สารออกฤทธิ์ทางชีวภาพของราเอนโดไฟต์ที่แยกจากพลู *Piper betle* Linn.

นางอนุรีย์ ชันบุญ



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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# BIOACTIVE COMPOUNDS FROME ENDOPHYTIC FUNGI ISOLATED FROM *Piper betle* Linn.

Mrs.Anuree Khanbun



A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Biotechnology
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|---|---|
| Thesis Title | BIOACTIVE COMPOUNDS FROM ENDOPHYTIC FUNGI ISOLATED FROM *Piper betle* Linn. |
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นางอนุรีย์ ขันบุญ: สารออกฤทธิ์ทางชีวภาพของราเอนโดไฟต์ที่แยกจากพลู *Piper betle* Linn.(BIOACTIVE COMPOUNDS FROM ENDOPHYTIC FUNGI ISOLATED FROM *Piper betle* Linn.) อาจารย์ที่ปรึกษา : ศ.ดร.โสภณ เรืองสำราญ, อาจารย์ที่ปรึกษาร่วม รศ.ดร.ประกิตติ์สิน สีหนนทน์ 107 หน้า. ISBN 974-17-6096-5.

งานวิจัยนี้ทำการแยกสารออกฤทธิ์ทางชีวภาพที่สร้างจากราเอนโดไฟต์ที่แยกจากใบพลู *Piper betle* Linn. โดยเก็บตัวอย่างจากจังหวัดกรุงเทพฯ, ปทุมธานี,ระยองและนครศรีธรรมราช มาคัดแยกราโดยผ่านวิธีฆ่าเชื้อพื้นที่ผิวภายนอกและวางบน Potato Dextrose Agar สามารถแยกราเอนโดไฟต์ได้ 32 ไอโซเลต จากการทดสอบเบื้องต้นในการสร้างสารออกฤทธิ์ทางชีวภาพของราเอนโดไฟต์ในการยับยั้งจุลินทรีย์ พบว่าราเอนโดไฟต์ไอโซเลต PBL004 มีฤทธิ์ต้านจุลินทรีย์ทดสอบได้ดีคือออกฤทธิ์ยับยั้ง *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922 และ *Candida albicans* ATCC 10231 เมื่อทำการจัดจำแนกสายพันธ์ โดยศึกษาลักษณะทางสัณฐานวิทยา และการวิเคราะห์ลำดับนิวคลีโอไทด์ในบริเวณ internal transcribed spacer ของ rDNA พบว่าราเอนโดไฟต์ไอโซเลต PBL004 คือ *Fusarium proliferatum* เมื่อทำการศึกษาเพื่อแยกสารออกฤทธิ์ทางชีวภาพ โดยเลี้ยงในอาหารเหลว malt czapek broth แยกสารบริสุทธิ์จากน้ำหมักด้วยวิธีทางโครมาโทกราฟีและการตกผลึก และหาสูตรโครงสร้างของสารโดยอาศัยสมบัติทางกายภาพและเทคนิคทางสเปกโทสโกปี พบว่าได้ของผสม CB1 ซึ่งมีโครงสร้างหลัก คือ 7-butyl-6,8-dihydroxy-3(*R*)-pent-11-enylisochroman-1-one ลักษณะของแข็งเม็ดทรงกลมสีขาว มีจุดหลอมเหลว 158 -159 $^{0}$C นำของผสม CB1 มาทดสอบฤทธิ์ทางชีวภาพในการยับยั้งจุลินทรีย์ทดสอบ พบว่าของผสม CB1 มีฤทธิ์ยับยั้ง *Bacillus subtilis* ATCC6633 และ *Candida albicans* ATCC 10231 มีค่า MIC เท่ากับ 3.81 และ 1,000 µg/ml มีฤทธิ์ยับยั้ง herpes simplex virus type I ATCC VR-260 มีค่า $IC_{50}$ เท่ากับ 6.25 µg/ml และมีฤทธิ์ยับยั้งเซลล์มะเร็งชนิด HEP-G2 (มะเร็งตับ), SW620 (มะเร็งลำไส้), KATO-3 (มะเร็งกระเพาะอาหาร), BT474 (มะเร็งเต้านม) โดยมีค่า $IC_{50}$ เท่ากับ 6.8, 5.5, 7.4 และ 6.8 µg/ml ตามลำดับ เส้นใยราสามารถแยกสาร diglyceride ปริมาณ 1001.4 mg/L culture broth ซึ่ง diglyceride ประกอบด้วยกรดไขมัน 5 ชนิด คือ myristic acid (0.4%), palmitic acid (27.21%), stearic acid (16.42%), oleic acid (37.49%) และ linoleic acid (17.26%) ปริมาณ 4.0, 272.5, 164.4, 375.4 และ 172.8 mg/L culture broth ตามลำดับ

ลายมือชื่อนิสิต.....อนุรีย์ ขันบุญ.....

สาขาวิชา..เทคโนโลยีชีวภาพ.....ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา..2547..... ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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KEYWORD: ENDOPHYTIC FUNGI/ BIOACTIVE COMPOUNDS/ *Fusarium* / 7-BYTYL-6,8-DIHYDROXY-3(*R*)-PENT-11-ENYLISOCHROMAN-1-ONE

ANUREE KHABUN: BIOACTIVE COMPOUNDS FROM ENDOPHYTIC FUNGI ISOLATED FROM *Piper betle* Linn. THESIS ADVISOR: PROF. SOPHON ROENGSUMRAN, Ph.D., THESIS COADVISOR: ASSOC.PROF.PRAKITSIN SRIHANONTH, Ph.D. 107 pp. ISBN 974-17-6096-5

The purpose of this research was to isolate bioactive compounds from endophytic fungi isolated from Thai medicinal plant *Piper betle* Linn. Leaves samples were collected from Bangkok, Pathumthani, Rayong and Nakornsithammarat Province and 32 isolates of the endophytic fungi were obtain by surface sterilization technique and placed onto potato dextrose agar. Fungal endophytes were isolated from leaves. Thirty two fungal isolates were tested for the production of antimicrobial compounds. Fungal isolate PBL004 was chosen for the further study of bioactive compounds due to this isolate produced active compounds against *Bacillus subtilis* ATCC 6633, *Cacdida albican* ATCC 10231and *Escherichia coli* ATCC 25922. Based on morphology and nucleotide sequencing of ITS regions of rDNA, isolate PBL004 was identified as *Fusarium proliferatum*. Chromatographic techniques and crystallization were used to purify bioactive compounds from malt czapek culture broth of PBL004. Structure elucidation of the pure compound was investigated using physical properties and spectroscopic techniques. Mixture CB1, major structure was identified as 7-butyl-6,8-dihydroxy-3(R)-pent-11-enylisochroman-1-one, which was a round white solid (melting point 158 - 159 °C). Antimicrobial activities from this compound were tested. It was found that active against *Bacillus subtilis* ATCC 6633, *Candida albicans* ATCC 10231 with MIC 3.81 and 1000 μg/ml, respectively and Herpes simplex virus type I ATCC VR-260 with $IC_{50}$ 6.25 μg/ml and activity on cytotoxicity against to tumer cells; HEP-G2, SW620, KATO-3 and BT474 with $IC_{50}$ 6.8, 5.5, 7.4 and 6.8 μg/ml, respectively. Diglyceride (1001.4 mg/ml) were isolated from mycelium extracts culture broth. These driglyceride consists of five fatty acid, myristic acid (0.40%), palmitic acid (27.21%), stearic acid (16.42%), oleic acid (37.49%) and linoleic acid (17.26%) with weight value of 4.0, 272.5, 164.4, 375.4 and 172.8 mg/L culture broth, respe ctively.

Student's signature.....

Field of study..Biotechnology.....Advisor's signature.....

Academic year..2004.....Co-advisor's signature.....

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| $[\alpha]_D^{20}$ | = Specific rotation at 20 $^0$C and Sodium D line (589 nm) |
| ATCC | = American Type Culture Collection, Maryland, U.S.A. |
| °C | = degree Celsius |
| $^{13}$C-NMR | = carbon-13 nuclear magnetic resonance |
| cm | = centimeter |
| COSY | = $^1$H-$^1$H correlation spectroscopy |
| $\delta$ | = chemical shift |
| DEPT | = distortion enhancement by polarization transfer |
| d | = doublet (for NMR spectral data) |
| dd | = doublet of doublet (for NMR spectral data) |
| DMSO-d6 | = deuterated dimethylsulphoxide |
| dt | = doublet of triplets (for NMR spectral data) |
| $\varepsilon$ | = molar absorptivity |
| EIMS | = electron impact mass spectroscopy |
| eq | = equatorial |
| EtOAc | = ethylacet ate |
| g | = gram |
| HMBC | = $^1$H-$^{13}$C heteronuclear correlation |
| HMQC | = $^1$H-detected heteronuclear multiple quantum coherence |
| $^1$H-NMR | = proton nuclear magnetic resonance |
| Hz | = hertz |
| IR | = infared spectroscopy |
| L | = liter |
| $\mu$l | = microliter |
| $\lambda_{max}$ | = wavelength of maximum absorption |
| $[M+H]^+$ | = protonated molecular ion |
| m | = multiplet (for NMR spectral data) |
| MeOH | = methanol |
| MIC | = minimum inhibitory concentration |

mg = milligram
μg = microgram
MHz = megaheartz
ml = milliliter
mm = millimeter
NMR = nuclear magnetic resonance
ppm = part per million
s = singlet (for NMR spectral data)
SEM = scanning electron microscope
t = triplet (for NMR spectral data)
TLC = thin layer chromatography
UV = ultraviolet
MCzA = malt czapek agar
MCzB = malt czapek broth
MEA = malt extract agar
PDA = potato dextrose agar
PDB = potato dextrose broth
SDA = sabuoraud dextrose agar
YES = yeast extract sucrose agar
NA = nutrient agar
NB = nutrient broth
cfu = colony forming unit
pfu = plaque forming unit
$OD_{625}$ = wavelength at 625 namometer

# CHAPTER I
# INTRODUCTION

The advent of the development of drug resistance in human pathogenic bacteria among such microbes as *Staphylococcus* sp., *Mycobacterium tuberculosis*, *Streptococcus* sp. and others has prompted a search for more and better antibiotic. Together with this is increasing need for more and better antimycotics, especially as the human population is developing more fungal infections as a result of the AIDS epidemic and the increased numbers of patients with organ transplants , whole immune systems weakened. In addition, the world's arsenal is not large for the treatment of parasitic protozoan infections, e.g. malaria (Strobel, 2003). Increased efforts are therefore needed to develop and search for new drugs from natural bioresearches. Of the dug-producing microbes employed for pharmaceutical industry, fungi are probably not only the most important, but also the most poorly studied organism. Fungi play important role in biodiversity because they have world wide distribution and successfully exploit many different habitats (Hawksworth, 1991). Approximately 64,000 species of fungi have been described and estimated fungi are 1,600,000 species. Fungi have been surveyed in various sources as soil, marine, fresh water, litter, dung and decaying re mains of plants and animals. Living plant is an interesting source for screening of new microorganisms. Fungi from special source like plant may also produce novel compounds possessing biological activities (Charlie and Watkinson, 1994).

Endophytic fungi are microorganisms that commonly live in intercellular spaces of living plant hosts. They may provide their hosts with metabolites and other potentially useful bioactive compounds. The association of these microorganisms with higher plants ranges from mutualistic symbiosis or commensalism to borderline latent pathogenicity. Endophytes can be transmitted from one generation to next through the tissue of host seeds or vegetative propagules. Some grass endophytes appear to be transmited horizontally, external to host tissues, with their aerial spores (Strobel and Long, 1998).

In Thailand, there are few reports of endophytic fungi. Endophytic fungi were isolated from 81 Thai medicine plant species collected from forests in in four geographical regions of Thailand were examined for the presence of endophytic fungi with biological activity (Wiyakrutta *et al*., 2004). Studies by Mekkamol (1998) have shown that the endophytic fungi were isolated from leaves of *Tectona grandis* L. in plantations as well as in nature stands from northern Thailand.

In this research, *Piper betle* Linn. leaf was employed as a plant source of fungi because *P. betle* Linn. is used as thai medicinal plant, having pharmacological activities such as antimicrobial activity to bacteria in the mouth i.e. *Streptococcus viridans*, *S. mutans* and *Staphylococcus aureus*. Essential oils of *P. betle* Linn. contained phenolic compounds such as cavicol, cavibetol, carvacrol eugenol and allipyrocatechol. Thesed compounds are assumed could inhibit foodborne pathogens as well as food spoilage microorganisms (Jenie *et al*., 2001).

Objectives

1. To isolate the endophytic fungi found within healthy mature leaves of *Piper betle* Linn. from Bangkok, Pathumthani, Rayong and Nakornsithammarat Province.
2. To determine biological activities of the extracts from endophytic fungal cultures, including antibacterial and antifungal activities.
3. To identify a selected endophytic fungal isolate by using classification based on morphology and nucleotide sequence of ITS regions of rDNA.
4. To extract, isolate and purify the bioactive compounds of a selected endophytic fungal isolate.
5. To elucidate the structural formula of the isolated bioactive compounds.
6. To evaluate the biological activity of the bioactive compounds obtained.

