter, mostly smooth, tardily dehiscent, red-orange when ripe. Seeds are flat shape.

Figure 1 *Suregada multiflora* (A. Juss.) Baill. (Euphorbiaceae) – ขันทองพยาบาท

Distribution: In forest, open places. Flowering on March to May and fruiting on April to June.

Source in Thailand

Northern: Chiang Mai, Nan, Lampang, Phrae, Tak, Nakhon Sawan

North-eastern: Loei, Udon Thani

Eastern: Nakhon Ratchasima, Surin, Si Sa Ket, Ubon Ratchathani

South -western: Uthai Tani, Kanchanaburi, Phetchaburi, Prachuap Khiri Khan

Central: Saraburi, Bangkok (cultivated)

South-eastern: Sa Kaeo, Prachin Buri, Chon Buri, Chanthaburi, Trat

South: Chumporn, Ronong, Surat Thani, Phangnga, Phuket, Krabi, Nakhon Si Thamarat, Trang, Satun, Songkhla, Pattani, Narathiwat.

There are several reports for bioactive compound from *Suregada multiflora* (A. Juss.). Gelonin toxin from the seeds of *Suregada multiflora* (A. Juss.) Baill.was applied in several research to study cancer therapy. It was a Mr 30,000 single-chain protein that inactivated the ribosomal 60S subunit by cleaving rRNA adenine N-glycoside bonds in a sequence-specific fashion to stop protein synthesis (Stirpe *et al*,. 1980). GAP31 from the seeds of *Suregada multiflora* (A. Juss.) Baill. was inhibitory to viral proliferation (HIV, cytomegalovirus) (Bourinbaiar *et al*., 1996).; In addition, the crude extract MeOH–CH_2Cl_2 (1:1) of *Suregada multiflora* (A. Juss.) Baill. was found to exhibit selective cytotoxicity against different human cancer cell lines (Choudhary *et al*., 2004).

2.2 Association of the endophytic fungi and plant

Endophytic fungi colonize living plant tissues by penetration of fungul hyphae between plant cells. They may also grow intracellularly and must obtain nutrient materials through this intimate contact with the host. (Isacc, 1992). The relationship between fungi and its host plant may ranges from mutualistic symbiosis, or commensalism to borderline latent pathogen (Strobel *et al*., 1998). Results of their interaction are the increase in capacity of a plant to resist disease and the increase in

survival of plant from natural environment by producing bioactive substances (plant growth promoting, antibacterial, antifungal and insecticidal) to enhance the plant growth. For example, an aquatic plant, *Rhyncholacis penicillata*, could be in a river system in Southwest Venezuela where the harsh aquatic environment subjected the plant to constant beating by virtue of rushing waters, debris, and tumbling rocks and pebbles without any effects of pathogenic fungi. It was the result of Oocydin A, a novel antioomycetous compound from a strain of *Serratia marcescens* isolated from *R. penicillata* (Strobel *et al*., 1999). This was directly related to the endophyte's relationship with its higher plant host.

2.3 Study of secondary metabolites from the endophytic fungus

In the past, research on fungal endophyte was limited only for identification and classification, until paclitaxel (Taxol®), a natural bioactive diterpene derivative and a widely used anticancer drug, was isolated from the endophytic fungus, *Taxonomyces andreanae*, from Pacific yew (*Taxus brevifolia*) by Strobel *et al* in 1993.

Nowaday, the interest for fungal endophyte research is screening of secondary metabolites that exhibited interesting bioactivities such as antifungal, insecticidal, antimicrobial, antimalarial, anticancer, immunosuppressive, and antiviral activities. For examples, *Pestalotiopsis microspora*, a common rainforest endophyte was also isolated from the endangered tree *Torreya taxifolia* and produces several compounds that have antifungal activity, including pestaloside, an aromatic glucoside, and two pyrones, pestalopyrone and hydroxypestalopyrone (Lee *et al*., 1995). Cryptocin, a unique tetramic acid, was produced by *Cryptosporiopsis quercina* from *Tripterigeum wilfordii*, a medicinal plant native to Eurasia and displayed potent activity against *Pyricularia oryzae*, human pathogenic fungus (Lee *et al*., 2000). Tricin, a bioactive flavoniod with insecticidal activities, is a secondary metabolite of *Neotyphodium typhnium* isolated from blue grass (*Poa ampla*) (Ju *et al*., 1998). Taxol (paclitaxel), the anticancer drug from pacific yew bark, is secondary metabolite of *Taxomyces andranae* isolated from *Taxus brevifolia* (Strobel *et al*., 1993). Two novel human cytomegalovirus protease inhibitors, cytonic acids A and B, p-tridepside antivirus compound were isolated from

the endophytic fungus C*ytonaema* sp. obtained from *Quercus* sp. (Guo et al., 2000). Two compounds, pestacin and isopestacin, were secondary metabolite of *Pestalotiopsis microspora*, an endophyte isolated from a combretaceaous plant, Terminalia morobensis, growing in the Sepik River drainage of Papua New Guinea (Harper *et al*. 2003, Strobel *et al*., 2002). Both pestacin and isopestacin display antimicrobial activity as well as antioxidant activity. Subglutinols A and B, two inmonosuppresstive compounds were isolated from *Fusarium subglutinans*, an endophytic fungus of *Tripterygium wilfordii* (Lee *et al*., 1995). *Cryptosporiopsis quercina*, the fungal endophyte isolated from *Tripterygium wilfordii*, a chinese medicinal plant, produced cryptocandin, a cyclopeptide antifungal compound (Strobel *et al*., 1999). Phomoxanthones A and B, two novel xanthone dimmers with antimalarial activities were isolated from the endophytic fungus *Phomopsis* sp. BCC 1323 that isolated from *Tectona glandis* leaf (Isaka *et al*., 2001). The biological activities, sources, chemical compounds of secondary metabolites from fungal endophyte were summarized in Table A (in Appendix A).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

Chemical used in this study are as the following: boric acid (Merck, GR), ammonium tartrate (Merck, GR), sodium nitrate ($NaNO_3$) (BHD, AR), sodium chloride (NaCl) (Merck, GR), sodium hydrogen carbonate ($NaHCO_3$) (Merck, GR), sodium acetrate (NaOAc) (Sigma, AR), disodium hydrogen phosphate (Na_2HPO_4) anhydrous (Merck, GR), potassium dihydrogen phosphate (KH_2PO_4) anhydrous (Merck, GR), magnesium chloride ($MgCl_2$) (Merck, GR), calcium dinitrate [$Ca(NO_3)_2$] (Merck, GR), potassium nitrate (KNO_3) (Merck, GR), ferric chlorind ($FeCl_3$) (Merck, GR), manganese sulphate ($MnSO_4$) (Merck, GR), potassium iodide (KI) (Merck, GR), magnesium sulphate heptahydrate ($MgSO_4.7H_2O$) (Merck, GR), potassium chloride (KCl) (Riedeldehaen, AR), dipotassium hydrogen phosphate (K_2HPO_4) (Merck, GR), zinc sulphate heptahydrate ($ZnSO_4.7H_2O$) (Merck, GR), copper sulphate pentahydrate ($CuSO_4.5H_2O$) (Merck, GR), ferrous sulphate heptahydrate ($FeSO_4.7H_2O$) (Merck, GR), absolute ethanol (Merck, GR), 95% ethanol (industrial grade), liquid paraffin (specific gravity of 0.83-0.89, medicinal grade), dichloromethane (CH_2Cl_2) (Labscan, AR),ethyl acetate (EtOAc) (Labscan, Ar), phenol (C_6H_5OH) (Amersham, AR), Tris-HCl (Sigma, AR), EDTA (Sigma, AR), methylene blue (Sigma, AR), glycerol (Merck, GR), bromophenol blue (Sigma, AR), chloroform-D, 99.9 atom %D (Labscan, AR) and acetone-d_6, 99.9 atom %D (labscan, AR).

Molecular biology grade reagent used were deoxynucleotide triphosphate (dATP, dCTP, dGTP and dUTP) (FINNZYMES), *taq* DNA polymerase (FINNZYMES), *Pst*I (FINNZYMES) and LE agarose (Seakerm®, FMC).

3.1.2 Culture media

Culture media used for cultivation of endophytic fungi were corn meal agar (CMA) (Difco), potato dextrose agar (PDA) (Merck), Sabouraud's dextrose agar (SDA) (Merck), malt extract powder (Merck), yeast extract powder (Merck), soytone (Merck) and agar base (agar-agar ultrapure granulated) (Merck), yeast extract sucrose medium (agar and broth) (YES), malt Czapek medium (agar and broth) (MCz), and potato dextrose broth (PDB). The formular are shown in appendix B.

3.1.3 Plant sample

Healthy host plant (*Suregada multiflora* (A. Juss.) Baill.) was collected from Siriruckhachati Medicinal Plant Garden, Mahidol university, Nakhornpathom provinces. Fresh specimens were kept in a plastic bag and then immediately brought to the laboratory and processed with in 24 h after collection.

3.1.4 Equipments

3.1.4.1 Chromatography

3.1.4.1.1 Analytical thin-layer chromatography (TLC)

Thin-layer chromatography was carried out on silica gel 60 PF_{254} precoated plate. Detection was visualized under ultraviolet light at wavelengths 254 and 366 nm.

3.1.4.1.2 Column chromatography (CC)

Column chromatography was performed using Sephadex LH-20 (Phamacia Code No. 17-0090-01) and Silica gel 60H (Merck Code No. 7734) as packing materials.

3.1.4.2 Structure elucidation

Structures were elucidated by the interpretation of one and two dimensional NMR spectra. Additional spectroscopic techniques such as MS, UV-vis, FT-IR and optical rotation properties were also employed for the structural elucidation.

3.1.4.2.1 Nuclear magnetic resonance spectroscopy (NMR)

^{1}H NMR spectra were recorded on a Varian Gemini 2000 spectrometer operating at 200 MHz spectrometer (with $CDCl_3$, acetone-d_6 and DMSO-d_6 as solvent). ^{1}H NMR spectra of pure compounds and all other NMR measurements were performed on a Bruker AM-400 (400 MHz) or a Bruker AVANCE 600. NMR spectrometer was operated at 400 or 600 MHz (1H), and 100 or 150 MHz (^{13}C), respectively. NMR spectra of prue compounds were processed using Bruker software. They were calibrated using solvent signals (^{13}C: $CDCl_3$ 77.00 ppm,Acetone-d_6 (CD_3COCD_3) 29.8, 206.0 ppm and DMSO-d_6 (CD_3SOCD_3) 39.5 ppm) or a signal of the protion of the partly or not deuterated solvent (^{1}H: $CHCl_3$ in $CDCl_3$ δ 7.29 ppm, acetone in acetone-d_6 δ 2.05 ppm, water (H_2O) in DMSO-d_6 δ 2.8 ppm, DMSO in DMSO-d_6 δ 2.05 ppm and water (H_2O) in DMSO-d_6 δ 3.31 ppm). Structural assignments were based on the interpretation of the following NMR experiments: ^{1}H, ^{13}C, DEPT, ^{1}H-^{1}H COSY, ^{1}H-^{13}C direct correlation (HQMC) and ^{1}H-^{13}C kong-range correlation (HMBC).

3.1.4.2.2 Mass spectrometer (MS)

EI-MS spectra were obtained from a Finnigan Mat GCQ mass spectrometer.

Accurate mass was obtained from the time of flight (TOF) mass spectrometric technique, using a Micro TOF, Bruker daltonics by APCI ionization mode or ESI mode.

3.1.4.2.3 Ultraviolet-visible measurements (UV-vis)

UV-vis spectra were recorded on a Shimadzu UV-vis 2001s spectrophotometer.

3.1.4.2.4 Fourier transform infrared spectroscopy (FT-IR)

FT-IR spectra were recorded on a Perkin Elmer Spectrum One spectrophotometer. Samples for IR were examined using a Universal Attenuted Total Reflectance, solid or liquid (UATR-solid, UATR-liquid).

3.2 Methods

3.2.1 Isolation of endophytic fungi from *Suregada multiflora* (A. Juss.) Baill.

3.2.1.1 Surface sterilization and isolation

The branches and leaves of healthy plant were washed in running tap water to remove soil particles and sterilized by sequential immersion in 70% ethanol for 1 min and sodium hypochlorite solution (6 % available chlorine) for 5 min. Samples were washed in sterile water 2 times to remove surface sterilization agents before cut into small fragments, placed on water agar (WA) and incubated at 25 °C. Hyphal tipping was performed under microscope. The hyphal tip was transfered to half-strength PDA medium and incubated at 25 °C for 7 days. Culture purity was determinaed from colony morphology.

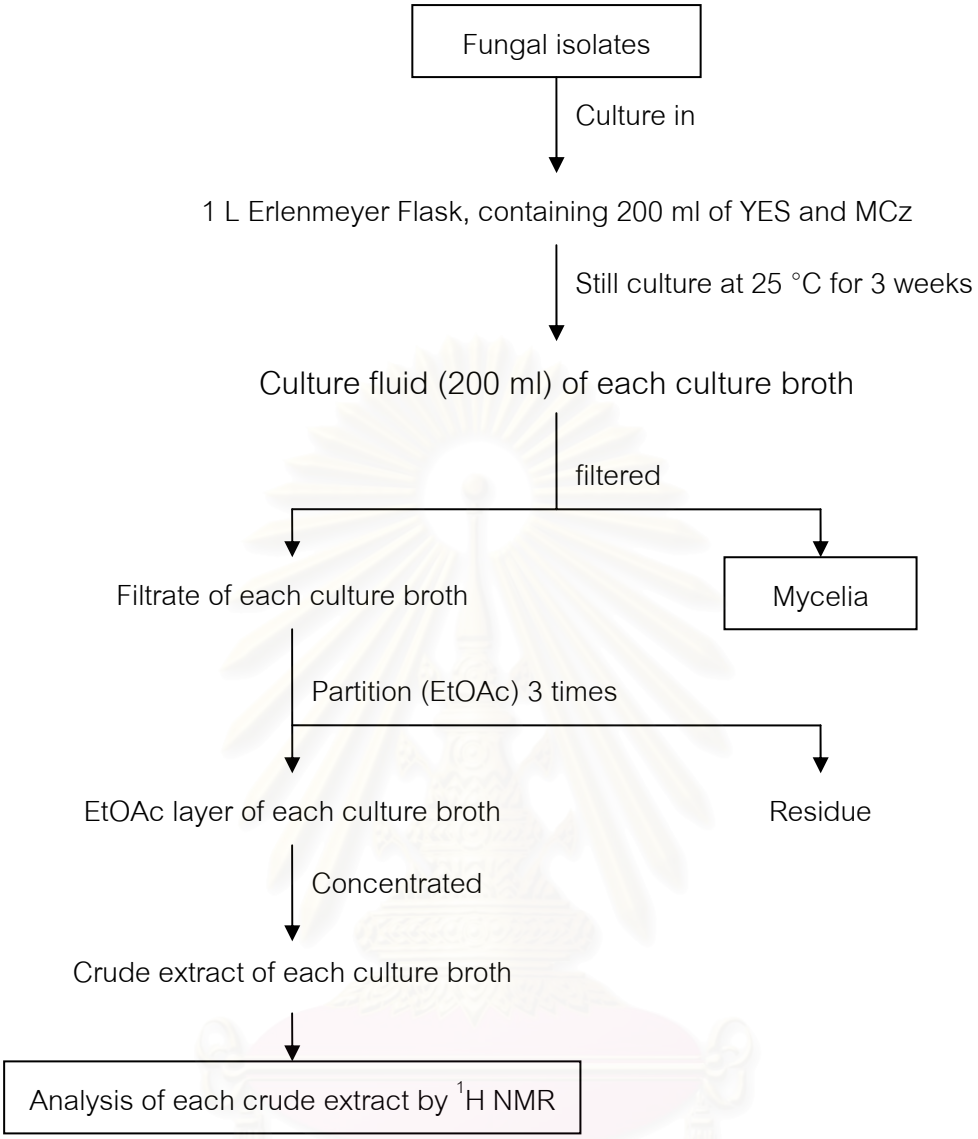
3.2.1.2 Deposition of fungi

All isolated were deposited at the MIM laboratory, Department of Microbiology, Faculty of Science, Mahidol University. The fungi were placed in distilled water for short-term storage and kept in 15 % glycerol at -70 °C for longer term storage.