# CHAPTER II

# REVIEW OF LITERATURE

## 2.1 Association of the endophytic fungi and plant

Endophytic fungi colonize living plant tissues by penetration of fungal hyphae between plants cells or may also grow intracellularly and must obtain nutrient materials through this intimate contact with the host. (Isaac, 1992). Long before the recent interest in endophytic fungal communities, basic methods for study of endophytes had been well established by those studying fungal disease of woody plants and interactions among fungal pathogens and other fungi resident within the same host. What differs about ongoing investigations of endophytic fungi, is that single causal organisms of disease have not been pursued, but all plant tissues have been surveyed on a systematic basis for culturable internal fungi in a manner analogous to ecological investigations of vegetation landscapes. During studies of various host plants, the internal fungi have been sampled in very methodical patterns with the goal of describing the spatial distributions and temporal sequences of these fungal assemblages. Investigators have tried to ascertain what are the underlying anatomical features, developmental attributes, host preferences, environmental and climatic cues, and geographical factors that determine colonization strategies of endophytic fungi (Petrini, 1991). Because of the inherent obstacles to direct observation and identification of fungi inside living plants, probing endophytic fungal communities has depended heavily on observation and correlative data complied from exhaustive isolations from plant tissue. As always, the interpretation of microbial community structure based on isolation organisms produces biased results related to the selective action of the isolation methods and sampling patterns.

The association of fungi with plant rangers from mutualistic symbiosis, or commensalisms to borderline latent pathogen (Strobel and Long, 1998). Results of their interaction are increase capacity of a plant to resist desease and increase survival of plant from natural environment by productin bioactive substance (plant growth promoting, antibacterial, antifungal and insecticidal) to enhance the plant growth.

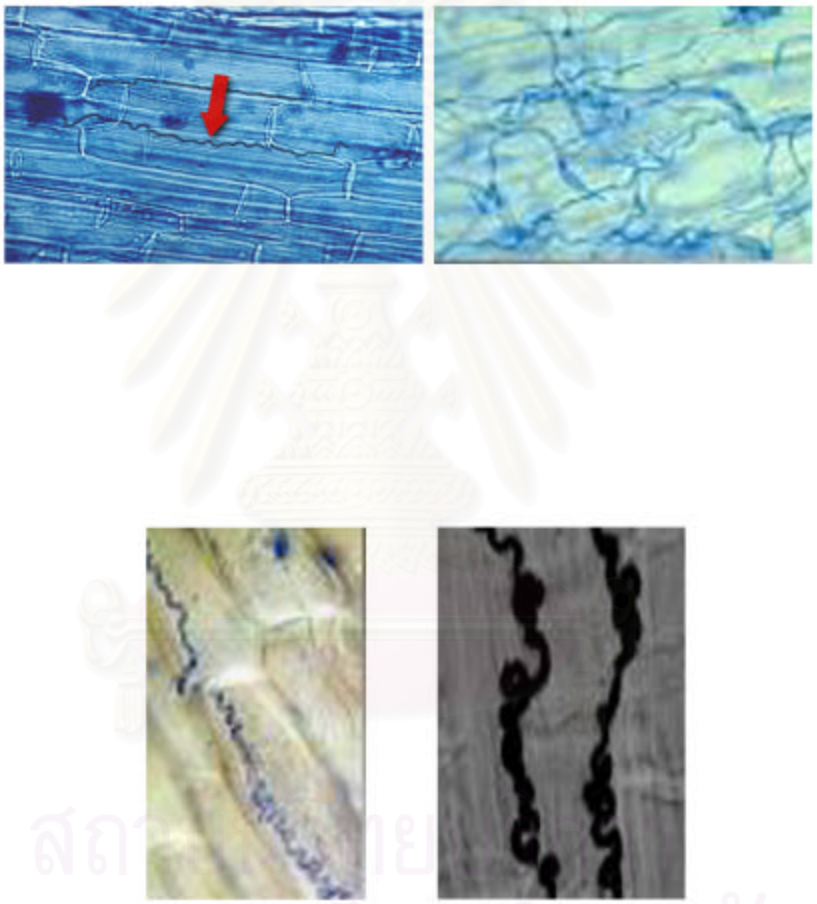


Figure 2.1 View through a microscope at an RTF leaf, showing the individual cells of the leaf. The arrow points at the Endophyte fungus growing between the cells

2.2 Study of secondary metablites from the endophytic fungus

Before 1993, research on fungal endophyte was limited only for identification and classification, until in 1993 Strobel *et al* isolated paclitaxel (Taxol®, anticancer drug) from the endophytic fungus *Taxomyces andreanae* from Pacific yew *Taxus brevifolia*. After that taxol was found from endophytic fungi, *Pestalotiopsis guepinii* from *Wollemia nobilis* (Strobel *et al.*, 1997), *Periconia* sp. from *Torreya grandifolia* (Li *et al.*, 1998), *Pestalotiopsis microspora* from *Taxus wallachina* (Metz *et al.*, 2000, Li *et al.*, 1998), *Tubercularia* sp. from *Taxus mairei* (Wang *et al.*, 2000), *Aspergillus niger* from *Taxus chinensis* (Wang *et al*., 2001), and *Stegolerium kukenani* from *Stegolepis guianensis* (Strobel *et al*., 2001),

The fungal endophyte research has focused on screening of secondary metabolites that exhibited interesting bioactivities such as antifungal, insecticidal, antimicrobial, antimalarial, anticancer, immunosuppressive and antiviral activities. For examples, *Cryptosporiopsis* cf *quercina*, the fungal endophyte isolated from *Tripterygium wilfordii*, a Chinese medicinal plant, produced cryptocandin, a cyclopeptide antifungal (Strobel *et al*., 1999). Tricin, a bioactive flavonoid with insecticidal activities, is a secondary metabolite of *Neotyphodium typhnium* isolated from blue grass (*Poaampla*) (Ju *et al*., 1998). *Phomopsis longicolla*, the endophytic funfus of the endangered mint *Dicerandra frutescens*, was found to produce dicerandrol A, B, C, the xanthones with antimicrobial activities (Wagenaar and Clardy, 2001). 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). Taxol (paclitaxel), the anticancer drug from pacific yew bark, is a secondary metabolite of *Taxomyces andranae* isolated from *Taxus brevifolia* (Strobel *et al*., 1995). Two novel *p*-tridepside antiviral compounds, cytonic acid A and B, were isolated from the endophytic fungus *Cytonaema* sp. Ovtained from *Quercus* sp. (Guo *et al*., 2000). The biological activities, sources, chemical compounds of secondary metabolites from fungal endophyte were summarized in Table 2.2

Table 2.1 The chemical compounds, sources, biological activities of secondary metabolites of endophytic fungi

| No. | Compounds | Endophytic fungi | Host plants | Biological activities | References |
|---|---|---|---|---|---|
| 1 | Taxol | *Taxomyces andreanae* | *Taxus brevifolia* | Anticancer | Strobel *et al.*, 2003, Stierle and Strobel,1995, Stierle *et al.*, 1993, Strobel and Stierle, 1993 |
| | | *Stegolerium kukenani* | *Stegolepis guianensis* | Anticancer | Strobel *et al.*, 2001 |
| | | *Aspergillus niger* | *Taxus chinensis* | Anticancer | Wang *et al.*, 2001 |
| | | *Tubercularia* sp. | *Taxus mairei* | Anticancer | Strobel *et al.*, 2003, Wang *et al.*, 2000 |
| | | *Pestalotiopsis microspora* | *Taxus wallachina* | Anticancer | Strobel *et al.*, 2003, Metz *et al.*, 2000, Li *et al.*, 1998, Strobel *et al.*, 1996 |
| | | | *Taxodium distichum* | Anticancer | Li *et al.*,*1996* |
| | | *Periconia* sp. | *Torreya grandifolia* | Anticancer | Li *et al.*, 1998 |
| | | *Pestalotiopsis guepinii* | *Wollemia nobilis* | Anticancer | Strobel *et al.*, 1997 |

Table 2.1 (continued)

| No. | Compounds | Endophytic fungi | Host plants | Biological activities | References |
|---|---|---|---|---|---|
| 2 | 1,3,5,7 cyclooctatetraene or [8]annulene | *Gliocladium* sp. | *Eucryphia cordifolia* | Antimicrobial | Stinson *et al.*, 2003 |
| 3<br>4 | Lactones 1893 A<br>Lactones 1893 B | Endophytic fungus No. 1893 | *Kandelia candel* | Cytotoxic | Chen *et al.*, 2003 |
| 5 | Pestacin | *Pestalotiopsis microspora* | Rainforest | Antioxidant and antimycotic | Harper *et al.*, 2003 |
| 6 | 7-Butyl-6,8-dihydroxy-3(*R*)-pent-11-enylisochroman-1-one | *Geotrichum* sp. | *Crassocephalum crepidioides* | Antimalarial, antituberculous and antifungal | Kongsaeree *et al.*, 2003 |
| 7 | 7-Butyl-15-enyl-6,8-dihydroxy-3(*R*)-pent-11-enylisochroman-1-one | | | | |
| 8 | 7-Butyl-6,8-dihydroxy-3(*R*)-pentylisochroman-1-one | | | | |

Table 2.1 (continued)

| No. | Compounds | Endophytic fungi | Host plants | Biological activities | References |
|---|---|---|---|---|---|
| 9 | Brefeldin A | *Paecilomyces* sp. and *Aspergillus clavatus* | *Taxus mairei and Torreya grandis* | Cytotoxic | Wang *et al.*, 2002 |
| 10 | Isopestacin | *Pestalotiopsis microspora* | *Terminalia morobensis* | Antifungal and antioxidant | Strobel *et al.*, 2002 |
| 11<br>12<br>13 | Preaustinoid A<br>Preaustinoid B<br>Alkaloid verruculogen | *Penicillium* sp. | *Melia azedarach* | Bacteriostatic | Santos and Rodrigues-Fo, 2002 |
| 14 | Ambuic acid | *Pestalotiopsis* spp., *Monochaetia* sp. | Rainforests | Antifungal | Li *et al.*, 2001 |
| 15<br>16 | Jesterone<br>hydrosy-jesterone | *Pestalotiopsis jesteri* | *Fragraea bodenii* | Antioomycete | Li *et al.*, 2001 |
| 17<br>18<br>19 | Preussomerin G<br>Preussomerin H<br>Preussomerin I | Mycelia sterile | *Atropa belladonna* | Antibacterial, antifungal and antialgal | Krohn *et al.*, 2001 |

Table 2.1 (continued)

| No. | Compounds | Endophytic fungi | Host plants | Biological activities | References |
|---|---|---|---|---|---|
| 20 | Preussomerin J | Mycelia sterile | *Atropa belladonna* | Antibacterial, antifungal and antialgal | Krohn *et al.*, 2001 |
| 21 | Preussomerin K | | | | |
| 22 | Preussomerin L | | | | |
| 23 | Dicerandrols A | *Phomopsis longicolla* | *Dicerandra frutescens* | Antibiotic and cytotoxic | Wagenaar and Clardy, 2001 |
| 24 | Dicerandrols B | | | | |
| 25 | Dicerandrols C | | | | |
| 26 | Microcarpalide | Unidentified endophytic fungus | *Ficus microcarpa* | Microfilament disrupting agent | Ratnayake *et al.*, 2001 |
| 27 | Nomofungin | Unidentified endophytic fungus | *Ficus microcarpa* L. | Microfilament disruptin agent and cytotoxic | Ratnayake *et al.*, 2001 |
| 28 | Isoprenylindole-3-carboxylic acid | *Collectotrichum* sp. | *Artemisia annua* | Antibactirial and antifungal | Lu *et al.*, 2000 |

Table 2.1 (continued)

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

Table 2.1 (continued)

| No. | Compounds | Endophytic fungi | Host plants | Biological activities | References |
|---|---|---|---|---|---|
| 38 | CR377, pentaketide | *Fusarium* sp. | *Selaginella pallescens* | Antifungal | Brady and Clardy, 2000 |
| 39 | Cytochalasin 1 | *Rhinocladiella* sp. | *Tripterygium wilfordii* | Cytotoxic | Wagenaar *et al.*, 2000 |
| 40 | Cytochalasin 2 | | | | |
| 41 | Cytochalasin 3 | | | | |
| 42 | Cytochalasin E | | | | |
| 43 | Cryptocandin | *Cryptosporiopsis* cf. *quercina* | *Tripterigeum wilfordii* | Antimycotic | Strobel *et al.*, 1999 |
| 44 | Geniculol | *Geniculosporium* sp. | *Teucrium scorodania* | Antialgal | Konig *et al.*, 1999 |
| 45 | Cytochalasin F | | | | |
| 46 | Sequoiatones A | *Aspergillus parasiticus* | *Sequoia sempervirens* | Antitumor | Stierle *et al.*, 1999 |
| 47 | Sequoiatones B | | | | |
| 48 | Terpendole M | *Neotyphodium lolii* | *Lolium perenne* | neurotoxins | Gatenby *et al*., 1999 |
| 49 | Tricin (1) | *Neotyphodium typhnium* | *Poa ampla* | Insecticidal | Ju *et al*., 1998 |
| 50 | 7-O-(B-D-glucopyranosyl) tricin | | | | |
| 51 | Isoorientin (3) | | | | |