3.2.2 Preliminarily screening for antimicrobial activities

3.2.2.1 Cultivation of fungal culture and preparation of crude extract

Each isolated were cultivated in 200 ml of yeast extract sucrose (YES) broth and 200 ml of malt Czapek (MCz) broth for three weeks at 25 °C in still condition. The mycelia were separated from culture broth by filtration, passed through four layers of cheese cloth. The filtrate was extracted with an equal volume of ethyl acetate (EtOac) 3 times and then evaporated to remove the solvent. The residue was dissolved in dichloromethane or methanol, and transferred to a vial. The crude extracts of each fungal isolate were examined by ^{1}H NMR analysis for preliminarily screening of interesting compounds. The extraction of each isolated are shown in Scheme 1.



Scheme 1 Experimental steps used to get crude extract from fungal cultures.

3.2.2.2 Determination of antimicrobial activities

Antimicrobial activities of 25 crude extracts and pure compounds were determined by disk diffusion test based on NCCLS M7-A8 method (1). Test microorganisms used in this study were *Staphylococcus aureus* ATCC 25923, methicillin resistant *S. aureus* (MRSA) (kindly provided by Associate Professor Dr. Pintip Pongpetch) and *C. albicans* ATCC 10231. Each bacterial strain was grown in tryptic soy broth at 37 ^{o}C for 2-3 h. *C. albicans* was grown on Sabouraud's dextrose agar (SDA) at 30 ^{o}C for 24 h and suspended in 0.85% NaCl. The turbidity of bacterial and yeast

suspensions were adjusted to match that of 0.5 McFarland standard (OD_{625} 0.1). A sterile cotton swab was used to apply each bacterial and yeast inocula onto the entire surface of the Mueller Hinton agar plate and SDA plate, respectively. Six sterile blank disks (6 mm diameter) were distributed evenly onto the inoculated agar surface. Ten µL of 100 mg/ml of test compound in DMSO and of 25.6 mg/ml of pure compound were applied to the blank disks.

For determination of synergistic activity, 10 µg of ampicillin in 10 µl of DMSO was applied to the blank disk placed onto the center of MRSA inoculated plate. One hundred µg of ketoconazole in 10 µl of DMSO and 25.6 µg of nystatin in 10 µl of DMSO were applied to the blank disk placed onto the center of *C. albicans* inoculated plate. After keeping at room temperature for 1 h, the test plates were incubated at 37 °C for 24 h. The inhibition zone around disk indicates antimicrobial activity of compound.

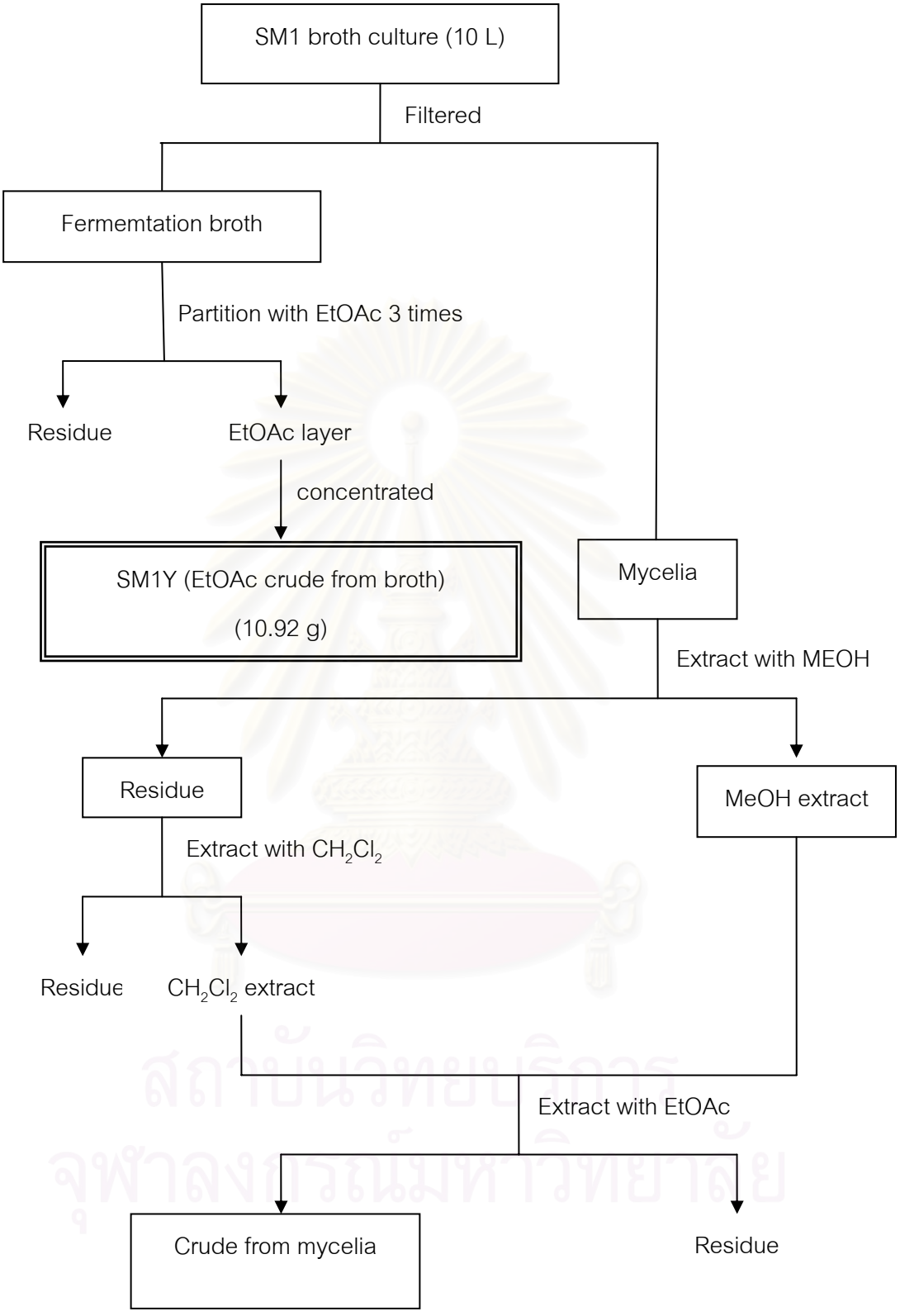
3.2.3 Cultivation of selected fungus, SM1

The selected fungus was grown on potato dextrose agar (PDA) medium for one week at 25 °C before cultivated in 1 L Erlenmeyer flask containing 200 ml of YES broth (50 flasks) for three weeks at 25 °C in still condition.

The culture broth was extracted as previously described in 3.4.1 and shown in Scheme 2.

3.2.4 Isolation of bioactive compounds from endophytic fungus isolate SM1

The crude extract designated as SM1Y was purified by gel filtration chromatography using Sephadex LH-20 (column 3.5 x 53 cm). MeOH was used as a mobile phase. Twenty three fractions were obtained, as shown in Table 2.



Scheme 2 Extraction of culture broth and mycelia of the fungus isolate SM1

Table 2 Fractions obtained from Sephadex LH-20 column chromatography of crude extract SM1Y

Fraction code	Weight (mg)
Fraction 1	3.2
Fraction 2	7.1
Fraction 3	14.9
Fraction 4	31.7
Fraction 5	62.4
Fraction 6	100.5
Fraction 7	275.1
Fraction 8	554.5
Fraction 9	1,137.0
Fraction 10	1,979.4
Fraction 11	1,905.2
Fraction 12	1,868.5
Fraction 13	1,175.4
Fraction 14	956.0
Fraction 15	439.7
Fraction 16	161.5
Fraction 17	65.4
Fraction 18	20.8
Fraction 19	7.4
Fraction 20	3.5
Fraction 21	2.3
Fraction 22	1.3
Fraction 23	1.7

Each fraction was examined by analysis of ^{1}H NMR spectrum data. ^{1}H NMR pattern showed that Fraction 11, Fraction 12 and Fraction 13 were interesting. Fraction

11, Fraction 12 and Fraction 13 were combined and purified by gel filtration chromatography using by Sephadex LH-20 (column 3.5 x 53 cm), eluted with MeOH. Twenty fractions were obtained and examined by TLC. Eight new fractions from combination were assigned in new code, as shown in Table 3.

Table 3 Fractions obtained from Sephadex LH-20 column chromatography of Fraction 11-13

Fraction code	Weight (mg)
SM1Y A	1,970.0
SM1Y B	1,170.0
SM1Y C	17.0
SM1Y D	12.0
SM1Y E	19.9
SM1Y F	113.0
SM1Y G	223.0
SM1Y H	717.4

^{1}H NMR pattern showed that Fraction SM1Y A was interesting. This fraction had high yield and was purified by gel filtration chromatography using Silica gel 60H (gel 7734) (column 2.5 x 30 cm) in CH_2Cl_2 : EtOAc 20% system. Fifty fractions were collected, examined by TLC and NMR. The fractions with silimar ^{1}H NMR pattern was combined. Fourteen new fractions from combination were assigned in new code, as shown in Table 4.

Table 4 Fractions obtained from Silica gel 60H column chromatography of Fraction SM1Y A

Fraction code	Weight (mg)
SM1Y A1	610.0
SM1Y A2	97.0
SM1Y A3	198.9
SM1Y A4	59.0
SM1Y A5	103.2
SM1Y A6	32.4
SM1Y A7	61.8
SM1Y A8	27.8
SM1Y A9	4.9
SM1Y A10	1.5
SM1Y A11	1.4
SM1Y A12	0.4
SM1Y A13	4.1
SM1Y A14	9.5

^{1}H NMR pattern showed that Fraction SM1Y A1 was interesting. Fraction SM1Y was selected for further purification based on its interesting ^{1}H NMR pattern and high yield. It was purified by gel filtration chromatography using Silica gel 60H (gel 7734) (column 2.5 x 25 cm) in Hexane : EtOAc 20% system. Fifty fractions were obtained and examined by TLC and NMR. The fractions with silimar ^{1}H NMR pattern were combined. Twenty eight new fractions from combination were assigned in new code, as shown in Table 5.

Table 5 Fractions obtained from Silica gel 60H column chromatography of Fraction SM1Y A1

Fraction code	Weight (mg)
SM1Y A1-1	2.9
SM1Y A1-2	2.9
SM1Y A1-3	1.5
SM1Y A1-4	6.7
SM1Y A1-5	6.5
SM1Y A1-6	4.6
SM1Y A1-7	3.3
SM1Y A1-8	48.8
SM1Y A1-9	21.0
SM1Y A1-10	9.0
SM1Y A1-11	7.9
SM1Y A1-12	15.2
SM1Y A1-13	63.6
SM1Y A1-14	16.8
SM1Y A1-15	13.6
SM1Y A1-16	37.8
SM1Y A1-17	12.6
SM1Y A1-18	11.1
SM1Y A1-19	23.9
SM1Y A1-20	23.2
SM1Y A1-21	17.3
SM1Y A1-22	8.6
SM1Y A1-23	6.1
SM1Y A1-24	31.1
SM1Y A1-25	2.1

Table 5 Fractions obtained from Silica gel 60H column chromatography of Fraction SM1Y A1 (continued)

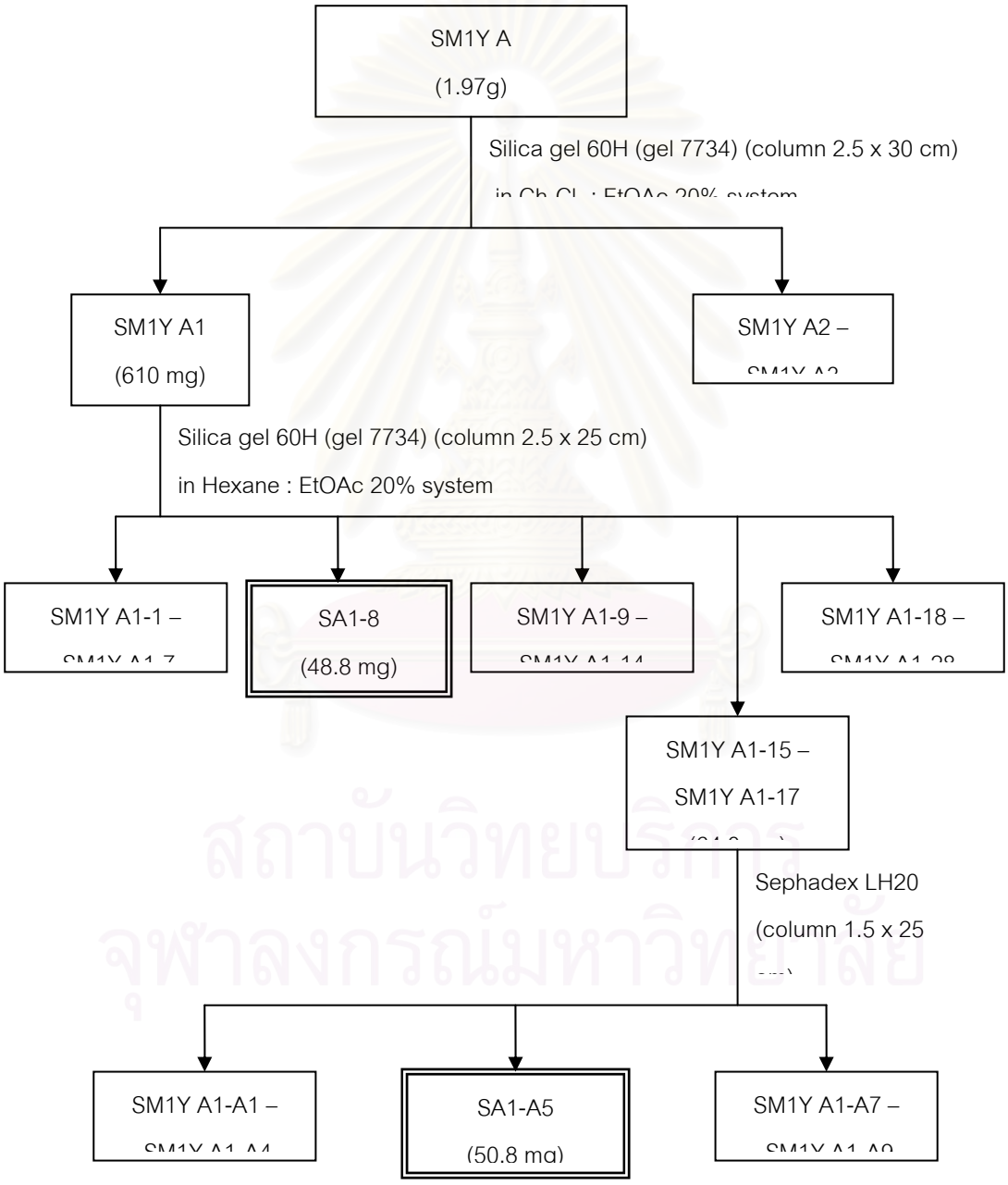
Fraction code	Weight (mg)
SM1Y A1-26	29.7
SM1Y A1-27	21.7
SM1Y A1-28	17.1

^{1}H NMR pattern showed that Fraction SM1Y A1-8 was a pure compound and Fraction SM1Y A1-15, SM1Y A1-16, and SM1Y A1-17 was interesting. They were combined and further purified by gel filtration chromatography using by Sephadex LH-20 (column 1.5 x 25 cm), eluted with MeOH. Fifty fractions were obtained, observed by TLC and NMR. The fraction with silimar ^{1}H NMR pattern were combined. Nine new fractions from combination were assigned in new code, as shown in Table 6.