Table 2.1 (continued)

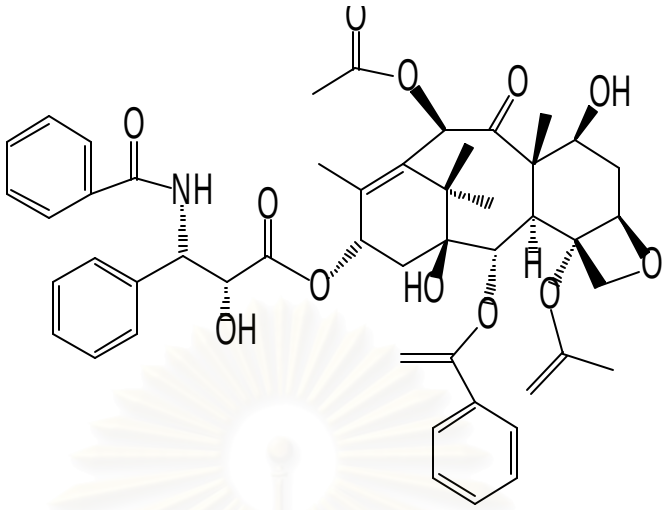
| No. | Compounds | Endophytic fungi | Host plants | Biological activities | References |
|---|---|---|---|---|---|
| 52 | 7-? -[a-L-Rhamnopyranosyl(1-6)-ß-D-glucopy-ranosyl]tricin | *Neotyphodium typhnium* | *Poa ampla* | Insecticidal | Ju *et al*., 1998 |
| 53 | Lolitrem B | *Acremonium lolii* | *Lolium perenne* | Neurotoxic | Berny *et al.*, 1997 |
| 54 | Leucinostatin A | *Acremoium* sp. | *Taxus baccata* | Antifungal and anticacer | Strobel *et al*., 1997 |
| 55<br>56<br>57<br>58 | Oreganic acid (1)<br>Trimethyester (2)<br>Desulfated analog (3)<br>Desulfated analog (4) | Endophytic fungus (MF 6046) | *Berberis oregana* | Anticancer | Jayasuriya *et al.*, 1996 |
| 59<br>60 | Pestalotiopsins A<br>Pestalotiopsins B | *Pestalotiopsis* sp. | *Taxus brevifolia* | - | Pulici *et al*., 1996 |
| 61<br>62 | (R)-mellein<br>(-)-mycorrhizin A | *Pezicula* sp. | Deciduous and coniferous trees | Fungicidal, herbicidal, algicidal and antibacterial | Schulz *et al*., 1995 |

Table 2.1 (continued)

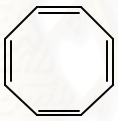
| No. | Compounds | Endophytic fungi | Host plants | Biological activities | References |
|---|---|---|---|---|---|
| 63 | 2-methoxy-4-hydroxy-6-methoxymethyl-benzaldehyde | *Pezicula* sp. | Deciduous and coniferous trees | Fungicidal, herbicidal, algicidal and antibacterial | Schulz *et al.*, 1995 |
| 64 | (+)-cryptosporiopsin | | | | |
| 65 | 4-epi-ethiosolide | | | | |
| 66 | Altersolanol A | *Phoma* sp. | *Taxus wallachiana* | Antibacterial | Yang *et al.*, 1994 |
| 67 | 2-hydroxy-6-methylbenzoic acid | | | | |
| 68 | Preussomerin D | *Hormonema dematioides* | Conifer wood | Antifungal | Polishook *et al.*, 1993 |
| 69 | Lolitrem C | *Acremonium lolii* | *Lolium perenne* | Neurotoxic and insect antifeedant | Rowan *et al.*, 1993 |
| 70 | Peramine R=H | | | | |
| 71 | Diacetylperamine R=Ac | | | | |
| 72 | Paxilline | | | | |
| 73 | Loline alkaloid | | | | |
| 74 | Ergovaline | | | | |

Table 2.1 (continued)

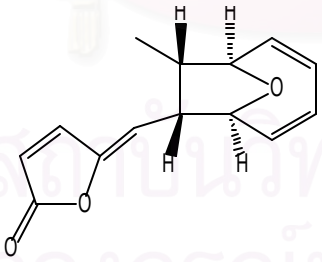
| No. | Compounds | Endophytic fungi | Host plants | Biological activities | References |
|---|---|---|---|---|---|
| 75 | Lysergic acid | *Acremonium coenophialun* | *Festuca arundinacea* | Toxin | Garner *et al.*, 1993 |
| 76 | Isolysergic acid | | | | |
| 77 | Pospalic acid | | | | |
| 78 | Lysergol | | | | |
| 79 | Lysergic acid amide | | | | |
| 80 | Lysergic acid diethyl-amide | | | | |
| 81 | Lycergic acid-2-propanolamide or (Ergonovine) | | | | |



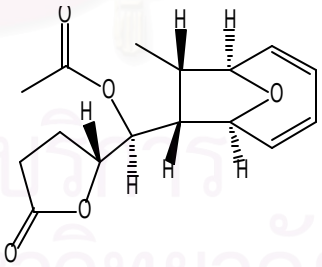
[1] Taxol



[2] 1,3,5,7 cyclooctatetraene or (8)-annulene

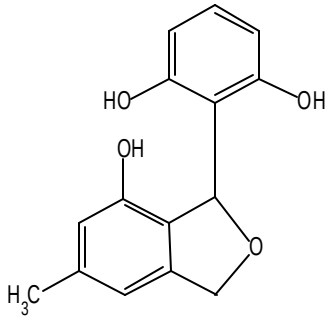


[3] Lactones 1893 A

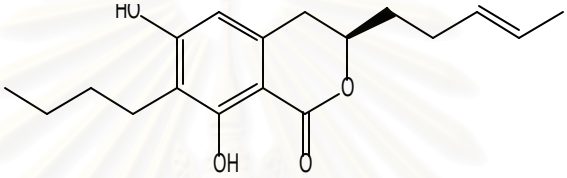


[4] Lactones 1893 B

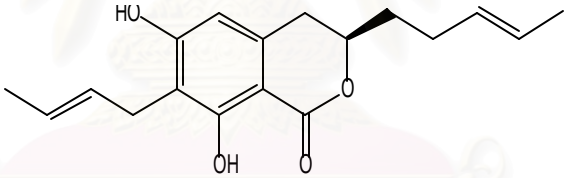
Figure 2.2 Structure of secondary metabolites of endophytic fungi



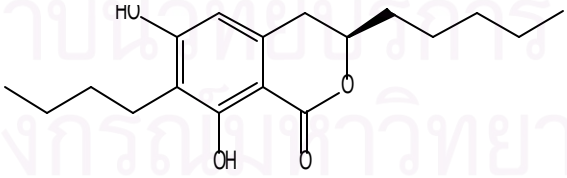

[5] Pestacin




[6] 7-Butyl-6,8-dihydroxy-3(R)-pent-11-enylisochroman-1-one



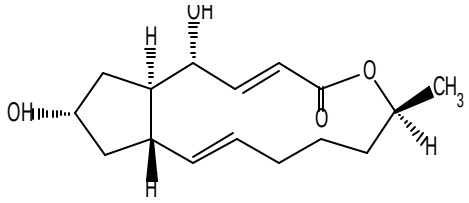

[7] 7-Butyl-15-enyl-6, 8-dihydroxy-3(R)-pent-11-enylisochroman-1-one



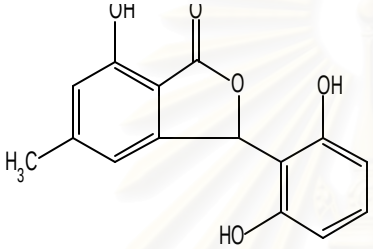

[8] 7-Butyl-6, 8-dihydroxy-3(R)-pentylisochroman-1-one

Dihydroisocumarins [6-8]

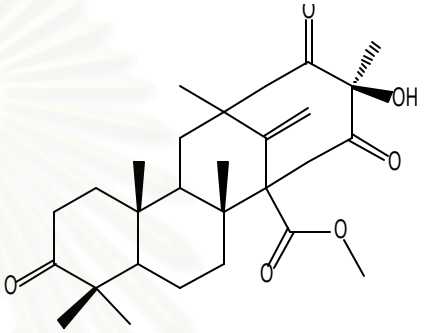
Figure 2.2 (continued)



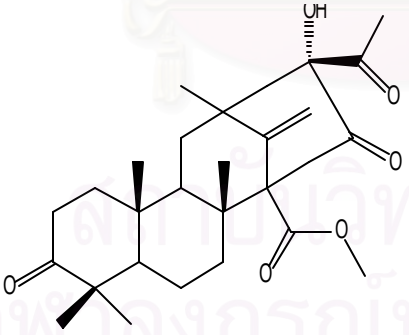
[9] Brefeldin A



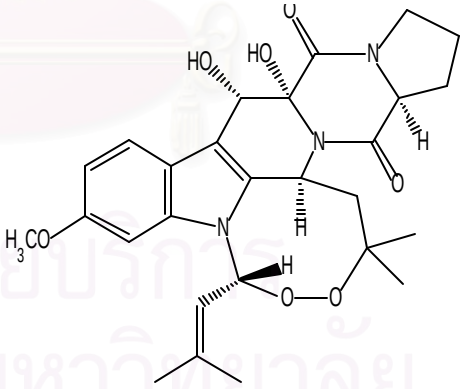
[10] Isopestacin



[11] Preaustinoid A

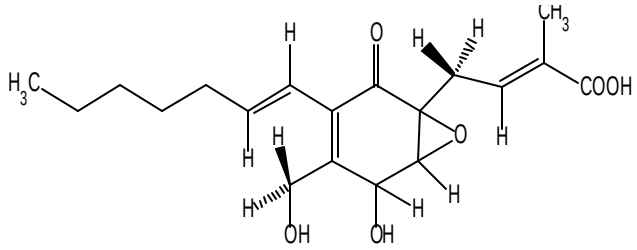


[12] Preaustinoid B

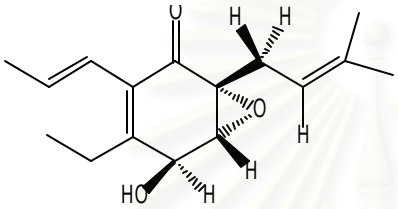


[13] Alkaloid verruculogen

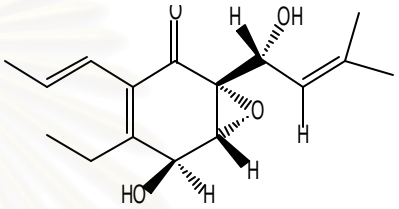
Figure 2.2 (continued)



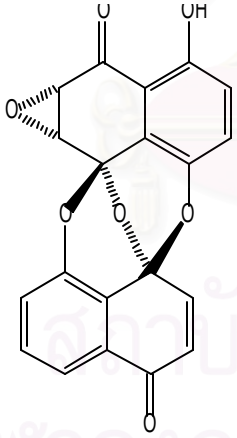
[14] Ambuic acid



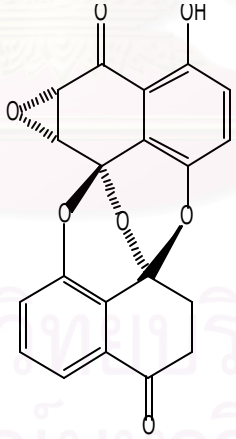
[15] Jesterone



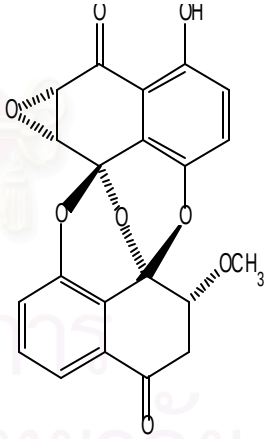
[16] Hydroxy-jesterone



[17] Preussomerin G

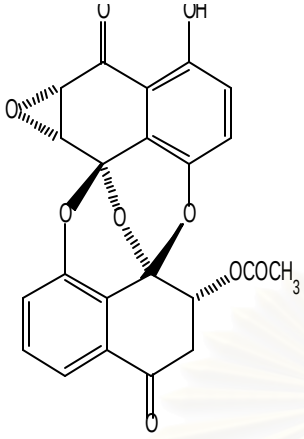


[18] Preussomerin H

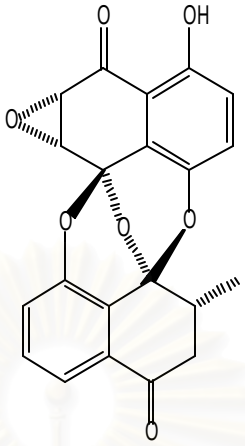


[19] Preussomerin I

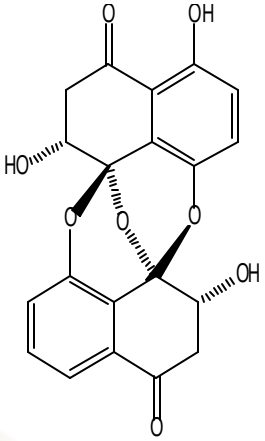
Figure 2.2 (continued)