Table 6 Fractions obtained from Sephadex LH-20 column chromatography of combined Fraction SM1Y A1-15 - SM1Y A1-17

Fraction code	Weight (mg)
SM1Y A1-A1	0.3
SM1Y A1-A2	0.7
SM1Y A1-A3	1.0
SM1Y A1-A4	20.0
SM1Y A1-A5	14.8
SM1Y A1-A6	36.0
SM1Y A1-A7	1.9
SM1Y A1-A8	0.8
SM1Y A1-A9	0.6

^{1}H NMR pattern showed that Fraction SM1Y A1-A5, and SM1Y A1-A6 were similar and pure compound. They were combined into Fraction SM1Y A1-A5. Fraction SM1Y A1-8 and Fraction SM1Y A1-A5 were designated as SA1-8 and SA1-A5 respectively. Both of them were analyzed by 2-D NMR and ^{13}C NMR, Mass spectrometer, Ultraviolet-visible measurements, Fourier transform infrared spectroscopy. Isolations of compound SA1-8 and S1A1-A5 are shown in Scheme 3.



Scheme 3 Isolation of compound SA1-8 and SA1-A5 from the fungus isolate SM1

3.2.5 Evaluation of biological activities

Antimalarial activity, anticancer activity and cancer chemoprevention were evaluated at the Chulabhorn Research Institute (CRI), Bangkok, Thailand.

3.2.5.1 Antimalarial activity

3.2.5.1.1 Parasite culture

Human erythrocytes (type O) infected with *Plasmodium falciparum* strain 94, (Chloroquine resistant) was maintained in continuous culture, according to the method described by Targer and Jensen (1976). RPMI 1640 culture medium (Gibco, USA) supplemented with 25 mM of HEPES (Sigma, USA), 40 mg/L gentamicin sulfate (Government Pharmaceutical Organization, Thailand) and 10 mL of human serum was used in continuous culture.

Before starting the experiment, *P. falciparum* culture was synchronized by using sorbital induced hemolysis according to the method of Lambors and Vanderberg (1979) to obtain only ring-infected cells and then incubated to 48 h prior to the drug testing to avoid effect of sorbitol.

The experiments were started with synchronized suspension of 0.5% - 1% infected red blood cell during ring stage. Parasites were suspended with culture medium supplemented with 15% human serum to obtain 10% cell suspension. The parasite suspension was put into 96-well microculture plate; 50 µL in each well and then added 50 µL of various test drug concentrations. These parasite suspensions were incubated for 48 h in the atmosphere of 5% CO_2 at 37°C.

After 48 h incubation, parasite culture was fixed by adding 0.25% (v/v) glutaraldehyde (Sigma) in phosphate buffer saline (PBS) and these were kept for DNA staining.

3.2.5.1.2 Parasite DNA staining and flow cytometric analysis

Before parasite DNA staining, 5×10^6 red blood cells from each glutaraldehyde-fixed sample were washed once with PBS and resuspended in PBS containing propidium iodide (PI) (Molecular probe) at a final concentration at 10 µg/mL, and held for at least 1 h in dark. The PI stained cell were excited with 488 nm. Red fluorescence was detected at 585 nm. Red blood cells were gated on the basis of their forward scatter and side scatter. For each sample, 30,000 cells were required, stored and analyzed. Precent parasitemia, fluorescence intensity, and any abnormal fluorescence pattern were obtained from an integrated fluorescence histogram betweem the test and the control sample (chloroquine hydrochloride, $IC_{50} = 2.98\times10^{-7}$ M).

3.2.5.1.3 Anticancer activity

Anticancer activity was assessed against 4 cancer cell line *in vitro*, including HepG2 (human hepatoblastoma carcinoma), A549 (human lung adenocarcinoma, non-small cell ATCC No. CCL-185), HuCCA-1 (human cholangiocarcinoma), and MOLT-3 (acute lymphoblastic leukemia). Cytotoxicity assay were performed by MTT assay (Mosmann, 1983).

3.2.6 Identification of the endophytic fungus isolate SM1

3.2.6.1 Conventional method

SM1 fungus was grown at 30 °C on potato dextrose agar (PDA) and on water agar with several small pieces of sterile banana leaf placed onto the medium. Colony morphology on PDA was examined. Fruiting bodies and fungal conidia appearing on the banana leaf pieces were examined by light microscopy.

3.2.6.2 Molecular method

The SM1 fungus was grown on potato dextrose broth. After cultivation for 7 days, 50 mg of fresh fungal mycelia were harvested, washed twice with normal saline solution, and homogenated in 250 µl of sterile water. The mycelial homogenate was applied evenly to an FTA card matrix, allowed to dry at room temperature, and a 2-mm disk containing the fungal mycelia was punched from the FTA card. Total cellular DNA of fungal mycelia on the disk was extracted and purified using the FTA® Plant Kit (Whatman®) according to the manufacturer's instruction. The disk was transferred to a PCR tube and the ITS1-5.8S-ITS2 ribosomal RNA gene region of fungal genomic DNA was amplified using the ITS5 (GGAAGTAAAAGTCGTAACAAGG) and ITS4 (TCCTCCGCTTATTGATATGC) primers (White *et al*., 1990). PCR amplification was performed in a 50 µl reaction volume which contained Taq PCR Master Mix (USB Corp., USA) using an automated thermal cycler (Mastercycler gradient, Eppendorf, Hamburg, Germany). The thermocycling program was as follows: 3 min at 95°C followed by 30 cycles of 50 s at 95°C, 40 s at 45°C and 50 s at 72°C, with a final extension period of 10 min at 72°C. The PCR products were gel purified and directly subjected to sequencing (Bioservice Unit, NSTDA, Bangkok, Thailand) in both directions primed with either of the two primers used to originally amplify the fragment. The DNA sequence of ITS1-5.8S-ITS2 rRNA gene obtained was used as query sequence to search for similar sequences in GenBank using BLASTN 2.2.102 (Altschul et al, 1997). DNA sequence similarity was determined by the ClustalW (1.82) multiple sequence alignment program (Thompson et al., 1994). Alignments and boundary approximations were adjusted manually using BioEdit v7.0.0.

CHAPTER VI

RESULT AND DISCUSSION

4.1 Isolation of endophytic fungi from *Suregada multiflora* (A. Juss.) Baill. and determination of antimicrobial activities

A total of 118 isolated endophytic fungi were isolated from surface sterilized fragments of branches and leaves of *Suregada multiflora* (A. Juss.) Baill. Nineteen isolates were cultivated in YES and/or MCz or mixed medium. The yields of total 25 crude extracts obtained from each fermented broth are shown in Table 7. All crude extracts were screened for interesting compounds by ^{1}H NMR analysis and anti-microbial activity test. Most of them showed antimicrobial activities, as shown in Table 8. Figure 2 and 3 showed activities of some crude extracts against tested bacteria and *Candida albicans*, respectively.

Table 7 The summary of yield of 25 isolated endophytic fungal cultures from *Suregada multiflora* (A. Juss.) Baill.

code	culture broth	yield (mg)/ 200 ml
SM1	YES	205.5
SM1	MCz	8.6
SM2	YES	64.8
SM2	MCz	26.0
SM4	MCz	9.6
SM7	MCz	8.0
SM7	YES	136.0
SM8	YES	42.8
SM8	MCz	25.0
SM13	MCz	33.2
SM14	YES	73.5

Table 7 The summary of yield of 25 isolated endophytic fungi from *Suregada multiflora* (A. Juss.) Baill. (continued)

code	culture broth	yield (mg)/ 200 ml
SM15B	M+Y	67.4
SM16	YES	16.2
SM16	MCz	28.7
SM20A	M+Y	23.5
SM20B	M+Y	13.6
SM21B	M+Y	24.6
SM22A	M+Y	22.2
SM22B	M+Y	31.5
SM23A	M+Y	27.8
SM23B	M+Y	295.2
SM25A	M+Y	39.3
SM33	MCz	51.8
SM35	YES	150.3
SM35	MCz	29.3

M+Y = the mixture broth of MCz (100 ml) and YES (100 ml)

Table 8 The summary of anti-microbial activities screening of 25 crude extract of endophytic fungi from *Suregada multiflora* (A. Juss.) Baill.

code	inhibition zone (mm)					
	Anti-*S.aureus*	Anti-MRSA	Anti-MRSA Amp	Anti-*C.albicans*	Anti-*C.albicans* Keto	Anti-*C.albicans* Nyst
SM1Y	7	8	8	8	0	0
SM1M	9	8	8	8.5	Syn	8

Table 8 The summary of anti-microbial screening of 25 crude extract of endophytic fungi from *Suregada multiflora* (A. Juss.) Baill. (continued)

code	inhibition zone (mm)					
	Anti-*S.aureus*	Anti-MRSA	Anti-MRSA Amp	Anti-*C.albicans*	Anti-*C.albicans* Keto	Anti-*C.albicans* Nyst
SM2Y	11	10	10	11.5	Syn	9
SM2M	9	9	9	7	Syn	7.5
SM4M	9	9	9	8	Syn	8
SM7M	8	8	8	6.5	0	0
SM7Y	7.5	7	7	7	7	6.5
SM8Y	8	8	8	8	8	7.5
SM8M	13	13	13	9	Syn	9
SM13M	7.5	8	8	9	Syn	7
SM14Y	7.5	8	8	0	Syn	8.5
SM15BMY	8	8	8	7	7	7
SM16Y	7.5	8	ND	6.5	Syn	ND
SM16M	13	12	12	8	Syn	8.5
SM20AMY	0	8	ND	7.5	Syn	ND
SM20BMY	9	8.5	8	8	Syn	8.5
SM21BMY	8	8	8	0	0	0
SM22AMY	8	7.5	7.5	7	7	7
SM22BMY	7	8	7.5	8	Syn	8
SM23AMY	10	9	9	10	Syn	9
SM23BMY	0	0	0	0	0	0
SM25AMY	8.5	8.5	9	9	Syn	9.5
SM33M	7	7	7.5	8	Syn	8
SM35Y	6.5	7	8	7	Syn	7.5
SM35M	7.5	8	9	10	Syn	9

ND = Not determined

Syn = Synergistic activity with ketoconazole

Amp = Ampicillin

Keto = Ketoconazole

Nyst = Nystatin

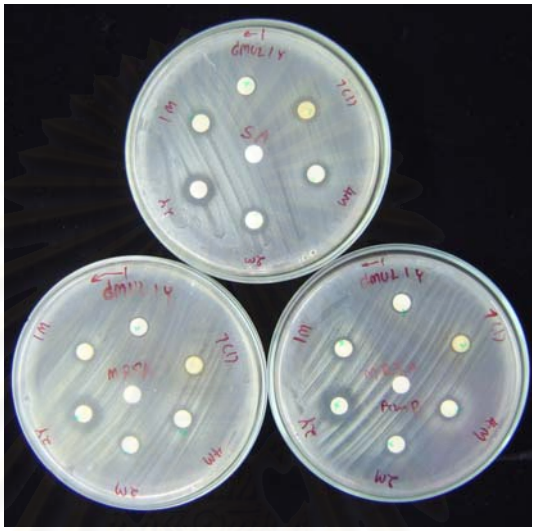


Figure 2 Inhibition zone of some crude extracts against tested bacteria

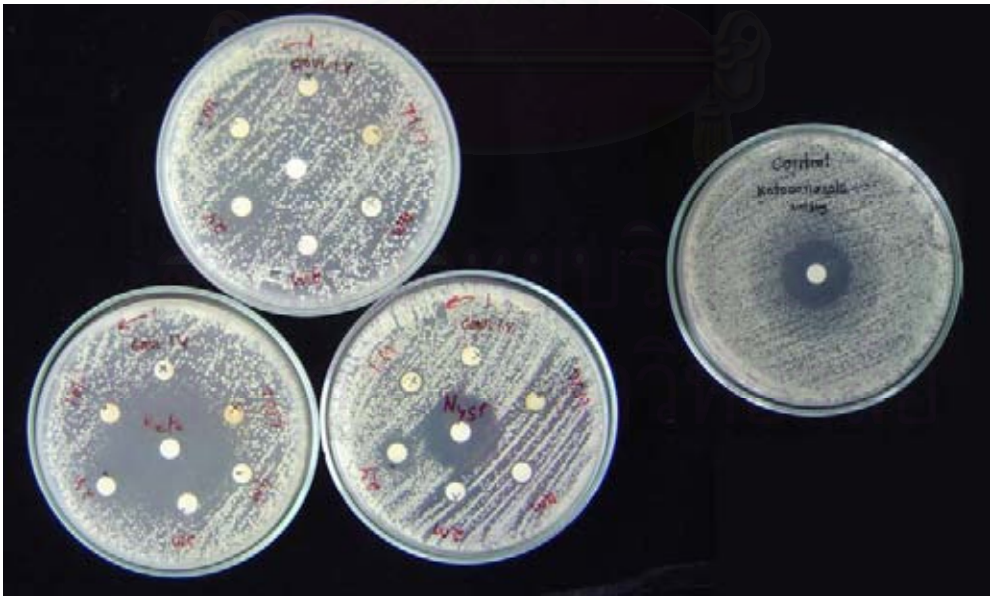


Figure 3 Synergistic activity of some crude extracts and ketoconazole against tested *Candida albicans*

From the result of anti-microbial activities, ^{1}H NMR spectral data, and crude extract yield, SM1 grown in YES broth was interesting and selected to culture in large scale (10 L) for Isolation of bioactive compounds.

4.2 Structure elucidation of the isolated compounds from endophytic fungus isolate SM1

There were two secondary metabolites, which were identified as 2-phenylethanol (SA1-8) 48.8 mg and new compound, 4-Methyl-5-oxo-2-propyl-tetrahydro-furan-3-carboxylic acid (SA1-A5) 50.8 mg, isolate from the ethyl acetate extract 10,923 mg of YES fermentation broth (10 L) of the endophytic fungus isolate SM1. The yield of 2-phenylethanol and new compound were 0.45% and 0.46% of ethyl acetate extract, respectively.

4.2.1 Structure elucidation of 2-phenylethanol (SA1-8)

Compound SA1-8 was obtained as light brown viscous liquid. The IR absorption spectrum (Figure C1 in Appendix C) showed characteristic bands at 3421.47 cm^{-1} (O-H stretching), 2915.30 cm^{-1} (C-H stretching), 1734.25 cm^{-1} (aromatic), and 700.06 cm^{-1} (C-H stretching). The UV spectrum in MeOH of compound SA1-8 (Figure C2 in Appendix C) exhibited λ_{max} at 223.50 (log ε 6.22) and 276 (log ε 5.69). The APCI-TOF mass spectrum of compound SA1-8 (Figure C3 in Appendix C) showed a couple of the $[M+H]^+$ ion peak at *m/z* 105.0689 $[C_8H_{10}O\text{-}H_2O+H]^+$ and *m/z* 121.0650 $[C_8H_{10}O\text{-}H]^+$

The 500 MHz ^{1}H-NMR spectrum of compound SA1-8 in $CDCl_3$ (Figure C4 in Appendix C) showed signals of proton in five positions, multiplet at δ 7.32-7.28 and 7.22 ppm; triplet at δ 3.82 and 2.84 ppm and doublet at δ 2.00 ppm, respectively.