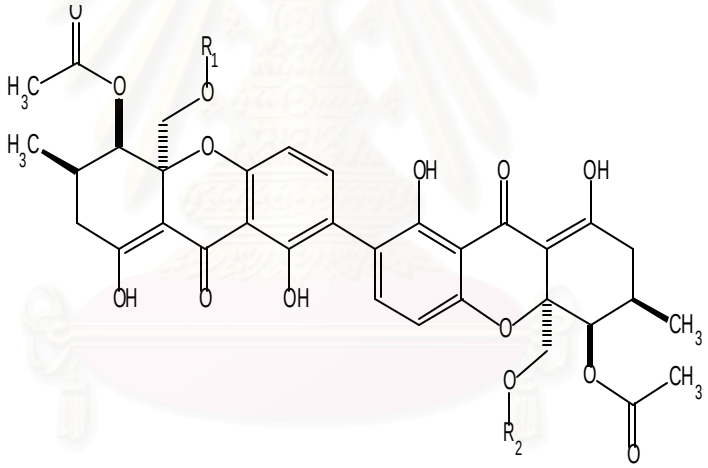
[20] Preussomerin J



[21] Preussomerin K



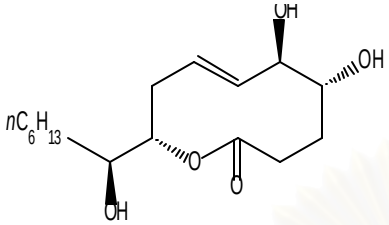
[22] Preussomerin L



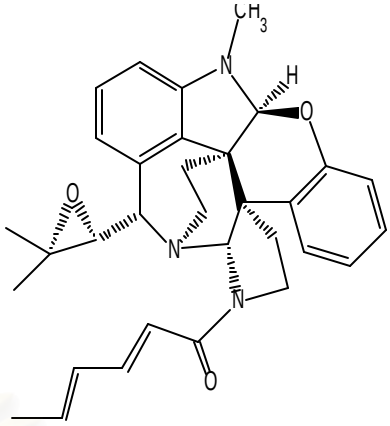
[23] Dicerandrols A, R1=R2=H
[24] Dicerandrols B, R1=Ac, R2=H
[25] Dicerandrols C, R1=R2=Ac



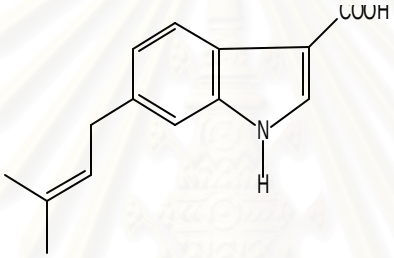
Figure 2.2 (continued)



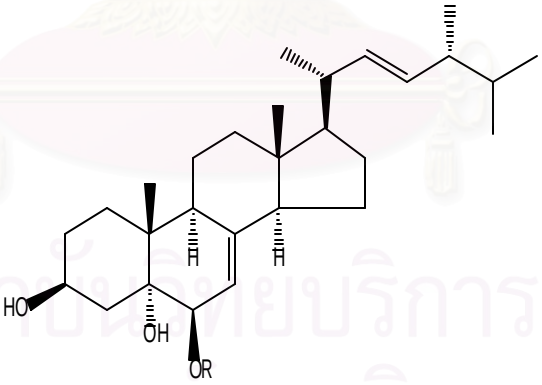
[26] Microcarpalide



[27] Nomofungin



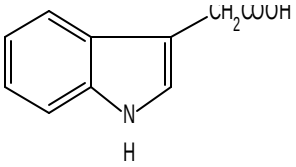
[28] Isoprenylindole-3-carboxylic acid



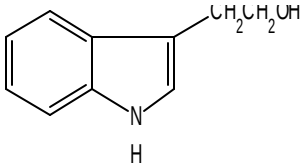
[29] 3beta, 5alpha-Dihydroxy-6beta-acetoxy-ergosta-7, 22-diene, R=$COCH_3$

[30] 3beta, 5alpha-Dihydroxy-6beta-phyenylacetyloxy-ergosta-7, 22-diene, R=$COCH_2C_6H_5$

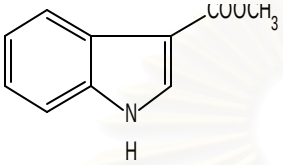
Figure 2.2 (continued)



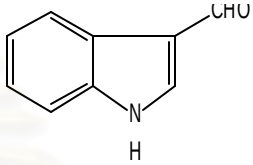
[31] Indole-3-acetic acid (IAA)



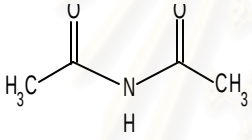
[32] Indole-3-ethanol (IEtOH)



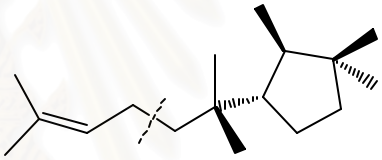
[33] Methylindole-3-carboxylate



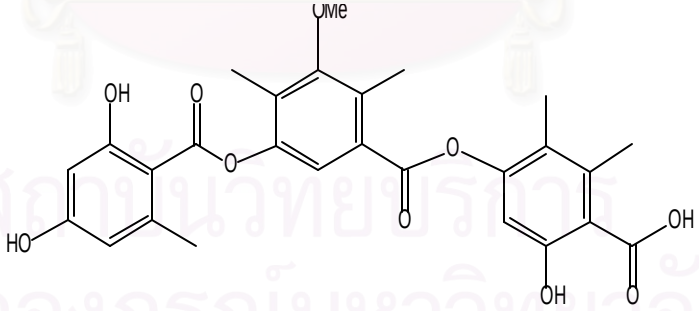
[34] Indole-3-carboxaldehyde



[35] Diacetamide

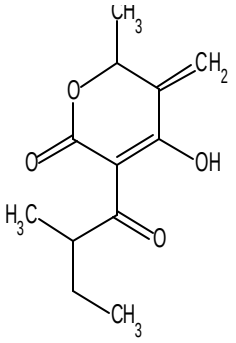


[36] Cyclonerodiol

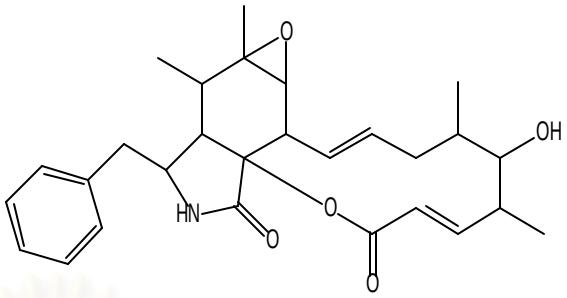


[37] Colletotric acid

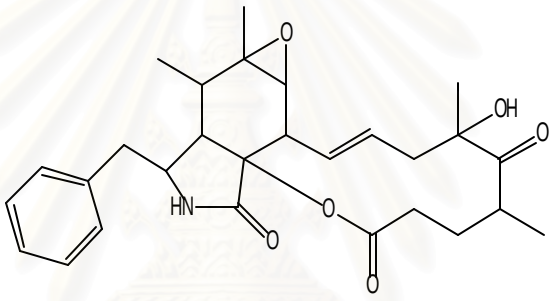
Figure 2.2 (continued)



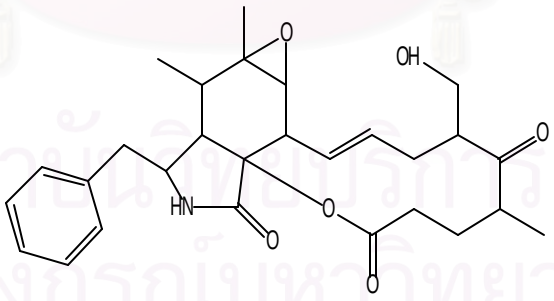

[38] CR377, pentaketide




[39] Cytochalasin 1

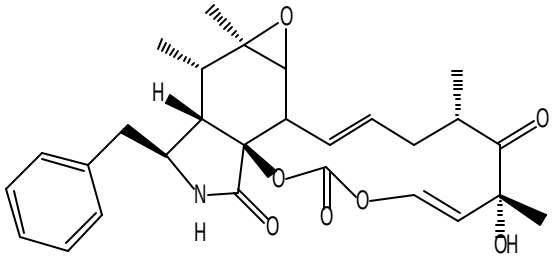



[40] Cytochalasin 2

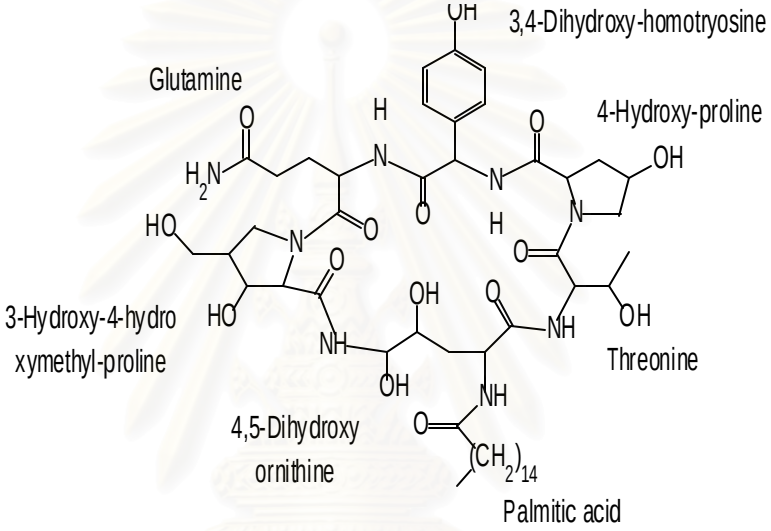



[41] Cytochalasin 3

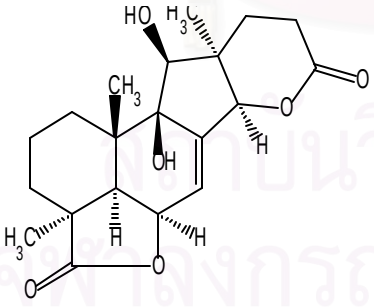
Figure 2.2 (continued)



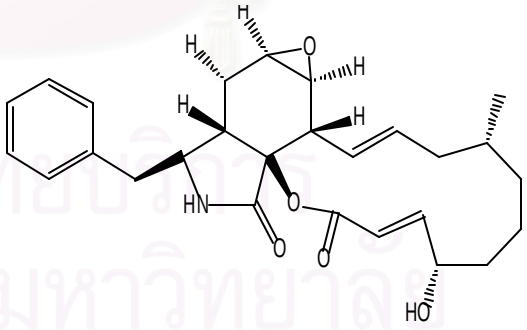
[42] Cytochanlasin E




[43] Cryptocandin

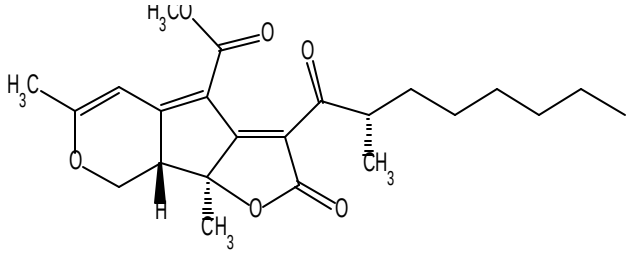


[44] Geniculol

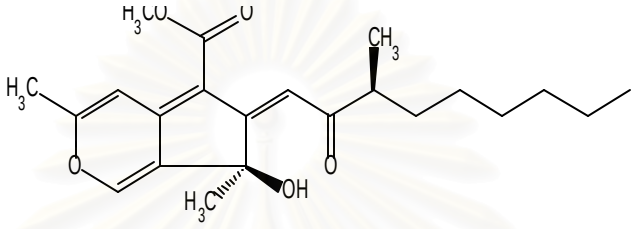


[45] Cytochalasin F

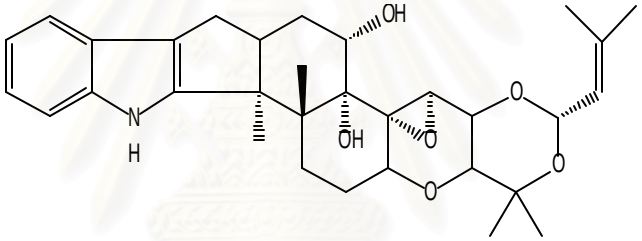
Figure 2.2 (continued)



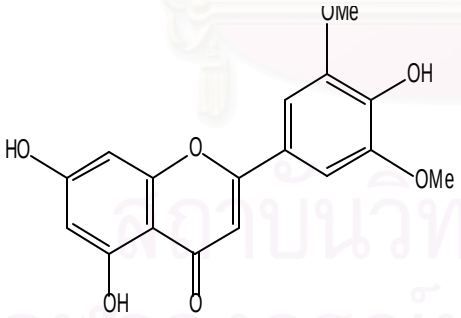
[46] Sequoiatones A



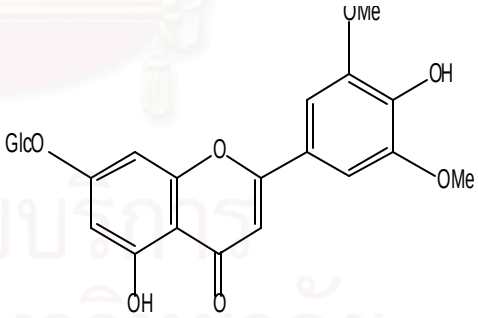
[47] Sequoiatones B



[48] Terpendole M

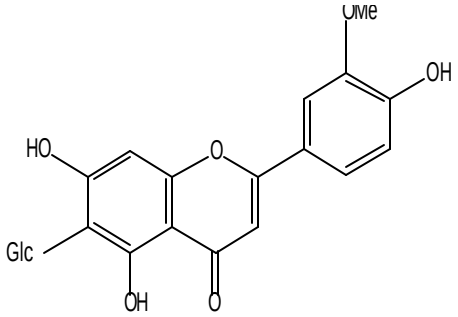


[49] Tricin

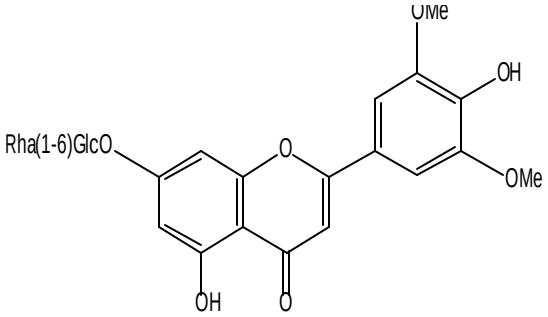


[50] 7-O-(B-D-glucopyranosyl)tricin

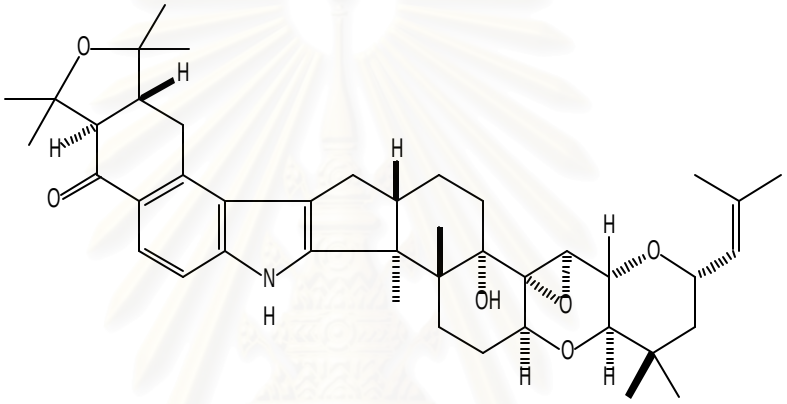
Figure 2.2 (continued)



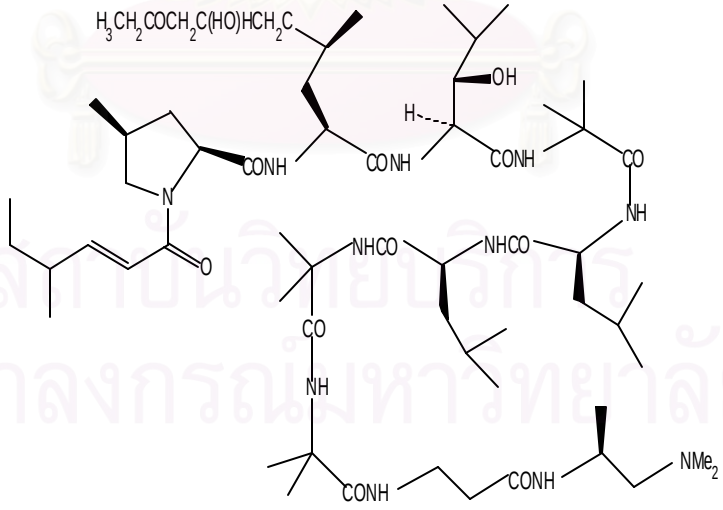

[51] Isoorientin




[52] 7-?-[a-L-Rhamnopyranosyl(1-6)-ß-D-glucopy-ranosyl]tricin

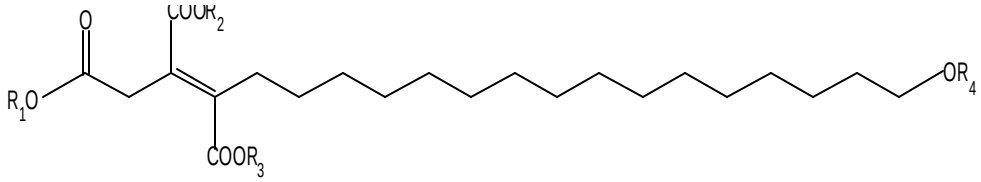



[53] Lolitrem B

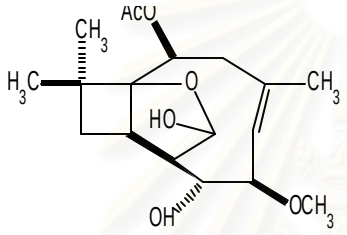



[54] Leucinostatin A

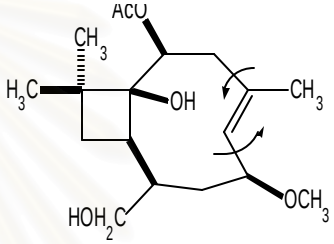
Figure 2.2 (continued)



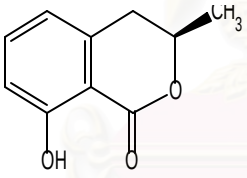
[55] 1: Oreganic acid, R1=R2=R3=H, R4=SO3H
[56] 2: Trimethyester, R1=R2=R3=CH3, R4=SO3H
[57] 3: Desulfated analog, R1=R2=R3=CH3, R4=H
[58] 4: Desulfated analog, R1=R2=R3=R4=H



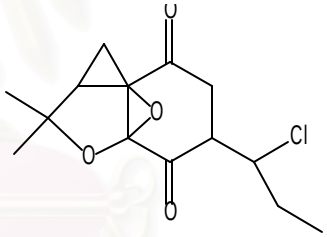
[59] Pestalotiopsins A



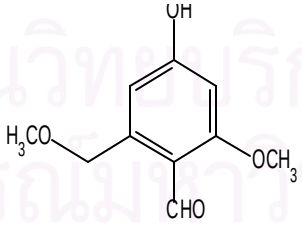
[60] Pestalotiopsins B



[61] (R)-mellein

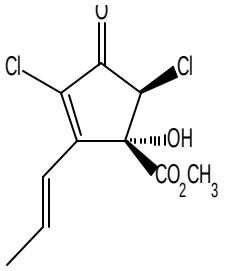


[62] (-)-mycorrhizin A

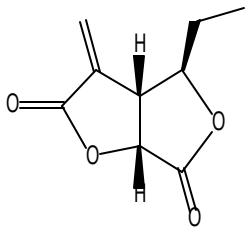


[63] 2-methoxy-4-hydroxy-6-methoxymethyl-benzaldehyde

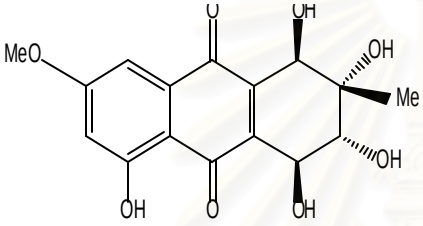
Figure 2.2 (continued)



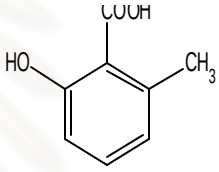
[64] (+)-cryptosporiopsin



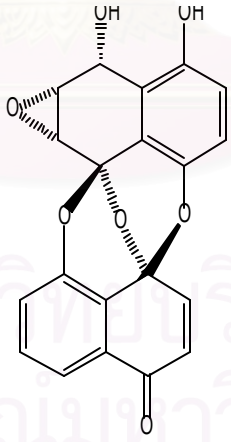
[65] 4-epi-ethiosolide



[66] Altersolanol A

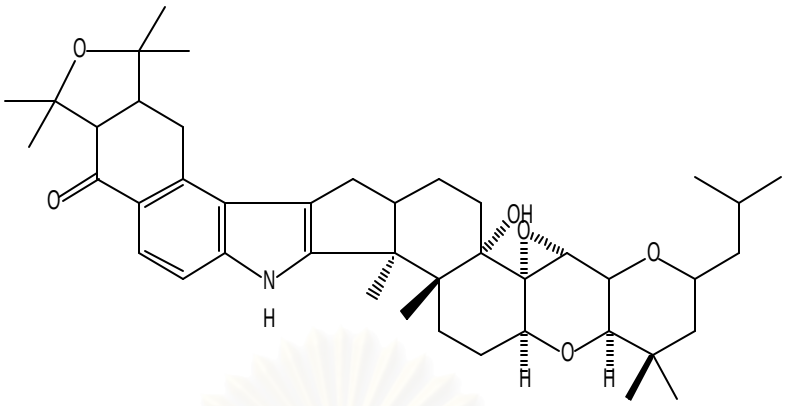


[67] 2-hydroxy-6-methyl benzoic acid

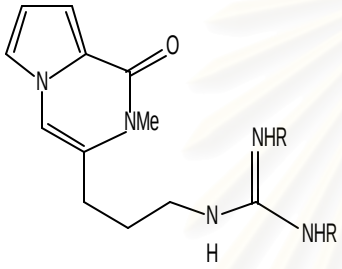


[68] Preussomerin D

Figure 2.2 (continued)

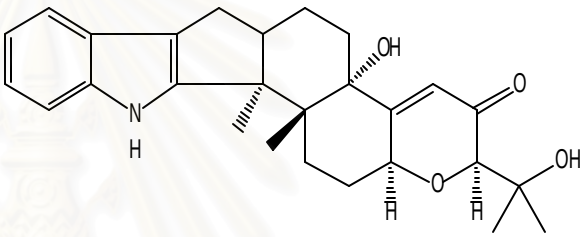


[69] Lolitrem C

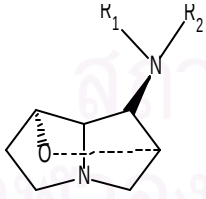


[70] Peramine R=H

[71] Diacetylperamine R=Ac

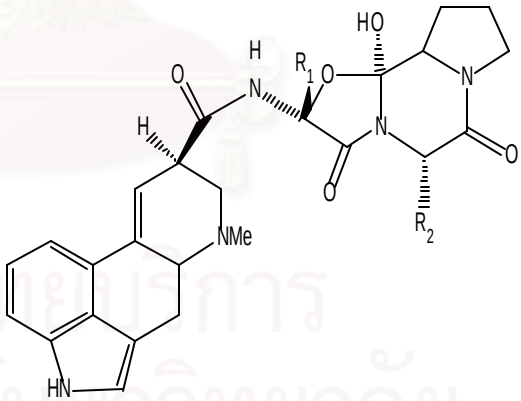


[72] Paxilline



[73] Loline alkaloid, $R_1$=H, Me,

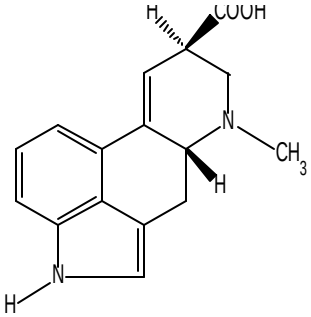
$R_2$= H, HCO, Ac



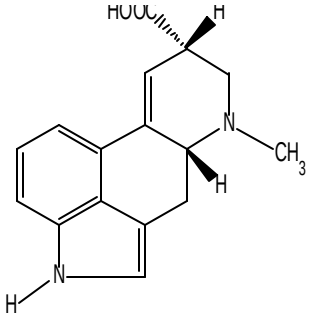
[74] Ergopeptine alkaloids

Ergovaline $R_1$=Me, $R_2$=i-Pr

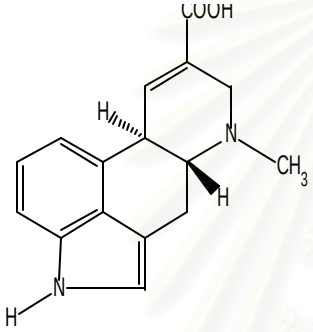
Figure 2.2 (continued)