The multiplicity at δ 7.32-7.28 and 7.22 ppm were multiplet in an aromatic region, which there were four methine proton signals at δ 7.32-7.28 ppm and one methine proton signals at δ 7.22 ppm. The multiplicity at δ 3.82 and 2.84 ppm were triplet which there were one methylene proton signal at δ 3.82 ppm and one methylene proton signal at δ 2.84 ppm.

The 125 MHz ^{13}C-NMR spectrum of compound SA1-8 in $CDCl_3$ (Figure C5 in Appendix C) showed six resonance positions, at δ 138.51, 129.02, 128.54, 126.43, 63.60 and 39.15 ppm, respectively.

The DEPT 135 and DEPT 90 spectra of compound SA1-8 in $CDCl_3$ (Figure C6 in Appendix C) classified carbon signals as two equivalent methine carbon signals at δ 129.02 ppm; two equivalent methine carbon signals at δ 128.54 and one methine carbon signals at δ 126.43 ppm; one methylene carbon signals at δ 63.60 ppm; one methylene carbon signals at δ 39.15 ppm, and one quaternary carbon signal at δ 138.51 ppm.

The HMQC spectrum of compound SA1-8 in $CDCl_3$ (Figure C7 in Appendix C) showed the chemical shift of proton signal at δ 2.00 ppm was not corresponding with any carbon signal.

The ^{1}H-^{1}H COSY spectrum of compound SA1-8 in $CDCl_3$ (Figure C8 in Appendix C) showed the coupling between methine proton signal at δ 7.32-7.28 ppm and methine proton signal at δ 7.22 ppm; the coupling between methylene proton signal at δ 3.82 ppm and methylene proton signal at δ 2.84 ppm; weakly coupling between methylene proton signal at δ 2.84 ppm and methine proton signal at δ 7.22 ppm.

The HMBC spectrum ($^nJ_{HC}$ = 8 Hz) of compound SA1-8 in $CDCl_3$ (Figure C9 in Appendix C) exhibited long-rang correlations of H (δ 2.84) to C-3 (δ 138.51), C-1 (δ 63.60), C-4 (δ 128.54), and C-8 (δ 128.54).

The ^{1}H-^{13}C long-rang correlations from HMBC spectrum of SA1-8 are summarized in Table 9. On the basis of these spectral data, SA1-8 is identified as 2-phenylethanol. The structure of SA1-8 is shown in Figure 4.

The NOESY experiment of compound SA1-8 in $CDCl_3$ was shown in Figure C10 in Appendix C.

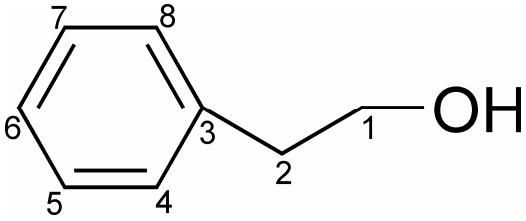


Figure 4 The structure of 2-phenylethanol (SA1-8)

Table 9 The ^{1}H, ^{13}C-NMR and HMBC spectral data of SA1-8 ($CDCl_3$).

position	SA1-8		
	δH (ppm), *mult*, (J in Hz)	δC (ppm)	Long-rang correlation in HMBC ($^{n}J_{HC}$ = 8 Hz) spectrum
1	3.82, *t*, 6.64	63.60	-
2	2.84, *t*, 6.62	39.15	C-1, C-3, (C-4,C-8)
3	-	138.51	-
4	7.32-7.28, *m*	128.54	C-2, C-3, C-5, C-6, C-8
5	7.32-7.28, *m*	129.02	C-3, C-4, C-6, C-7
6	7.22,*m*	126.43	C-4, C-5, C-7, C-8
7	7.32-7.28, *m*	129.02	C-3, C-5, C-6, C-8
8	7.32-7.28, *m*	128.54	C-2, C-3,C-4, C-6, C-7

Comparison the spectral data of compound SA1-8 to reported values in the Aldrich Library of ^{13}C-NMR and ^{1}H-NMR spectra indicated that SA1-8 is a known compound, 2-phenylethanol (Table 10)

Table 10 ^{1}H-NMR and ^{13}C-NMR spectral data of SA1-8 and 2-phenylethanol (Pouchert and Behnke 1993)

position	SA1-8		2-Phenylethanol (Pouchert and Behnke 1993)	
	δH (ppm), mult, (J in Hz)	δC (ppm)	δH (ppm), mult, (J in Hz)	δC (ppm)
1	3.82, *t*, 6.64	63.60	3.78, *t*	63.51
2	2.84, *t*, 6.62	39.15	2.80, *t*	39.12
3	-	138.51	-	138.50
4	7.29, *m*	128.54	7.28, *m*	128.44
5	7.31, *m*	129.02	7.24, *m*	128.94
6	7.22,*m*	126.43	7.20, *m*	126.31
7	7.31, *m*	129.02	7.24, *m*	128.94
8	7.29, *m*	128.54	7.28, *m*	128.44

4.2.2 Structure elucidation of 4-Methyl-5-oxo-2-propyl-tetrahydro-furan-3-carboxylic acid (SA1-A5)

Compound SA1-A5 was obtained as light brown viscous liquid. The IR absorption spectrum (Figure 22 in Appendix C) showed characteristic bands at 3525.32 cm^{-1} (carboxylic), 2934.78 cm^{-1} (C-H stretching), 1733.86 cm^{-1} (aromatic), 1182.44 cm^{-1} (C-H stretching), and 702.94 cm^{-1} (C-H stretching). The UV spectrum in MeOH of compound SA1-A5 (Figure C12 in Appendix C) exhibited λ_{max} at 223.50 (log ε 6.34). The APCI-TOF mass spectrum of compound SA1-A5 (Figure C13 in Appendix C) showed a couple of the $[M+H]^+$ ion peak at *m/z* 187.0959 $[C_9H_{14}O_4+H]^+$.

The 500 MHz ^{1}H-NMR spectrum of compound SA1-A5 in $CDCl_3$ (Figure C14 in Appendix C) showed signal of proton in seven positions, multiplet at δ 4.50, 2.99, 2.70, 1.75 and 1.51 ppm; doublet at δ 1.36 ppm and triplet at δ 0.97 ppm, respectively.

The multiplicity at δ 4.50 ppm was multiplet and was assigned as a methine proton. There were one multiplet methine proton signal at δ 2.99 ppm and one multiplet methine proton signal at δ 2.70 ppm. There was a non-equivalent methylene signals at δ 1.75 and 1.51 ppm. The multiplicity at δ 1.36 ppm was doublet which was of a methyl proton. The multiplicity at δ 0.97 ppm was triplet, belonging to a methyl proton.

The 125 MHz ^{13}C-NMR spectrum of compound SA1-A5 in $CDCl_3$ (Figure C15 in Appendix C) showed the signal of carbon in nine positions, at δ 176.76, 175.86, 79.17, 53.91, 39.79, 36.92, 18.66, 14.48, and 13.68 ppm, respectively.

The DEPT 135 and DEPT 90 spectra of compound SA1-A5 in $CDCl_3$ (Figure C16 in Appendix C) classified carbon signals as one methine carbon signal at δ 79.17 ppm; one methine carbon signal at δ 53.91 ppm; one methine carbon signal at δ 39.79 ppm; one methylene carbon signal at δ 36.92 ppm; one methylene carbon signal at δ 18.66 ppm; one methyl carbon signal at δ 14.48 ppm; one methyl carbon signal at δ 13.68 ppm; one quaternary carbon signal at δ 176.76 ppm and one quaternary carbon signal at δ 175.86 ppm.

The ^{1}H-^{1}H COSY spectrum of compound SA1-A5 in $CDCl_3$ (Figure C17 in Appendix C) showed the coupling between methine proton signal at δ 4.50 ppm and methine proton signal at δ 2.70 ppm; methine proton signal at δ 4.50 ppm and methylene proton signal at δ 1.75 ppm; methine proton signal at δ 2.99 ppm and methyl proton signal at δ 1.36 ppm; methine proton signal at δ 2.99 ppm and methine proton signal at δ 2.70 ppm; methylene proton signal at δ 1.75 ppm and methylene proton signal at δ 1.51 ppm; and methylene proton signal at δ 1.51 ppm and methyl proton signal at δ 0.97 ppm.

The HMQC, and NOESY spectrum of compound SA1-A5 in $CDCl_3$ were shown in Figure C19 and Figure C20 in Appendix C, respectively.

The ^{1}H-^{13}C long-rang correlations from HMBC spectrum of SA1-A5 (Figure C18 in Appendix C) are summarized in Table 11. The structure a new compound (SA1-A5) is shown in Figure 5.

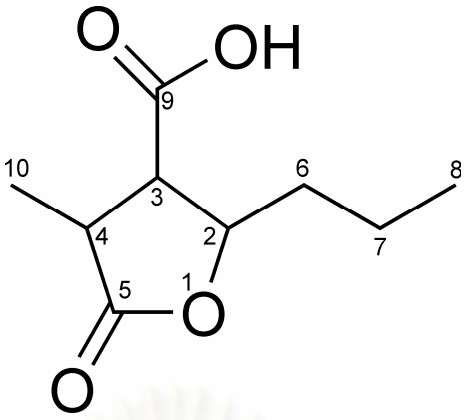


Figure 5 The structure of a 4-Methyl-5-oxo-2-propyl-tetrahydro-furan-3-carboxylic acid (SA1-A5)

Table 11 The ^{1}H, ^{13}C-NMR and HMBC spectral data of SA1-A5 in $CDCl_3$

position	SA1-A5		
	δH (ppm), *mult*, (J in Hz)	δC (ppm)	Long-rang correlation in HMBC ($^{n}J_{HC}$ = 8 Hz) spectrum
1	-	175.86	-
2	2.99, *m*	39.79	C-1, C-3, C-8, C-9
3	2.70, *dd*, 9.46	53.91	C-2, C-4, C-5, C-8, C-9
4	4.50, *ddd*, 3.98, 8.47	79.17	C-1, C-6, C-9
5	1.75, *m*	36.92	C-3, C-4, C-6, C-7
6	1.51, *m*	18.66	C-4, C-5, C-7
7	0.97, *t*	13.68	C-5, C-6
8	1.36, *d*	14.48	C-1, C-2, C-3, C-9
9	-	176.76	-

Comparison the ^{13}C-NMR spectral data of cyclic of compound SA1-A5 with γ-butyrolactone acid (**1**), 18R-hydroxydihydroalloprotolichesterinic acid (**2**), 18S-hydroxy dihydroalloprotolichesterinic acid (**3**) and 18S-hydroxyneodihydroprotolichesterinic acid (**4**) indicated that SA1-A5 was pentacyclic lactone compound, as shown in Teble 12.

Table 12 Comparison of ^{13}C-NMR spectral data of compound SA1-A5 with γ-butyrolactone acid (**1**),18R-hydroxydihydroalloprotolichesterinic acid (**2**), 18S-hydroxy dihydroalloprotolichesterinic acid (**3**), and 18S-hydroxyneodihydroprotolichesterinic acid (**4**) .

position	δC (ppm) of				
	SA1-A5	Compound 1	Compound 2	Compound 3	Compound 4
1	175.86	177.60	177.50	178.90	177.80
2	39.79	36.60	36.40	36.00	36.80
3	53.91	51.70	51.80	52.00	51.50
4	79.17	77.40	77.50	79.30	78.60
5	36.92	31.10	31.20	32.10	32.30
8	14.48	14.50	12.80	13.90	14.90
9	176.76	173.60	170.10	168.70	169.50

4.3 Biological activities of SA1-8 and SA1-A5

In this study the compound SA1-8 (48.8 mg) was lost for 45.6 mg by an accident. It remained 3.2 mg. So, this compound was not enough for biological activities screening, however there are several research that reported it's antimicrobial properties. SA1-8 or 2-phenylethanol (phenethyl alcohol; PEA) was known to inhibit the growth of several bacteria, particularly gram-negative organisms. For examples, 2-phenylethanol inhibited a range of *Escherichia coli*, and *Pseudomonas aeruginisa* at a concentration of 0.2 % (2,000 mg/L), and 0.46 % (4,600 mg/L), respectively (Fraud *et al*., 2003). It also inhibited a range of gram-positve cocci, such as *Mycobacterium smegmatis*, and *Mycobacterium phlei* at a concentration of 0.2 % (2,000 mg/L) (Fraud *et al*., 2003). 2-phenylethanol directly damaged to the cytoplasmic membrane of *Bacillus cereus*, and *Bacillus megaterium* at concentration up to 0.5 % (5,000 mg/L) (Silva et al., 1976) and completely inhibited the development of the spore septum of *Bacillus cereus* at a concentration of 0.35% (3,500 mg/L) (Remsen et al., 1966). In addition 2-phenylethanol

can inhibit the growth of several species of molds. For examples, completely inhibited the growth of *Asperillus niger*, *Penicilium notatum*, and *Neurospora crassa* at a concentration of 0.2 % (2,000 mg/L); *Rhizopus nigricans* at a concentration of 0.4 % (4,000 mg/L) (Lester G., 1965).

4.3.1 Antimalarial activity

The isolated compound (SA1-A5) was tested for antimalarial activity against *Plasmadium falciparum* 94 with the assay method described by Trager and Jensen (1976) method. The antimalarial result are classified as negative activity ($>10^{-5}$ M).

4.3.2 Cytotoxic activity

The isolated compound (SA1-A5) was tested for primary screening cytotoxic activity against MOLT-3 (acute lymphoblastic leukemia), A549 (human lung adenocarcinoma), Hep G2 (human hepatoblastoma carcinoma), and HuCCA-1 (human cholangiocarcinoma). Etoposide and doxorubicin were used as a positive control. SA1-A5 are classified as inactive activity (>50 µg/ml).

4.3.3 Antimicrobial activities

It was found that the isolated compounds (SA1-8 and SA1-A5) exhibited no antimicrobial activity.

4.4 Identification of the endophytic fungus isolate SM1

4.4.1 Conventional method

The SM1 PDA culture was initially white to smoke-grey with fluffy, aerial mycelium and became olivaceous-grey on the surface, with thick aerial mycelium. Reverse side of the colony was dark slate-blue, as shown in Figure 6. The colony

covered the medium surface (90 mm diameter Petri dish) after 2 days of incubation at 30 °C. On banana leaf pieces, the SM1 fungus produced pycnidia that were covered with mycelium, as shown in Figure 7. The conidia in the conidiomata were initially hyaline and aseptate, as shown in Figure 8. Contents of conidia were granular, as shown in Figure 9. A single median septum formed in aging brown conidia, as shown in Figure 10. These structures corresponded with the typical *Lasiodiplodia* morphology.

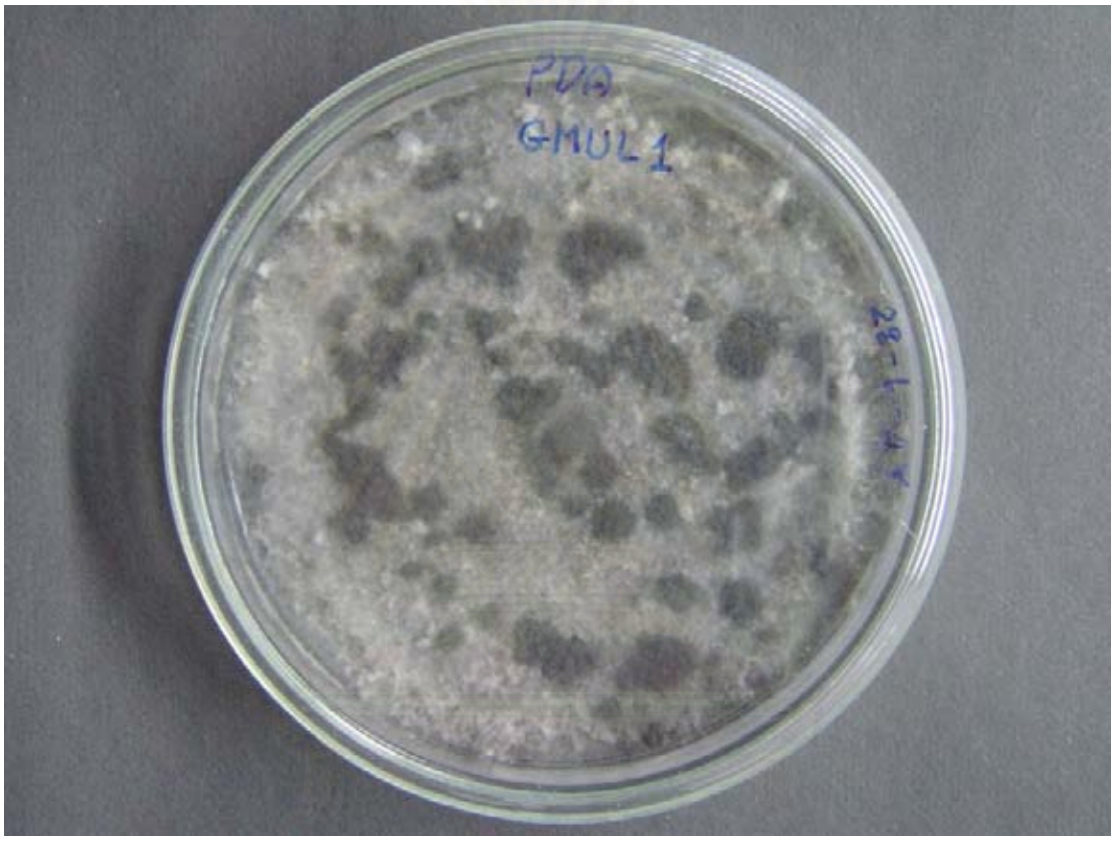


Figure 6 Colony morphology of endophytic fungus isolate SM1 (17 days) on potato dextrose agar (PDA).

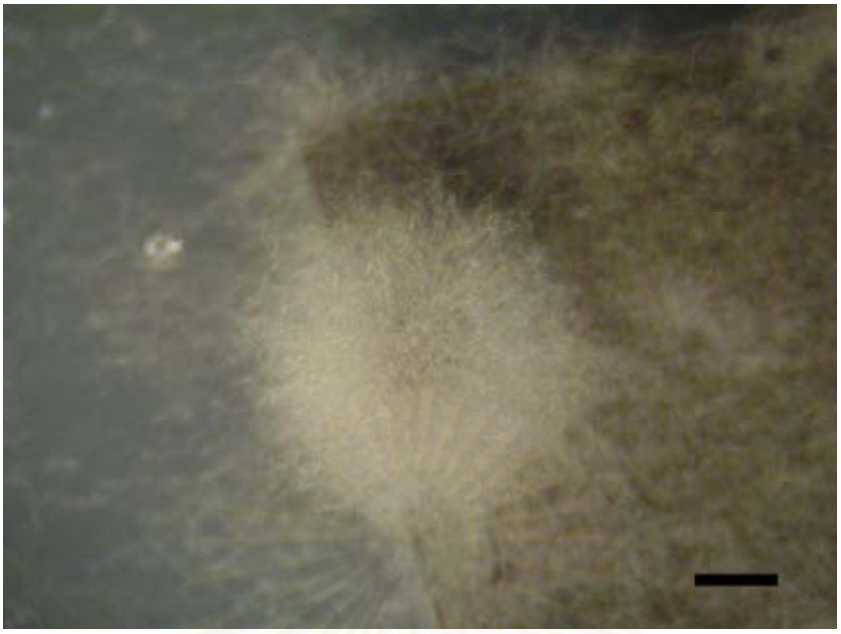


Figure 7 Pycnidium of endophytic fungus isolate SM1 formed in culture on banana leaf, covered with mycelium (Bar size = 1 mm)

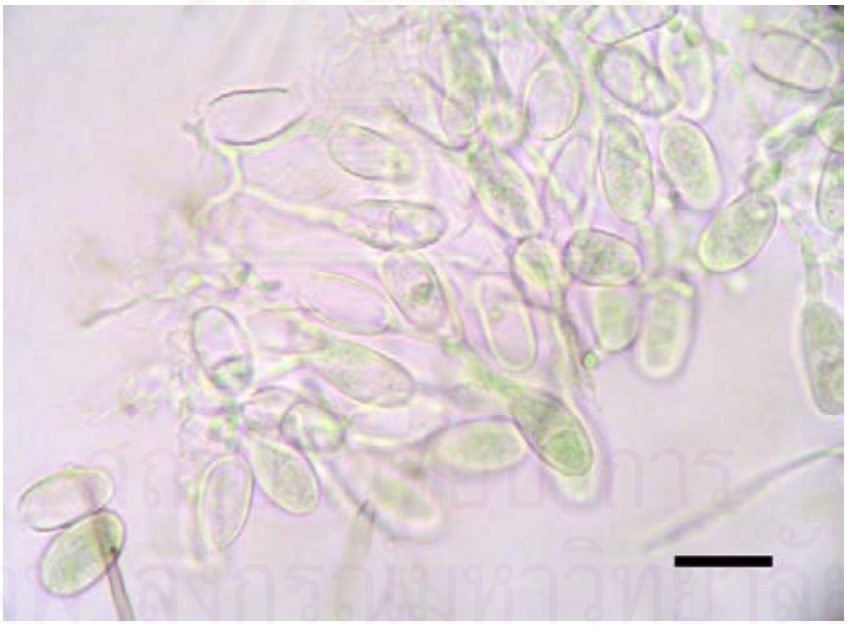


Figure 8 Hyaline immature conidia of endophytic fungus isolate SM1 (Bar size = 20 μm)

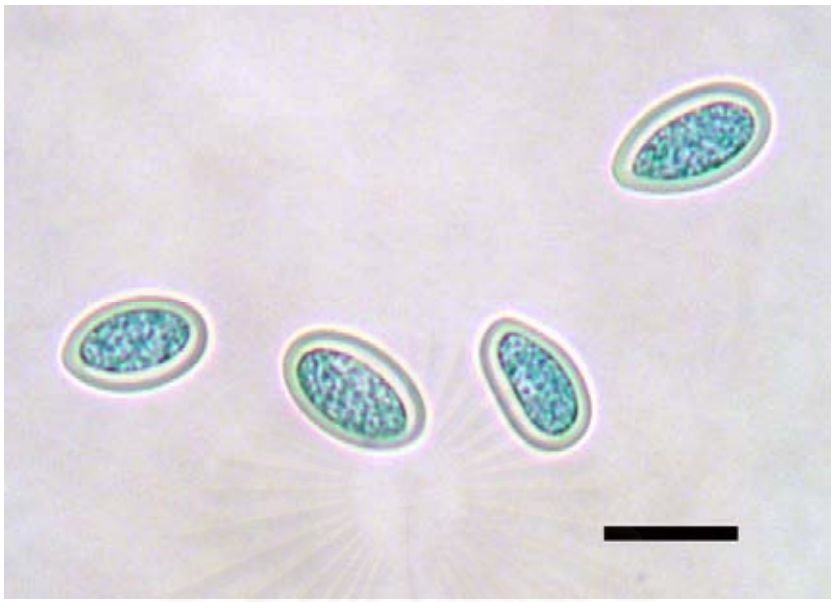


Figure 9 Hyaline immature conidia with granule contents of endophytic fungus isolate SM1 (Bar size = 20 µm)

Figure 10 Mature brown conidia with one septum showing the longitudinal striations (arrow) of endophytic fungus isolate SM1 (Bar size = 20 µm)

4.4.2 Molecular method

Sequencing of the PCR product amplified from chromosomal DNA of isolate SM1 resulted in a 496 bp fragment. This comprised partial of the 18S sequence, complete ITS1-5.8S-ITS2 sequences, and partial of the 28S sequence, as shown in Figure 11. The complete ITS1-5.8S-ITS2 sequences of the SM1 fungus was used as the query sequence to search for similar sequences from GenBank. It was found that DNA sequence of the ITS1-5.8S-ITS2 rRNA gene of SM1 was perfectly matched (100% homology) with that of *Botryosphaeria rhodina* (the teleomorph of *Lasiodiplodia theobromae*) and was 99.78% homology with *L. pseudotheobromae*. This suggests that SM1 fungus is *Lasiodiplodia* sp. and possibly an anamorph of *Botryosphaeria rhodina*.

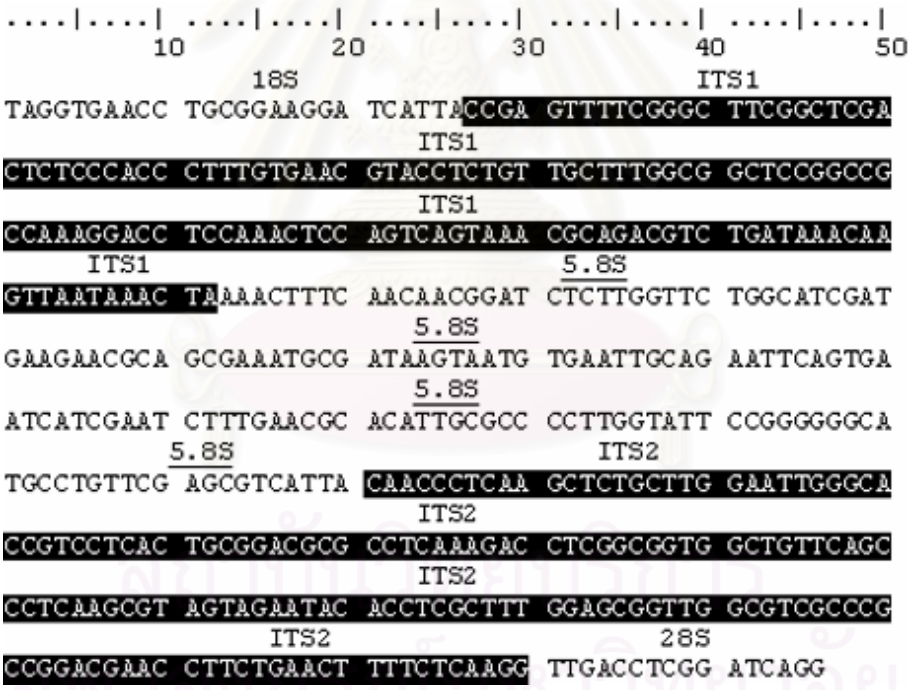


Figure 11 Nucleotide sequences of the partial 18S sequence, complete ITS1-5.8S-ITS2 sequences, and partial 28S sequence of the isolate SM1

CHAPTER V

CONCLUSION

The objective of this thesis was the investigation of secondary methabolites produced by endophytic fungus isolate from *Suregada multiflora* (A. Juss.) Baill., the medicinal plant collected from Siriruckhachati Medicinal Plant Garden, Mahidol university. Biological activities of the isolated compounds were also evaluated.

The endophytic fungus isolate SM1 was isolated from the surface-sterilized bark of *Suregada multiflora* (A. Juss.) Baill. (Euphorbiaceae). In the present investigation, two compounds were isolated from YES culture of endophytic fungus isolate SM1 and elucidated as 2-phenylethanol (SA1-8), and a new compound, 4-Methyl-5-oxo-2-propyl-tetrahydro-furan-3-carboxylic acid (SA1-A5). The yields of them were 0.45% and 0.46% of ethyl acetate extract, respectively. Both of them were studied for biological activity assays and any reported for bioactivity property in this experiment. Based on the microscopic morphology and nucleotide sequencing of ITS1-5.8S-ITS2 sequences of rDNA, endophytic fungus isolate SM1 was identified as *Lasiodiplodia* sp., possibly an anamorph of *Botryosphaeria rhodina* (100 % sequence homology).

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APPENDICES

APPENDIX A

Table A The Chemical compounds, sources, biological activities of bioactive compounds of endophytic fungi.

No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
1	Taxol	*Taxomyces andreane*	*Taxus brevifolia*	Anticancer	Stroble *et al*., 2003, Stierle and Stroble, 1995, Stierle *et al*., 1993, Stroble and stierle, 1993
		Stegolerium kukenani	*Stegolepis guianensis*	Anticancer	Stroble *et al*.,2001
		Aspergillus niger	*Taxus chinenis*	Anticancer	Wang *et al*., 2001
		Tubercularia sp.	*Taxus mairei*	Anticancer	Stroble *et al*., 2003, Wang *et al*., 2000
		Pestalotipsis microspora	*Taxus wallachina*	Anticancer	Stroble *et al*., 2003, Metz *et al*., 2000, Li *et al*., 1998, Stroble *et al*., 1996
			Taxodium distichum	Anticancer	Li *et al*., 1996

Tabie A (continued)

No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
2 3	Lactone 1893 A Lactone 1893 B	*Endophytic fungus* No. 1893	*Kandelia candel*	Cytotoxic	Chen *et al*.,2003
4	Brefeldin A	*Paecilomyces* sp. and *Aspergillus clavatus*	*Taxus mairei* and *Torreya grandis*	Cytotoxic	Wang *et al*., 2002
5 6 7 8	Cytochalasin 1 Cytochalasin 2 Cytochalasin 3 Cytochalasin E	*Rhinocladiella* sp.	*Tripterigium wilfordii*	Cytotoxic	Wagenaar and Clardy, 2000
9	Terpendole M	*Neotyphodium lolii*	*Lolium perenne*	Neurotoxins	Gatenby *et al*., 1999
10	Lolitrem B	*Acremonium lolii*	*Lolium perenne*	Neurotoxins	Berny *et al*., 1997
11	1,3,5,7 cyclooctatetraene or [8]annulene	*Gliocladium* sp.	*Eucryphia cordifolia*	Antimicrobial	Stinson *et al*., 2003
12	Colletotric acid	*Colletotrichum gloeosporioides*	*Artemisia mongolica*	Antimicrobial	Zou *et al*., 2000
13	Pestacin	*Pestalotiopsis microspora*	Rainforest	Antioxidant and antimycotic	Harper *et al*., 2003

Tabie A (continued)

No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
14 15 16	7-Butyl-6,8-dihydrocxy-3(R)-pent-11-enylisochroman-1-one 7-Butyl-15-enyl-6,8-dihydroxy-3(R)-pent-11-enylisochroman-1-one 7-Butyl-6,8-dihydroxy-3(R)-pentylisochroman-1-one	*Geotrichum* sp.	*Crassocephalum crepidiodes*	Antimalarial, antituberculous and antifungal	Kongsaeree *et al*., 2003
17	Isopestacin	*Pestalotiopsis microspora*	*Terminalia morobensis*	Antifungal and antioxidant	Stroble *et al*., 2002
18 19	Jesterone Hydrosy-jesterone	*Pestalotiopsis jesteri*	*Fragraea bodenii*	Antioomycete	Li *et al*., 2001