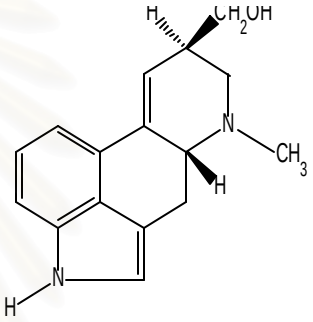
[75] Lysergic acid



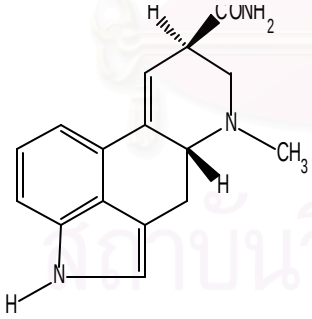
[76] Isolysergic acid



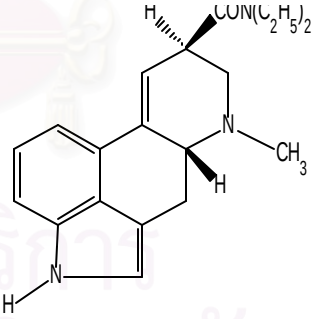
[77] Paspalic acid



[78] Lysergol

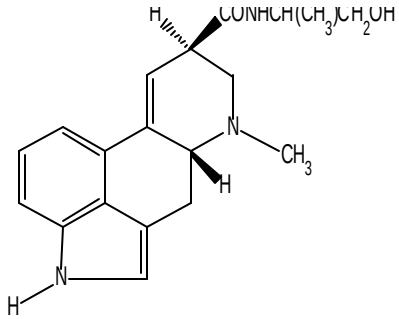


[79] Lysergic acid amide



[80] Lysergic acid diethyl amide

Figure 2.2 (continued)



[81] Lysergic acid-2-propanolamide (Ergonovine)

Figure 2.2 (continued)

2.3 Plant soure of endophytic fungi

*Piper betle* Linn. Piperaceae that Common Name is betel vine or phlu in Thai name. *P. betle* Linn. require a cool climate and high humidity during their life span of 2 - 3 years. If the plants are exposed to extreme heat, the leaves become dark green and brittle. If a cool climate and shade are created in the garden, the leaves will be light green. Woody climber with adventitious roots at swollen nodes. Leaf simple, alternate, cordate, 8-12 cm wide, 12-16 cm long, with description odor and spicy taste. Inflorescence in axillary spike; flowers unisexual, white. Fruit globose berry. It is often chewed in combination with the betel nut (*Areca catechu),* as a stimulatory. Extract of *P. betle* Linn. was essential oils that contained phenolic compounds such as cavicol, cavibetol, carvacrol eugenol and allipyrocatechol,it acts as a stimulant upon the central nervous system and produces a mild euphoria.It also contains an alkaloid called arecoline, which can usually due to excessive or immoderate use over a long period of time produce squamous cell carcinoma of the mouth, a form of skin cancer. Frequent use also stains the mouth, gums and teeth deep red, caused by the added catechu gum (Jenie *et al*., 2001)., http://www.itmonline.org/arts/kava.htm and http://www.fftc.agnet.org/library/data/rh/rh2003004a/rh2003004a.pdf

.*P. betle* Linn. is shown in Figure 2.2 and samples of phenolic compounds from *P. betle* Linn. is shown in Figure 2.3

Figure 2.3 *Piper betle* Linn.

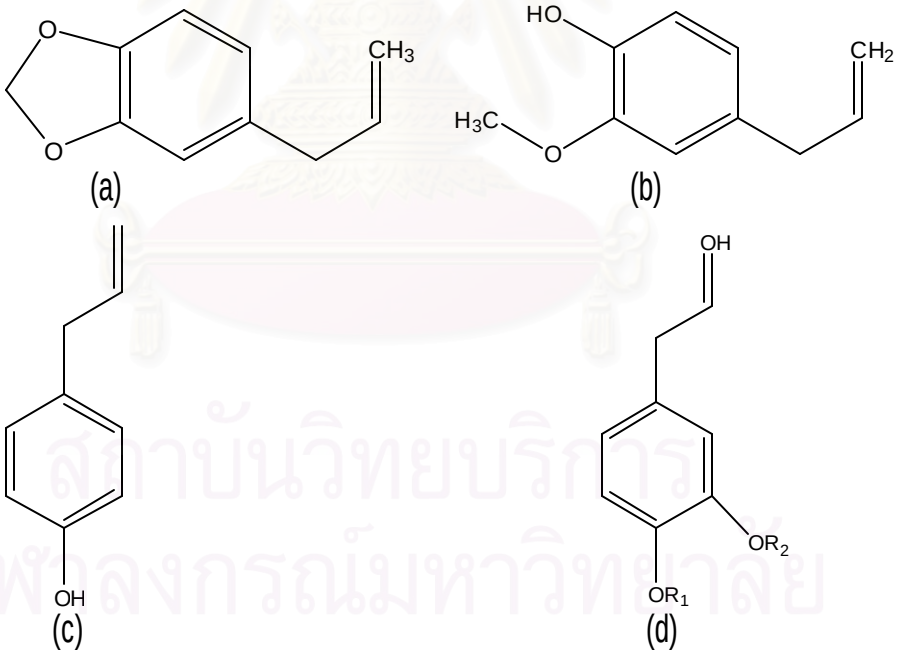



Figure 2.4 Phenolic compounds from *Piper betle* Linn., (a) safrol, (b)eugenol, (c)cavicol and (d) allpyrocatechol

# CHAPTER III

# MATERIALS AND METHODS

## 3.1 Source of plant sample

Mature healthy leaves of *Piper betle* Linn. were collected from Bangkok, Pathumthani, Rayong and Nakornsithammarat Province. The leaf samples were kept in a plastic bag. Fresh specimens were processed with in 24 hours of collection.

## 3.2 Culture media

Culture medium used for isolation, cultivation, and morphology observation of endophytic fungi Potato Dextrose Agar (PDA) was used as.

The medium for growing bacteria was nutrient medium (agar and broth). The formula for media is shown in Appendix A.

## 3.3 Isolation and culture of endophytic fungi

The leaf samples were washed in running tap water and dried in laminar air flow. From each leaf, the leaf was 1 $cm^2$ segments from the middle lamina (midway between the petiole and leaf tip and midway between the midvein and margin) leaf segments were surface in 95% ethanol for 1 min, in sterilized by sequential immersion Clorox® (5% available chlorine) for 5 min, in rinsed twice with sterile distilled water and allowed to surface-dry on sterile filter paper (Blodgett et al, 2000).

The sterile leaf segments were placed in petri dishes containing Potato Dextrose Agar (PDA). The Petri dishes were incubated at room temperature (25-30 $^oC$) and examined periodically for fungi mycelium from leaves under stereomicroscope. Outgrowing mycelia were purified and transferred into Petri dishes containing PDA. They were incubated for 14 days at room temperature and purity was determined by colony morphology. Fungal isolates were used for further study.

3.4 Determination of antimicrobial activities for screening of the isolate endophytic fungi

Screening of the isolated endophytic fungi for antimicrobial activities against test microorganisms were determined by using modifies agar diffusion method as described by Weaver, Angel, and Botlomley (1994) and Joseph, Dave, and Shah (1998).

3.4.1 Screening for antimicrobial activities

The tested microorganisms for antimicrobial activities are listed in Table 3.1

Table 3.1 Test microorganisms were used for antimicrobial activities

| Type of microorganisms | Reference strains |
|---|---|
| Tested bacteria | |
| Gram positive rod bacterium | *Bacillus subtilis* ATCC 6633 |
| Gram positive cocci bacterium | *Staphylococcus aureus* ATCC 25923 |
| Gram negative rod bacterium | *Escherichia coli* ATCC 25922 |
| Gram negative rod bacterium | *Pseudomonas aeruginosa* ATCC 27853 |
| Pathogenic yeast | *Candida albicans* ATCC 10231 |

3.4.2 Screening by isolated endophytic fungi for antimicrobial activities

A. Preparation of isolated endophytic fungi for antimicrobial activities testing

The isolated endophytic fungi were grown at room temperature (25-30 ◦C) for 2 week, in Malt Czapek Agar (MCzA), Malt Extract Agar (MEA), Saborouad Dextrose Agar (SDA) and Yeast Extract Sucrose Agar (YES). A small piece ( 7 mm diameter disks using a flamed cork hole borer) of the grown fungus were subjected for antimicrobial activities.

B. Preparation of bacterial inoculum for testing of antimicrobial activities

Bacteria were grown on Nutrient Agar (NA) for 24 hr at 37 ◦C. Single colonies were inoculated in to 5 ml of Nutrient broth (NB) and incubated at 37 ◦C for 2-6 hr, depending on the growth rate. The turbidity of the bacterial suspension was adjusted with NB to match the turbidity of a 0.5 McFarland ($OD_{625}$ = 0.1).

C. Preparation of yeast inoculum for testing of antimicrobial activities

Yeasts were grown on SDA for 24 h at room temperature (25-30 ◦C). Selected fresh single colonies were inoculated into 5 ml of SDA and incubated at room temperature for 2-3 hr, depending on the growth rate. The turbidity of the yeast suspension was adjusted with SDB to match the turbidity of a 0.5 McFarland ($OD_{625}$ = 0.1).

D. Inoculation of the tested plate

Sterile cotton applicators were immersed in the inoculum suspension and pressed lightly against the tube wall to remove excess moisture. Culture media NA and SDA were inoculated test microorganism by streaking across the entire surface. This was repeated twice, turning the plate 60 degree between each streaking. The surface of the medium was allowed to dry for 3-5 minutes.

E. Determination of activities against the bacterial and fungi of isolate endophytic fungi

The isolated endophytic fungi were grown at room temperature (25-30 ◦C) for 2 week on plate containing MCzA, MEA, SDA and YES. A small piece ( 7 mm diameter disks using a flamed cork hole borer) of the grown fungus was placed on NA and SDA previously inoculated with a test microorganism. After leaving at room temperature 1 h the bacterial and fungi plates were incubated at 37 ◦C for 24 hr. Clear zone due to inhibition of growth of the test microorganism around the endophytic fungus indicated its activity against the bacterial and fungi.

3.5 Identification of endophytic fungi

Fungal isolated, which has the best activity from screening test, was chosen for identification of fungi.

3.5.1 Morphological identification

A. Microscopical features

The microscopic analyses were based on morphological structure by light microscopy on an Olympus CH2 research microscope using a 40x dry objective. Specimens were mounted in lactophenol-cotton blue for observations of spores and other characteristics, and then identified. Identification of the isolate fungi were done as descried by Barnett and Hunter (1998), Ellis (1971), Subramanian (1971), and Sutton (1980).

B. Macroscopical features

Colony characteristic of specimens such as shape, size, color, margin, pigment, and others were studied using stereomicroscope (Leica model MZ6).

3.5.2 Molecular Identification

Sequences of internal transcribed spacer (ITS) regions of rDNA (Figure 3.1) from isolated endophytic fungi were sent for identification by molecular methods at the Asian Natural Environmental Science Center, The University of Tokyo, Japan.

A. DNA extraction

Genomic DNA was prepared from the fresh mycelium by homogenization in 1.5 ml tubes with a FastPrep FP120 homogenizer (Savant, faxmingdale, NY, USA) and followed by extraction with cetyltrimethylammonium bromide (CTAB) as described in Zhou et al. (1999). Fungal DNA extract was applied in CTAB buffer (2% CTAB, 0.1 M

Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl and 0.5% 2-mercaptoethanol) at 65 ◦C for 1 h, extracted with chloroform-isoamyl alcohol (25:24:1, v/v) then extracted with phenolchloroform-isoamyl alcohol mixture (24:1, v/v) twice. Fungal DNA was precipitated with isopropanol and centrifuged at 8000 rpm for 5 min. Fungal DNA was dissolved in 100 μl TE buffer (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA) and kept at -30 ◦C for further study.

B. ITS amplification

The ITS region of isolated endophytic fungus was amplified with the primers ITS1f (Gardes and Bruns, 1993), and ITS4 (White et al., 1990). Twenty microliters of reaction mixture contained 5 ng template DNA, 0.2 mM each dNTP, 1xPCR buffer, 1.5 mM Mg2, 0.5U Ampli Taq Gold (Ampli Taq Gold kit; Ferkin Elmer, Branchburg, NJ, USA) and 0.5 μM of the primer pair. The amplification reactions were performed in a thermal cycler (TP 3000; Takara Shuzo, Tokyo, Japan). Amplification was started at 94 ◦C for 9 min, followed by 38 cycles of a denaturing step at 94 ◦C for 1 min, an annealing step at 51 ◦C for 1 min, and an extension step at 72 ◦C for 1 min, and ended with an additional 5-min extension step at 72 ◦C (Kanchanaprayudh et al., 2003).