Tabie A (continued)

No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
20 21 22 23 24 25	Preussomerin G Preussomerin H Preussomerin I Preussomerin J Preussomerin K Preussomerin L	*Mycelia sterile*	*Atropa belladonna*	Antibacterial, antifungal and antialgal	Krohn *et al*., 2001
26 27 28	Dicerandrol A Dicerandol B Dicerandol C	*Phomopsis longicolla*	*Dicerandra frutecens*	Antibiotic and cytotoxic	Wagenaar and Clardy, 2001

Tabie A (continued)

No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
29	Isoprenylindole-3-carboxylic acid	*Collectotrichum* sp.	*Artemisia annua*	Antibacterial and antifungal	Lu *et al*., 2000
30	3beta, 5alpha-Dihydroxy-6beta-acetoxy-ergosta-7,22-diene				
31	3beta,5alpha-Dihydroxy-6beta-phyenylacetyloxy-ergosta-7,22-diene				
32	Indole-3-actetic acid (IAA)	*Epichloe/Neotyphodium spp.*	Grasses	Antifungal	Yue *et al*., 2000
33	Indole-3-ethanol (IEtOH)				
34	Methylindole-3-carboxylate Indole-3-carboxaldehyde				
35					
36	Diacetamide				
37	Cyclonerodiol				

Tabie A (continued)

No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
38	Preussomerin D	*Hormonema dematioides*	*Conifer wood*	Antifungal	Polishook *et al*., 1993
39	Ambuic acid	*Pestalotiopsis* spp., *Monochaetia* sp.	Rainforest	Antifungal	Li *et al*., 2001
40 41 42	Preauatinoid A Preauatinoid B Alkaloid verruculogen	*Penicillium* sp.	*Melia azedarach*	Bacteriostatic	Santos and Rodrigues-Fo, 2002
43	Cryptocandin	*Cryptosporiopsis* cf. *quercina*	*Tripterigeum wilfordii*	Antimycotic	Stroble *et al*., 1999

APPENDIX B

1. Media

1.1 Yeast Extract Sucrose Agar (YES)

Yeast Extract	20.0 g
Sucrose	150.0 g
Distilled water up to	1 L

Composition of Yeast Extract Sucrose (YES) is similar to YEA but not supplemented with agar.

1.2 Malt Czapek Broth (MCz)

Czapek stock solution A	50 ml
Czapek stock solution B	50 ml
Sucrose	30.0 g
Malt Extract	40.0 g
Distilled water up to	1 L

Czapek stock solution A

$NaNO_3$	4.0 g
KCL	1.0 g
$MgSO_4.7H_2O$	1.0 g
$FeSO_4.7H_2O$	0.02 g
Dissolved in distilled water up to	100 ml
Keep in a refrigerator	

Czapek stock solution B

K_2HPO_4	2.0 g
A solution	1.0 g
B solution	1.0 g
Dissolved in distilled water up to	100 ml
Keep in a refrigerator	

A solution

$ZnSO_4.7H_2O$	1.0 g
Dissolved in distilled water up to	100 ml

B solution

$CuSO_4.5H_2O$	1.0 g
Dissolved in distilled water up to	100 ml

1.3 Sabouraud's Dextrose Agar (SDA)

Dextrose	40.0 g
Neopeptone	10.0 g
Distilled water up to	1 L

Composition of Sabouraud's Dextrose Broth (SDB) is similar to SDA but not supplemented with agar.

1.4 Potato Dextrose Agar (PDA)

Potato	200.0 g
Dextrose	20.0 g
Distilled water up to	1 L

Composition of Potato Dextrose Broth (PDB) is similar to PDA but not supplemented with agar.

1.10 Water Agar

Agar	13.0 g
Distilled water up to	1 L

1.11 Corn Meal Agar

Corn meal	30.0 g
Agar	13.0 g
Distilled water up to	1 L

2. Reagent and buffer for DNA amplification by PCR

2.1 Lysis buffer

Tris-HCl (pH 7.2)	50.0 mM
EDTA	50.0 mM
SDS	3%
2-mercaptoethanol	1%

2.2 Chlorofrom : TE-saturated phenol 1:1 (v/v)

2.3 TE for resuspending pellet

Tris-HCl	10.0 mM
EDTA	0.1 mM

2.4 Gel loading buffer

Bromophenol blue	0.25%
Sucrose in water	40% (w/v)

Store temperature at 4 °C

2.5 5-X Tris-Borate-EDTA (TBE)

Tris base	54.0 g
Boric acid	27.5 g
0.5 M EDTA pH 8.0	20ml

The working solution was 1X TBE, diluted with four volume of distilled water.

2.6 10X Buffer

Tris-HCl pH 9.0	100 ml
KCL	500.0 mM
Triton X-100	1%

2.7 2 mM dNTP (dATP, dCTP, dGTP, dTTP mix)

dATP	100.0 mM
dCTP	100.0 mM
dGTP	100.0 mM
dTTP	100.0 mM

Mixed equal volume of each dNTP to get 25 mM dNTP, then dilute to 2 mM dNTP with sterile double distilled water.