C. DNA Sequencing

$ITS_{1f-4}$ regions were amplified from the representative sample of isolated endophytic fungus. Amplified $ITS_{1f-4}$ fragments were cloned using pT7 Blue vectors (Novagen, Madison, WI, USA) and transformed into *Escherichia coli* strain XL1-Blue MRF. Legation and transformation were performed according to the manufacturer's protocol. Plasmid DNA was extracted from positive clones and sequenced with a Thermo Sequence Pre-mixed Cycle Sequencing kit (Hitachi) using the T7 and M13 forward primers labeled with Texas Red (Hitachi) in and SQ-5500E sequencer (Kanchanaprayudh et al., 2003).

$ITS_{1f-4}$ sequences were automatically aligned with fungi ITS sequences obtained from GenBank DNA database (http://www.ddbj.nig.ac.jp)

Primers for amplification and sequencing of ITS region ad ITS2 sequence of rRNA gene.

ITS1f CTTGGTCATTTAGAGGAAGTAA

ITS4 TCCTCCGCTTATTGATATG

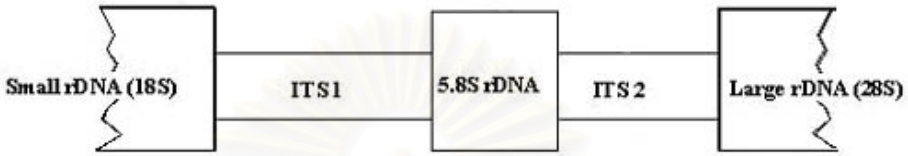



Figure 3.1 ITS regions of rDNA (Kanchanaprayudh et al., 2003)

3.6 Fungal cultivation and metabolite extraction

3.6.1 Fungal cultivation

The endophytic fungal had activity to bacteria or fungi was grown on MCzA at room temperature (25-30 ◦C) for 1 week. The agar culture was then cut into 7 mm diameter disks by a flamed cork hole borer. Five disk was inoculated into 250 ml Erlenmeyer flasks containing 100 ml of Malt Czapek Broth (MCzB) each. The culture was incubated for 3 weeks at room temperature (25-30 ◦C) under static condition.

3.6.2 Metabolite extraction

The culture broths was extracted with EtOAc and dried and kept for bioassays.

3.6.3 Application of cultural condition on diglyceride production with four substrates in Czapek broth and Potato dextrose broth.

The endophytic fungus isolate PBL004 was grown on MCzA at room temperature (25-30 ◦C) for 1 week. The agar culture was then cut into 7 mm diameter disks by a flamed cork hole borer. Five disks were inoculated into 250 ml erlenmeyer flasks containing 100 ml of 5 mediums ; four substrate in czapek broth(rice bran czapek, betel

vine leaves blended czapek, yeast extract czapek, yeast extract and malt extract at 1:1 ratio with czapek) and PDB. The cultures were incubated at room temperature under static for 21 days. The fungal mycelia were extracted with EtOAc. The flasks of fungal mycelium were extracted on sonicate at room temperature for 6 hours. The extracted solvent was filtered through filter paper (Whatman No. 4). The filtrate was then evaporated by using a rotary evaporator (Eyela, N-N series, Japan) at 35 ◦C. Isolation of diglyceride in crude EtOAc from mycelium extract the method is shown in Scheme 3.2

3.7 Determination of metabolites profile of the extracts from endophytic fungi

A few milligrams of the culture broth extracts and mycelium extracts in the section 3.5.2 were dissolved with methanol for determination of metabolites profile of the extracts by using TLC technique that is described in later.

Analytical thin-layer chromatography (TLC)

| | |
|---|---|
| Technique | : one dimension ascending |
| Adsorbent | : silica gel $F_{254}$ coated on aluminium sheet (E. Merck) |
| Layer thickness | : 250 μm |
| Distance | : 5 cm |
| Temperature | : laboratory temperature (25-30 ◦C) |
| Detection | : 1. Visual detection under ultraviolet light at wavelengths 254 and 365 nm |
| | 2. Visual detection in iodine vapour |

3.8 Determination of growth profile of culture filtrate from fungal isolate

Fungal endophyte PBL004 was grown on MCzA at room temperature (25-30 ◦C) for a week. The agar culture was then cut into 7 mm diameter disks by a flamed cork hole borer. Five disks were inoculated into 250 ml Erlenmeyer flasks containing 100 ml of MCzB medium. The cultures were incubated at room temperature (25-30 ◦C) under static for 21 days.

Mycelium dry weights were obtained by harvesting the mycelium on pre-activated (at 80 ◦C for 24 hr), and pre-weighted Whatman no. 1 filter paper. The mycelium was dried at 80 ◦C for 24 hr in an oven and weighted again. The difference between initial and final weight was take as dry weight.

3.9 Fungal cultivation for study metabolites of bioactive compounds

Endophytic fungal isolate PBL004 was chosen for metabolites studies and was cultivated on MCzA at room temperature (25-30 ◦C) for 1 week. The agar culture was then cut into 7 mm diameter disks using a flamed cork hole borer. Five pieces of agar culture were inoculated into 250 ml Erlenmeyer flasks containing 100 ml of malt Czapek broth. The flasks were incubated on static at room temperature for 21 days. Several flasks of culture were prepared to obtain 10 L of MCzB.

3.10 Chromatographic techniques

3.10.1 Analytical thin-layer chromatography (TLC)

| | |
|---|---|
| Technique | : one dimension ascending |
| Adsorbent | : silica gel $F_{254}$ coated on aluminium sheet (E. Merck) |
| Layer thickness | : 250 μm |
| Distance | : 5 cm |
| Temperature | : laboratory temperature (25-30 ◦C) |
| Detection | : 1. Visual detection under ultraviolet light at wavelengths 254 and 365 nm.<br>2. Visual detection in iodine vapour |

3.10.2 Gas Chromatography

Fatty acid were analyzed on 3 mm glass column of 15% DEGS on 60/80 mesh chromasorb S AW at 180 $^{o}$C using Shimadzu 15A Gas Chromatography (Shimadzu Ltd.,Kyoto)

3.11 spectroscopy

3.11.1 UV-VIS spectrometer

UV-VIS spectra were recorded on a Shimacsu UV160A Spectrometer in MeOH.

3.11.2 Fourier Transform infrared Spectrophotometer (FT-IR)

The FT-IR spectra were recorded on a Perkin-Elmer Model 1760X Fourier Transform Infrared Spectrophotometer. Solid samples were generally examined by incorporating the sample with potassium bromide (KBr) to form a pellet. Spectra of liquid samples were recorded as thin film on a sodium chloride (NaCl) cell.

3.11.3 Mass spectra

The mass spectra were recorded on a Fisons Instrument Mass Spectrometer Model Trio 2000 in EI mode at 70 eV.

3.11.4 Nuclear Magnetic Resonance Spectrometer (NMR)

The $^{1}$H-NMR (400 MHz), $^{13}$C-NMR (100 MHz), DEPT, COSY, HMQC, HMBC, NOESY, and TOCSY spectra were recorded on an OxFord Model YH400 spectrometer.

Deuterated solvents; chloroform-*d* ($CDCl_3$) was used in NMR experiments. Reference signals were the signals of residual protonated solvents at $\delta$ 7.30 ppm ($^{1}$H) and 77.0 ppm *t* ($^{13}$C) for $CDCl_3$ and 3.35 ppm ($^{1}$H) and 49.0 ppm *sept* ($^{13}$C) for $CD_3OD$.

3.12 Melting point

Melting points were examined using a Fisher-John melting point apparatus.

3.13 Optical rotation

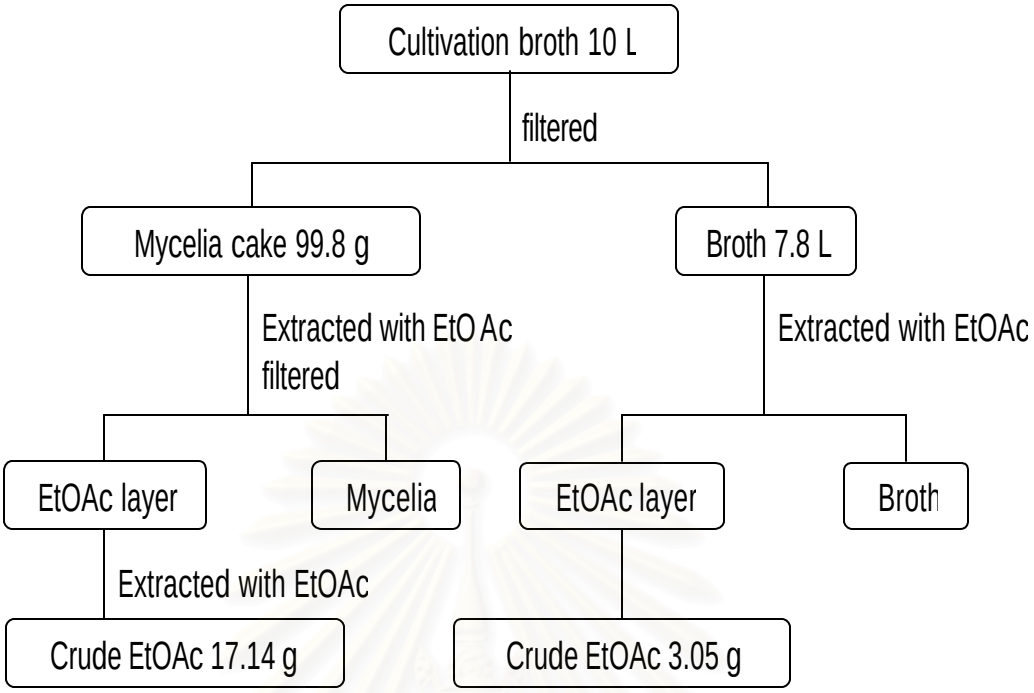
Optical rotations were measured on a Perkin Elmer 341 polarimeter, using a sodium lamp at wavelength 589 nm.

3.14 Solvent

All solvents used in this research such as hexane, chloroform ($CHCl_3$), ethyl acetate (EtOAc) and methanol (MeOH) were commercial grade and were purified prior to use by distillation.

3.15 Extraction

The cultivation broth of Isolate PBL004 10 L was extracted by EtOAc and filtered through a filter paper (Whatman No. 4). The filtrate was extracted by EtOAc. EtOAc layer was colledcted and concentrated to dryness under reduced pressure at 35 ◦C. The crude EtOAc extract from broth was obtained as a mixture of brown solid and dark brown viscous liquid (3.05 g). Mycelia cake was extracted 2 liters EtOAc and filtered through filter paper (Whatman No. 4). The EtOAc layer was collected and concentrated to dryness under reduced pressure at 35 ◦C. The crude EtOAc extract of mycelia cake was obtained as a dark brown oil liquid (17.14 g). The extraction of the cultivation broth and mycelia of the endophytic fungus isolate PBL004 is shown in Scheme 3.1