APPENDIX C

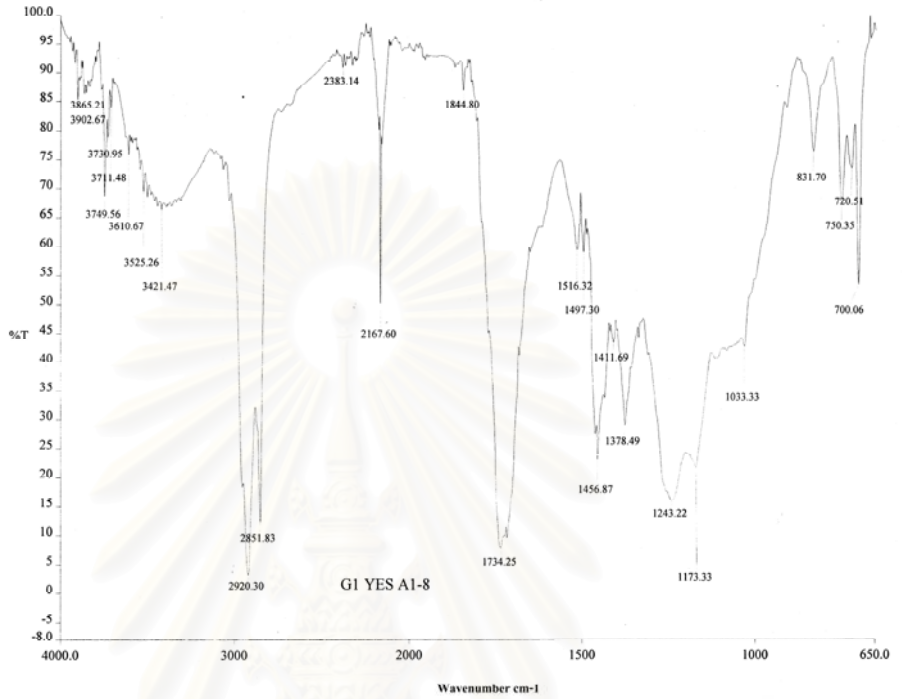

Figure C1 IR spectrum of compound SA1-8

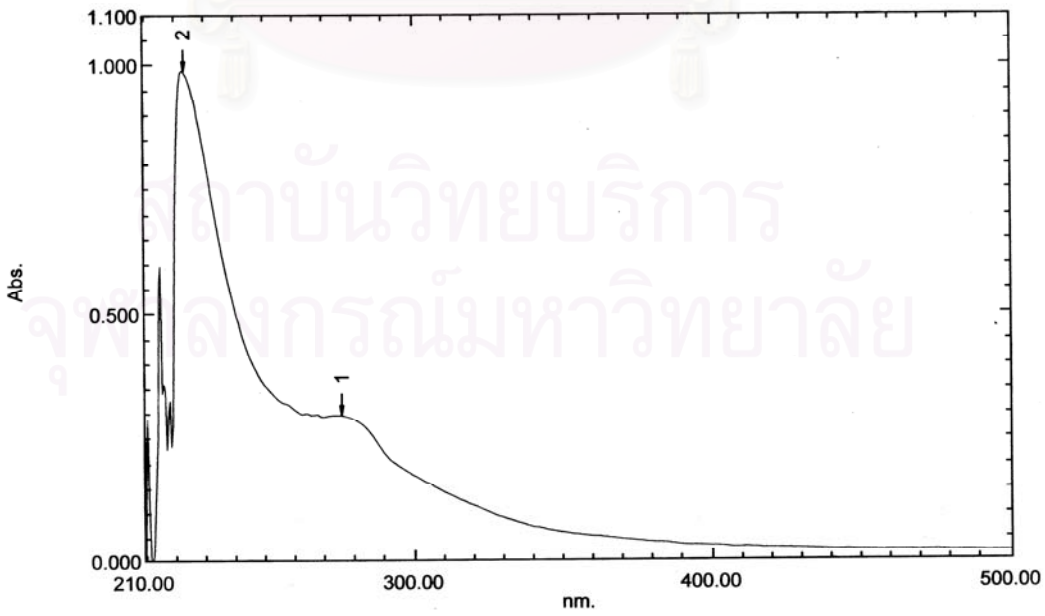

Figure C2 UV spectrum in MeOH of compound SA1-8

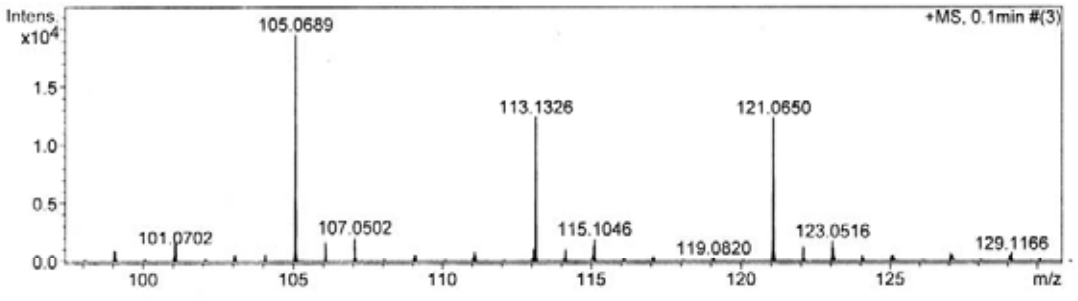

Figure C3 APCI-TOF mass spectrum of compound SA1-8

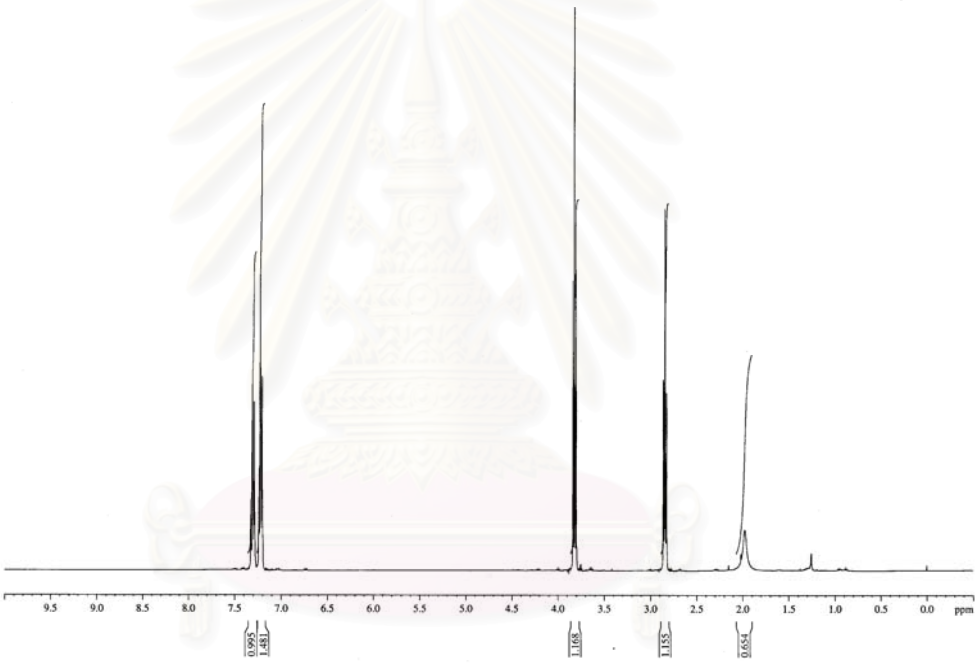

Figure C4 ^{1}H-NMR spectrum of compound SA1-8 in $CDCl_3$

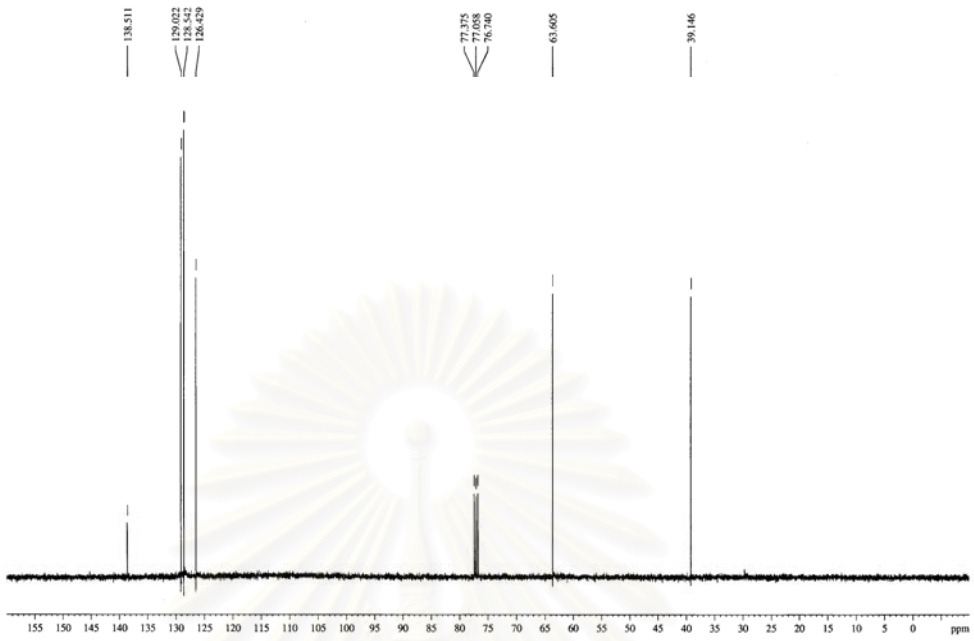

Figure C5 ^{13}C-NMR spectrum of compound SA1-8 in $CDCl_3$

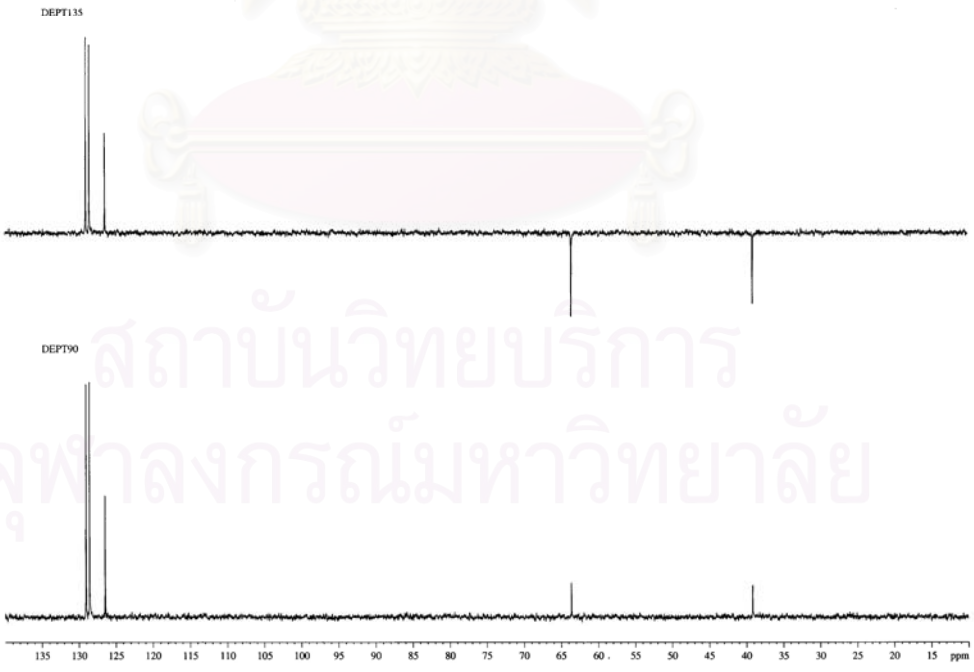

Figure C6 Depts spectra of compound SA1-8

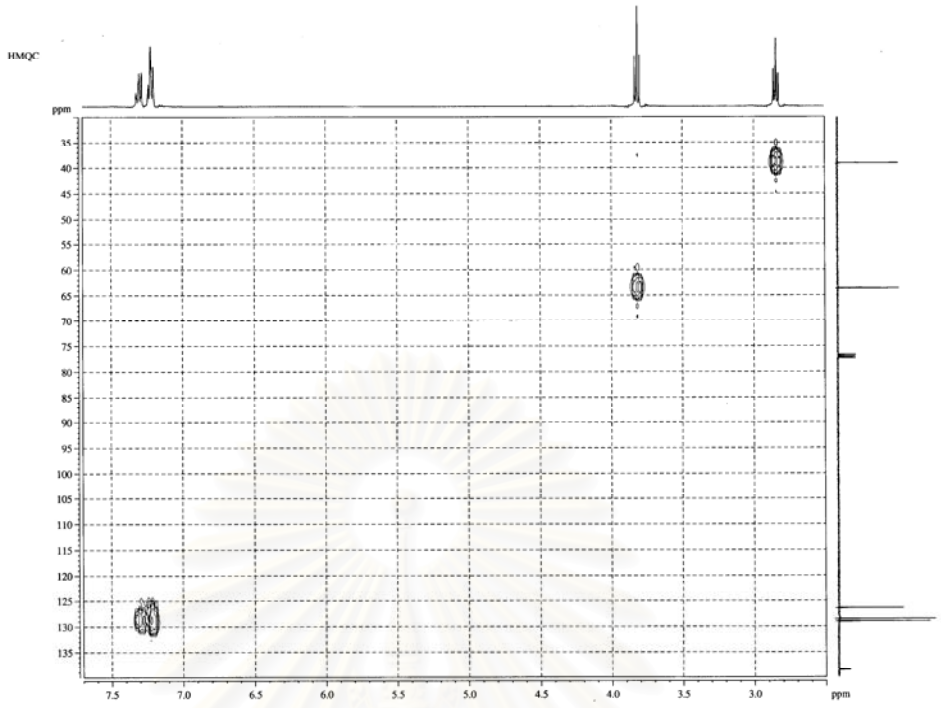

Figure C7 HMQC spectrum of compound SA1-8

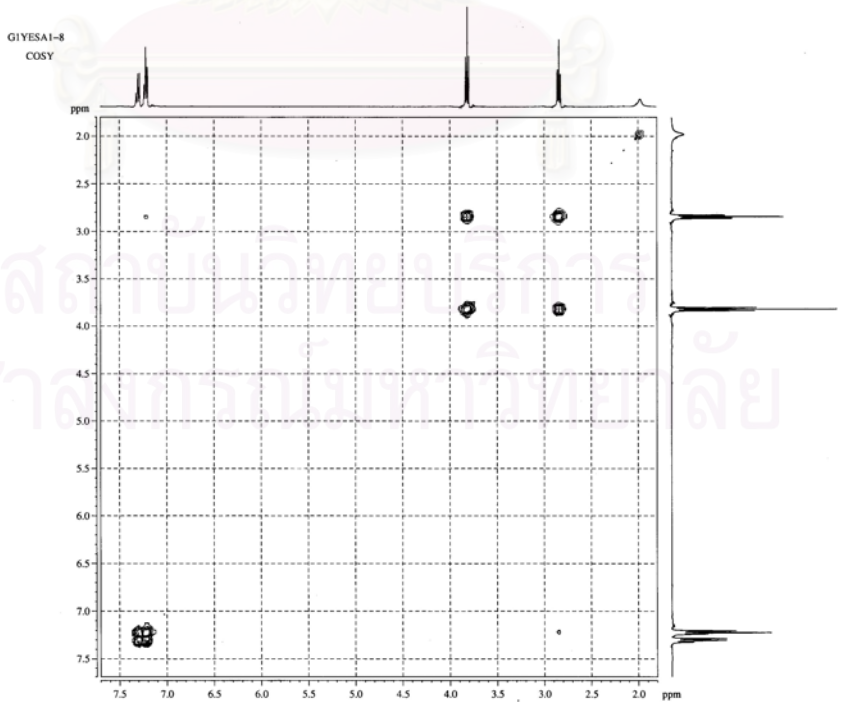

Figure C8 COSY spectrum of compound SA1-8

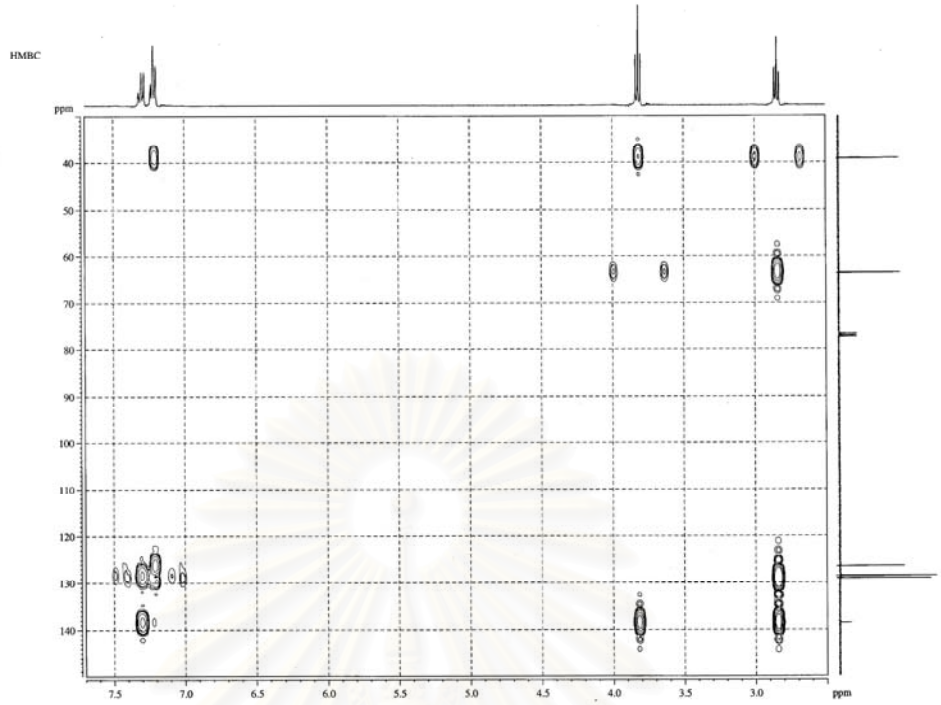

Figure C9 HMBC spectrum of compound SA1-8

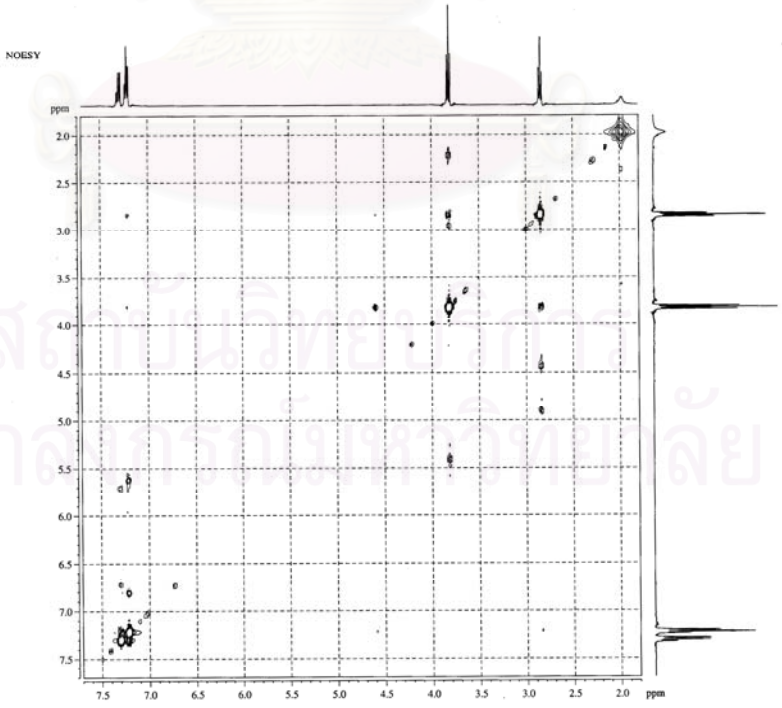

Figure C10 NOESY spectrum of compound SA1-8

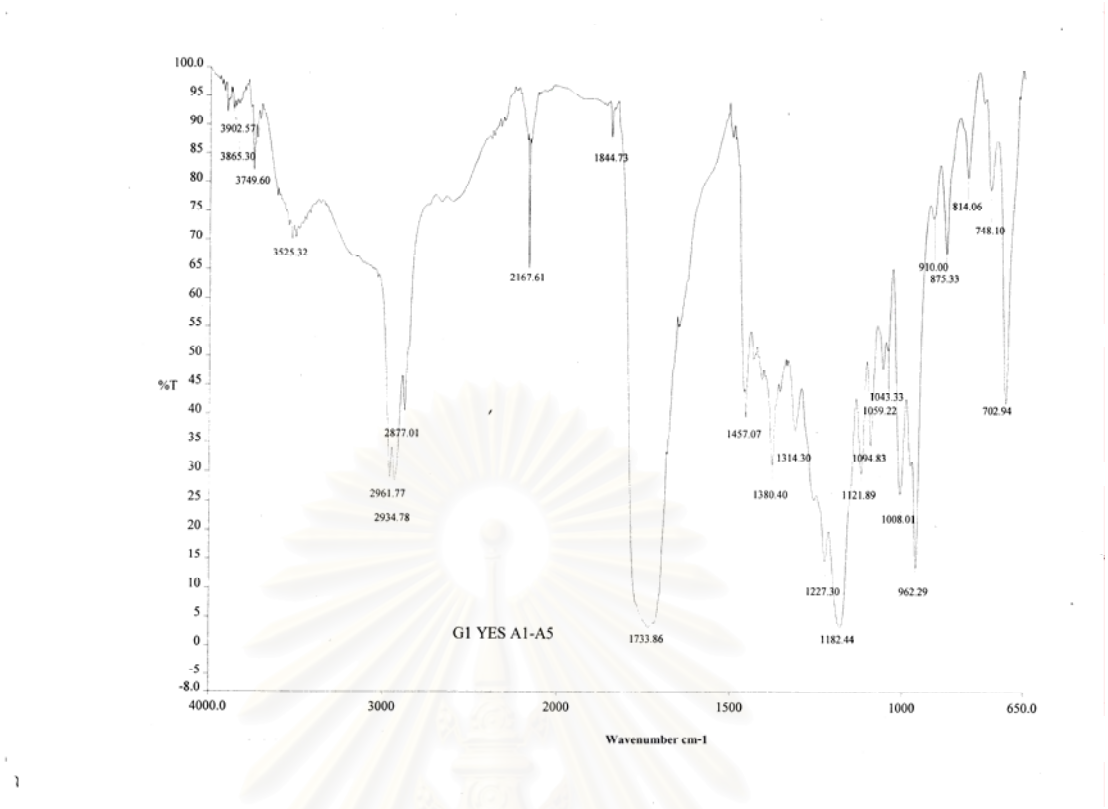

Figure C11 IR spectrum of compound SA1-A5

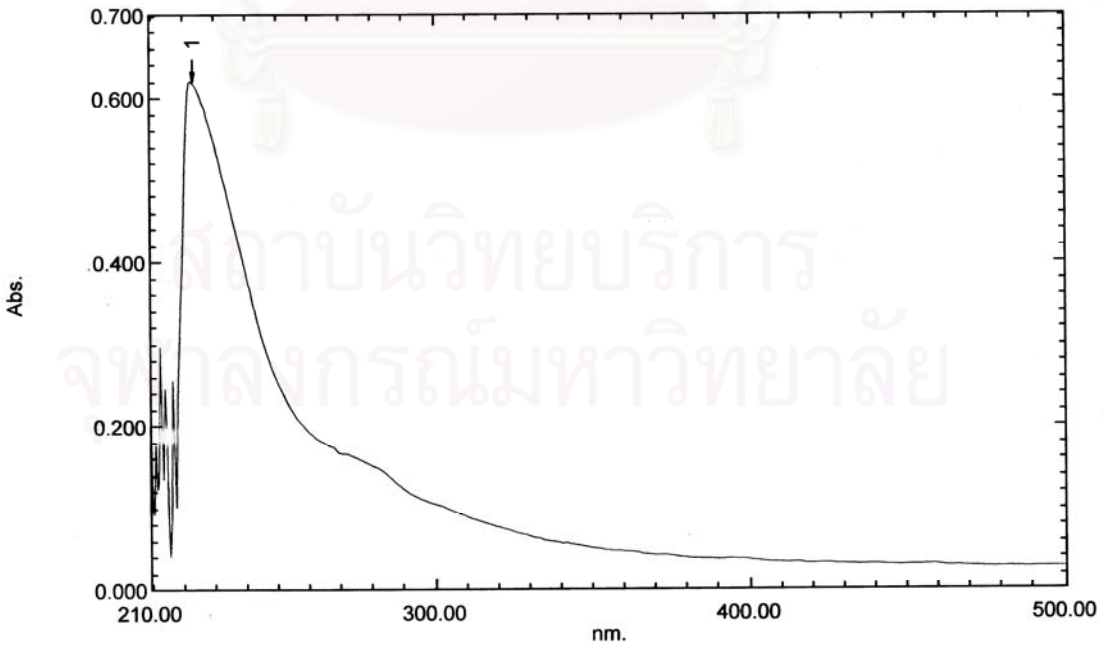

Figure C12 UV spectrum in MeOH of compound SA1-A5

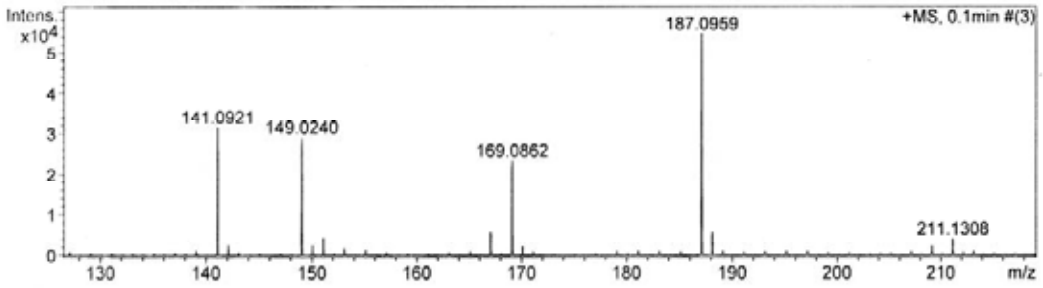

Figure C13 APCI-TOF mass spectrum of compound SA1-A5

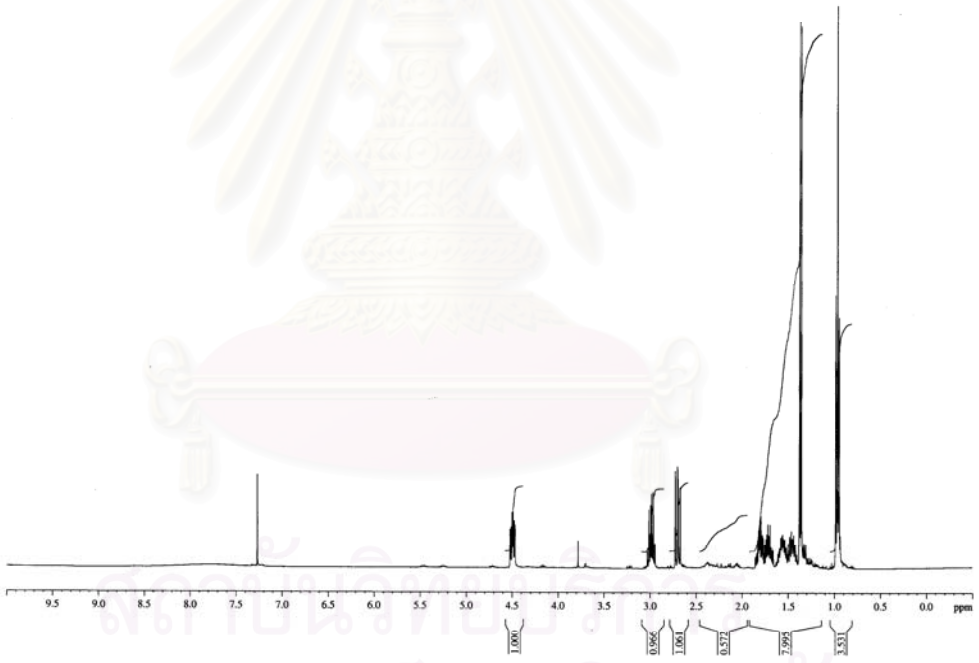

Figure C14 ^{1}H-NMR spectrum of compound SA1-A5 in $CDCl_3$

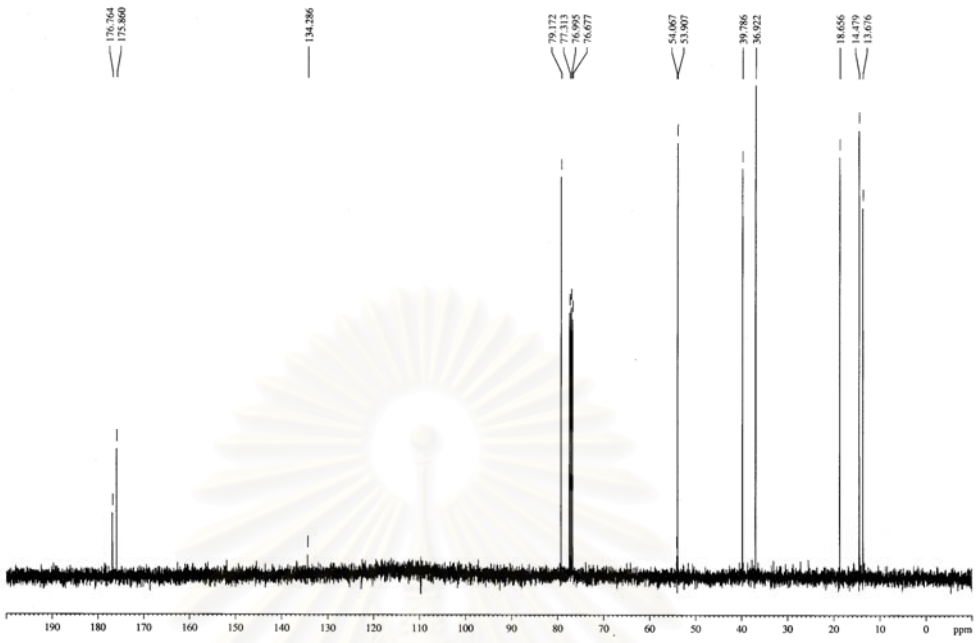

Figure C15 ^{13}C-NMR spectrum of compound SA1-A5 in $CDCl_3$

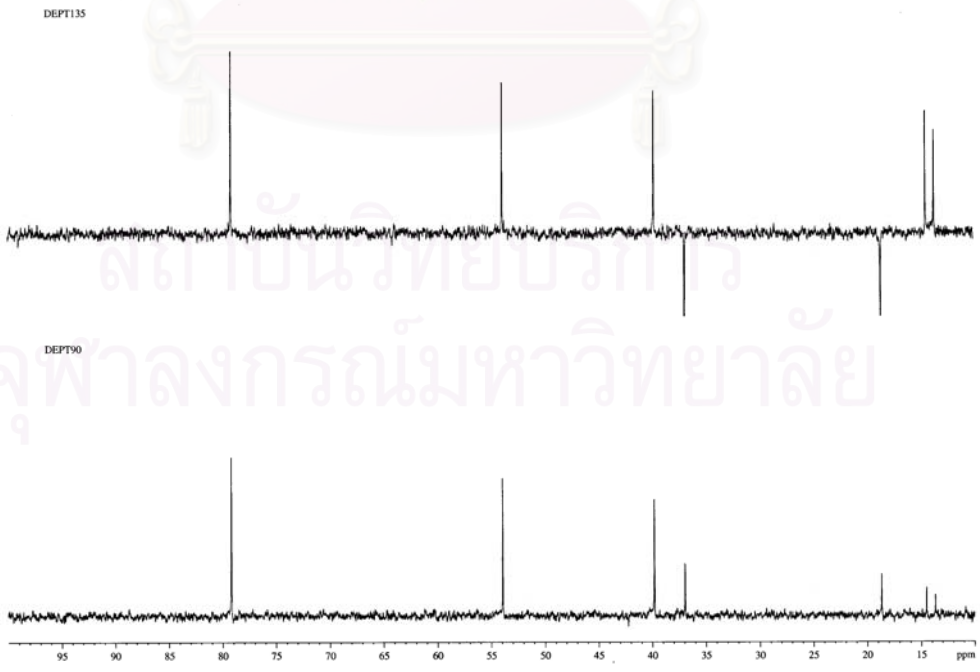

Figure C16 Depts spectra of compound SA1-A5

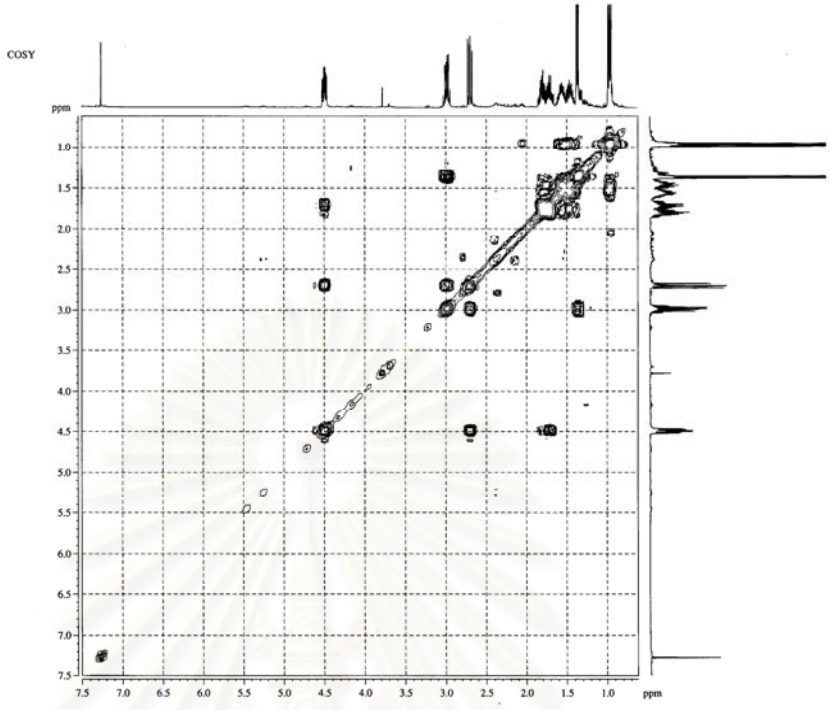

Figure C17 COSY spectrum of compound SA1-A5

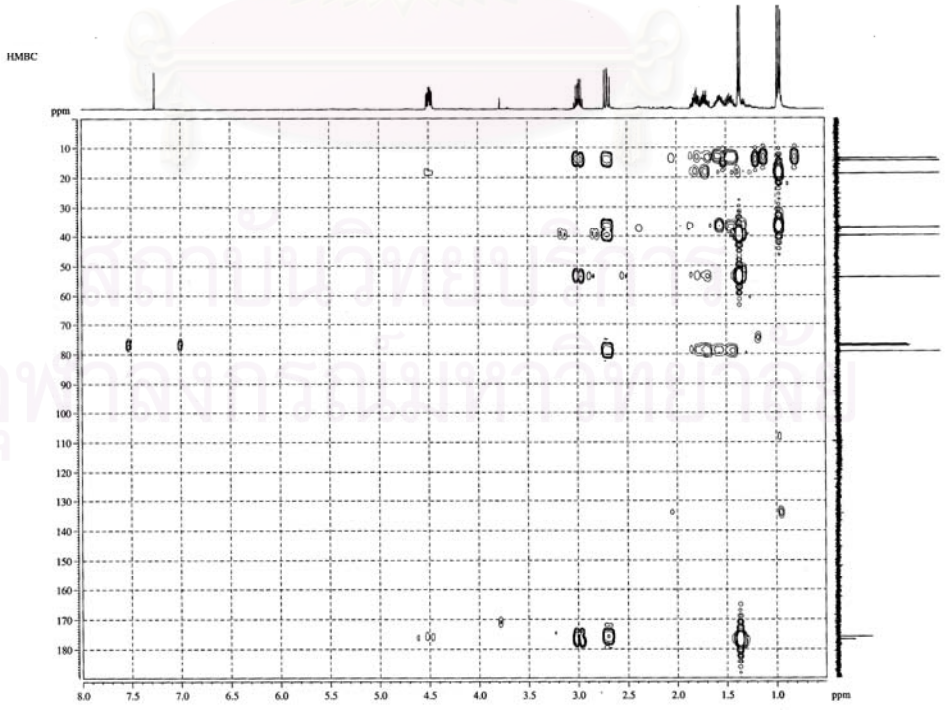

Figure C18 HMBC spectrum of SA1-A5

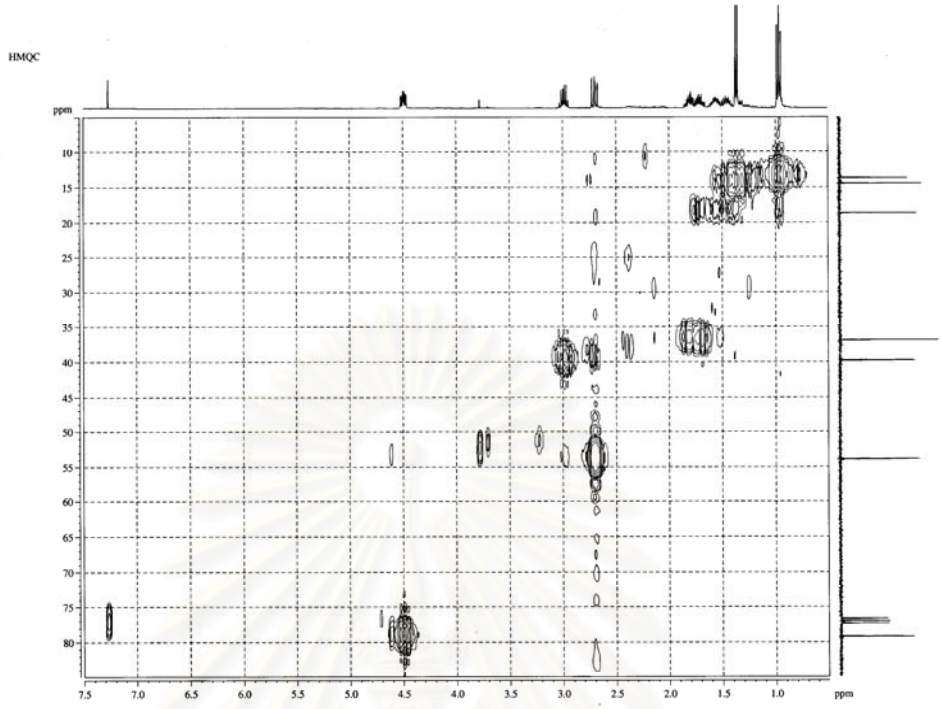

Figure C19 HMQC spectrum of compound SA1-A5

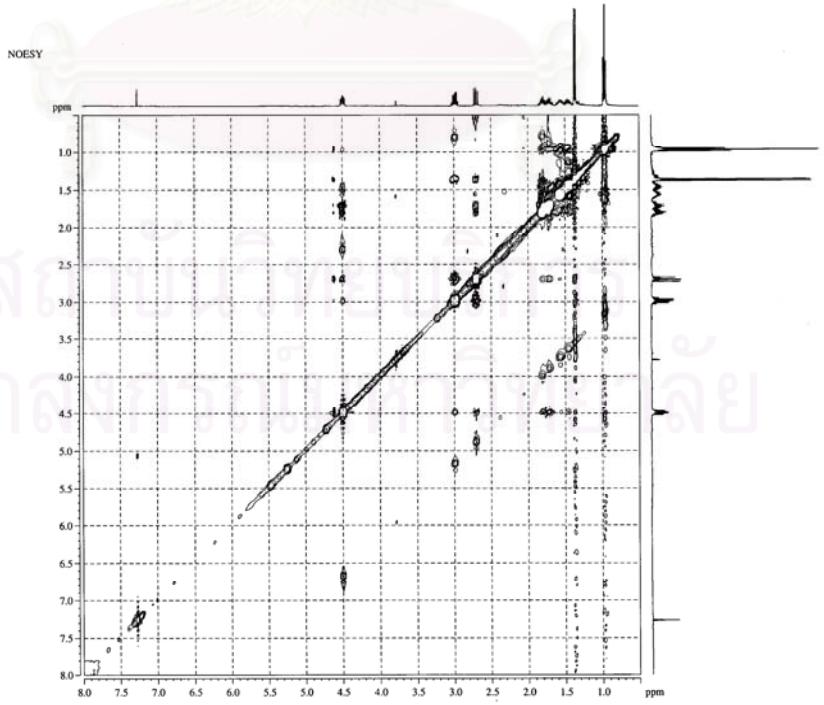

Figure C20 NOESY spectrum of compound SA1-A5

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