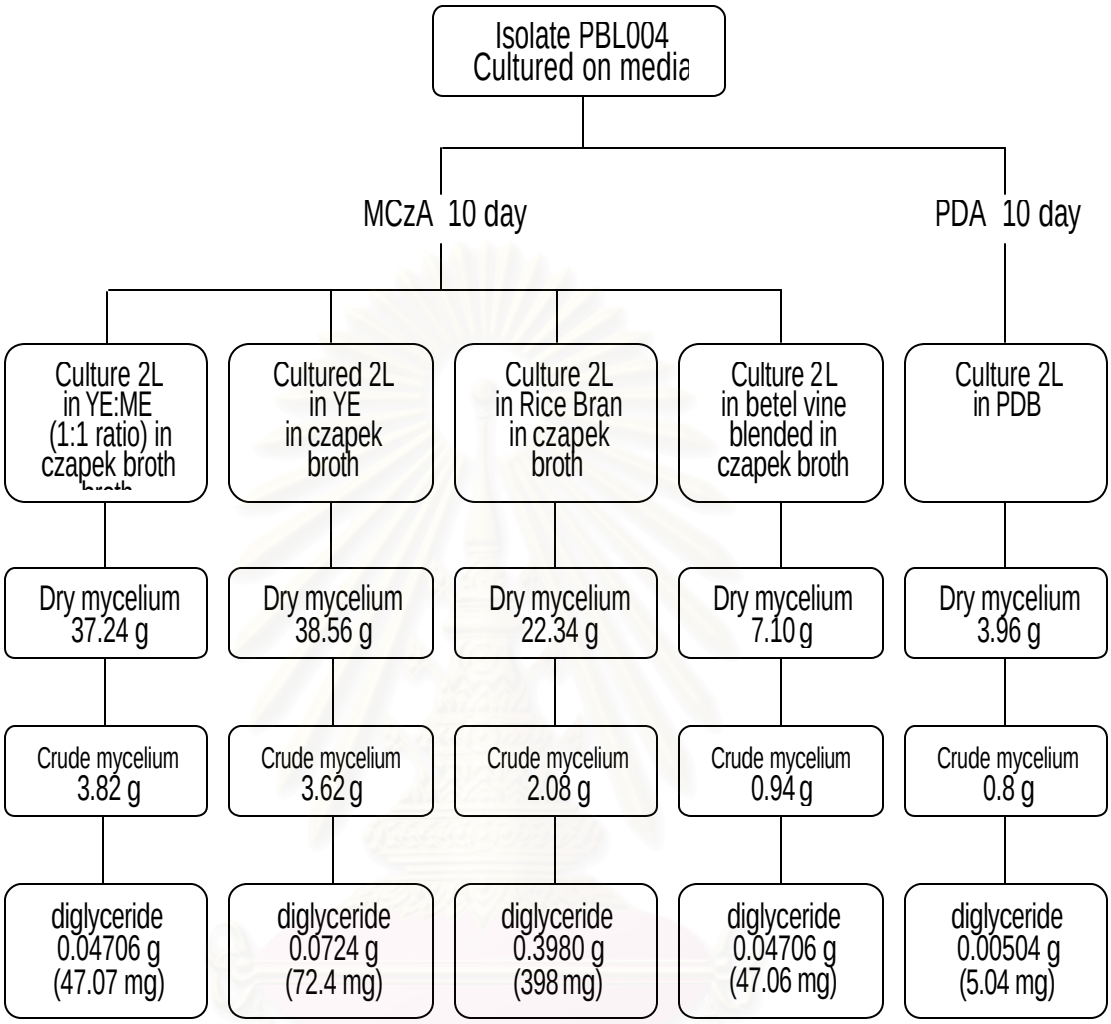



Scheme 3.1 Diagram of crude extraction of culture broth and mycelia of endophytic fungus isolate PBL004

3.16 Isolation of chemical compounds of endophytic fungus isolate PBL004

3.16.1 Isolation of chemical compounds in crude EtOAc from mycelium extract

The crude EtOAc from endophytic fungus isolate PBL004 mycelia extract (6.45 g) was subjected to column chromatography (silica gel, 22 g), using wet packing loading method. Eluents of increasing polarity from hexane to MeOH were used. Gradient elution with hexane, hexane and ether mixtures, ether, ether and MeOH mixtures, and MeOH were used. Fractions (50 ml each) were collected and examined. Fraction combination was by TLC on Silica gel plates with hexane, hexane and ether mixtures, ether, ether and MeOH mixtures, and MeOH as the developing solvent. Fractions with the same TLC pattern were pooled and dried. The results from the isolation of EtOAc crude of mycelia extracts were presented in Table 3.2. The biological activity of each pool fraction was examined and described in Chapter 4.




Remark : YE = Yeast Extract, ME = Malt Extract

Scheme 3.2 Diagram comprise production of diglyceride of endophytic fungal isolate PBL004 cultured in various media ;YE:ME (1:1 ratio) in czapek broth, yeast extract in czapek broth, rice bran in czapek broth , betel vine blended in czapek broth and potato dextrose broth

Table 3.2 Components of separation of EtOAc crude from mycelia extract from isolated PBL004

| Fraction Code | Eluents | Fraction No. | appearance | Weight (mg) |
|---|---|---|---|---|
| M001 | 100% hexane - 5 % ether in hexane | 0-6 | Light yellow oily | 4530 |
| M002 | 5 % ether in hexane | 7-9 | Amorphous white solid in light yellow oily | 40 |
| M003 | 10 % ether in hexane | 10-11 | Yellow Red brawn | 50 |
| M004 | 100 % ether | 12-14 | Red brawn wax | 93 |
| M005 | 100 % ether | 15-23 | Red brawn oily | 206 |
| M006 | 10 % MeOH in ether | 24-31 | brawn oily | 85 |
| M007 | 30 % MeOH ether | 32-55 | Brawn viscous liquid | 130 |

3.16.1.1 Isolation of EtOAc from mycelia extracts of fraction code M001 from isolate PBL004

The EtOAc crud e of mycelia extracts of fraction code M001 4.53 g was separated by column chromatography (silica gel, 14 g) using eluents of increasing polarity from hexane, to 100 % ether. The results from the separation of EtOAc crude of mycelia extracts (fraction code) were presented in Table 3.3.

Table 3.3 The results from isolation of EtOAc crude from mycelia extract isolate PBL 004 (Recolumn of fraction code M001)

| Fraction Code | Eluents | Fraction No. | appearance | Weight (mg) |
|---|---|---|---|---|
| Mr001 | 100% hexane | 0-60 | Colorless oily | 3800 |
| Mr002 | 10 % ether in hexane | 61-77 | Light yellow oily | 550 |
| Mr003 | 100 % ether | 78-86 | Light yellow oily | 220 |

Chemical compound was isolated from the mycelium of the endophytic fungus isolate PBL004. Mixture M1 (58.46% yield of the EtOAc extract ) was obtained from fraction code Mr001 as a colorless viscous liquid. Chemical structure of compound was determined by analyzed of spectroscopic data, FT-IR, $^{1}$H-NMR, $^{13}$C-NMR and GC as well as by comparison their spectral data with those of published values.

Mixture M1 is shown in Figure B 2 – B4 in Appendix B

$^{1}$H-NMR : $\delta$H (ppm), 400 MHz, 5.34 (m, broad), 5.26 (m, broad), 4.29 (dd, *J* = 4.0, 8.0 Hz), 4.14 (m), 2.32 (m), 2.01 (d, *J* = 5.6 Hz), 1.60 (s, broad), 1.28 (m, broad), 0.91 (t, *J* = 8.4 Hz) ppm.

$^{13}$C-NMR : $\delta$C (ppm), 100 MHz, 173.2, 172.8, 130.2, 130.0, 129.9, 129.7, 129.6, 128.1, 127.9, 77.4, 77.1, 77.7, 68.9, 62.1,62.0, 34.17, 34.0, 33.8, 31.9, 31.5, 29.8, 29.7, 29.6, 29.4, 29.3, 29.2, 29.2,29.1, 29.1, 29.0, 28.9, 28.8, 27.2, 27.1, 24.8, 22.7,14.1 ppm.

FT IR : $\upsilon$ max cm$^{-1}$ (Kbr): 3466 (broad), 2925 and 2854 (s), 1743 (s), 1465 (m), 1245 and 757 (w).

### 3.16.2 Isolation of bioactive compounds in crude EtOAc from broth culture extract isolate PBL004

The crude EtOAc from endophytic fungus isolate PBL004 culture broth extract (3.05 g) was subjected to column chromatography (silica gel, 40 g), using dry loading method. Eluents of increasing polarity from hexane and EtOAc mixtures to MeOH were used. Gradient elution with hexane and EtOAc mixtures, EtOAc, EtOAc and MeOH mixtures, and MeOH were used. Fraction (50 ml each) was collected, and examined. Fraction combination was by TLC on Silica gel plate with hexane, hexane and EtOAc mixtures, EtOAc, EtOAc and MeOH mixtures, MeOH as the developing solvent. Fractions with the same TLC pattern were pooled and dried. The results from the separation of EtOAc crude extract of culture broth extracts is presented in Table 3.4.

The EtOAc crude of culture broth extract of fraction code B008 (360 mg) was purified by crystallization with a mixture of EtOAc and hexane. It was dissolved in EtOAc until saturation, and then hexane was added. The solution was left standing at room temperature. The procedure for crystallization of compound CB1 from fraction code B008 is shown in scheme 3.3

Table 3.4 Characteristic fraction of isolation of EtOAc from cultivation broth extract isolate PBL004

| Fraction Code | Eluents | Fraction No. | appearance | Weight (mg) |
|---|---|---|---|---|
| B001 | 100% hexane | 1-5 | Amorphous white solid in light yellow oily | 10 |
| B002 | 3% EtOAc in hexane | 6-20 | Light yellow oily | 20 |
| B003 | 5% EtOAc in hexane | 21-23 | Yellow wax | 24 |
| B004 | 5% EtOAc in hexane | 24-28 | Light yellow oily | 26 |
| B005 | 10% EtOAc in hexane | 29-32 | Yellow oily | 12 |
| B006 | 12% EtOAc in hexane | 33-35 | Amorphous white solid in light yellow oily | 20 |
| B007 | 12% EtOAc in hexane | 36-38 | Yellow oily | 18 |
| B008 | 12% EtOAc in hexane | 39-47 | Round white solid | 360 |
| B009 | 14% EtOAc in hexane | 48-52 | Yellow oily | 50 |
| B010 | 14% EtOAc in hexane | 53-62 | Yellow oily | 40 |
| B011 | 14% EtOAc in hexane | 63-65 | Red brown oily | 18 |
| B012 | 16% EtOAc in hexane | 66-75 | Red brown oily | 21 |
| B013 | 20% EtOAc in hexane | 76-89 | Red brown oily | 25 |
| B014 | 30% EtOAc in hexane | 90-100 | Red brown oily | 30 |
| B015 | 40% EtOAc in hexane | 101-109 | Red brown oily | 80 |
| B016 | 50% EtOAc in hexane | 110-119 | Red brown viscous liquid | 140 |
| B017 | 70% EtOAc in hexane | 120-126 | Red brown viscous liquid | 151 |
| B018 | 100% EtOAc | 127-132 | Red brown viscous liquid | 102 |
| B019 | 5% MeOH in EtOAc | 133-141 | brown viscous liquid | 156 |
| B020 | 10% MeOH in EtOAc | 142-165 | brown viscous liquid | 381 |
| B021 | 20% MeOH in EtOAc | 166-177 | Black viscous liquid | 375 |
| B022 | 30% MeOH in EtOAc | 178-190 | Black viscous liquid | 304 |
| B023 | 50% MeOH in EtOAc | 191-212 | Black viscous liquid | 332 |
| B024 | 100% MeOH | 213-240 | Black viscous liquid | 306 |

EtOAc crude of culture broth extracts of fraction code B008 (360 mg)

Crystallization
EtOAc and hexane

Mixture CB1 (220 mg)

Scheme 3.3 Isolation of compound from EtOAc crude of culture broth extract of fraction code B008

3.17 Physical properties of isolated mixture CB1

Chemical compound was isolated from the culture broth of the endophytic fungus isolate PBL004. Mixture CB1 (220 mg, 7.21% yield of the EtOAc extract ) was obtained from fraction B008 as a round white solid. Chemical structure of compound was determined by analyzed of spectroscopic data, UV, FT-IR, EI-MS, $^{1}$H-NMR, $^{13}$C-NMR and 2D-NMR as well as by comparison their spectral data with those of published values.

Mixture CB1 is shown in Figure B5 – B15 in Appendix B

UV : $\lambda_{max}$ nm (log$\varepsilon$ ) in methanol 275 sh(4.2) (Figure B14 in Appendix B)

FT IR : $\upsilon$ max cm$^{-1}$: 3178 (br), 2925 (m), 2357 (w), 1614 (s), 1446 (s), 1264 (s), 1123 (s), 695 (m) (Figure B13 in Appendix B)

EI- MS : *m/z* 70eV; 304(15), 261(15). 115(14), 91(18), 85(45), 83(68), 77(27), 67(20) (Figure B15 in Appendix B)

$^{1}$H-NMR : $\delta$H (ppm), 400 MHz, in $CDCl_3$; 0.96(3H, t, *J*=7.2), 1.42 (2H, q, *J*=7), 1.55(2H, q, *J*=7.6), 1.67(3H, d, *J*=6), 1.94(2H, m), 2.23(2H, m), 2.66(2H, t, *J*=7.6), 2.84(2H, ddd, *J*=3.6, 16.4), 4.54(1H, m), 5.44(1H, m), 5.48(1H, m), 6.25(1H, s), 11.42(1H, s) (Figure B5 in Appendix B)

$^{13}$C-NMR : $\delta$C (ppm), 100 MHz, in $CDCl_3$; 14(q), 17.9(q), 22.3(t), 22.8(t), 27.8(t), 30.9(t), 32.9(t), 34.7(t), 78.5(d), 101.7(s), 102.3(s), 106.5(s), 114.8(s), 126.4(d), 129.5(d), 138.2(s), 160(s), 170.4(s) (Figure B6 in Appendix B)

2D-NMR : $\delta$H, $\delta$C (Figure B8-B12 in Appendix B)

3.18 Biological activities test

3.18.1 Test for antimicrobial activities

3.18.1.1 Test for antimicrobial activities of the crude extracts

Antimicrobial activity was performed against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *C. albican* ATCC 10231.

3.18.1.2 Test for antimicrobial activities of compounds

Evaluation of the antimicrobial activities of pure compounds were determined by the antimicrobial susceptibility test broth microdilution method (Woods and Washington, 1995). Antimicrobial activity was performed against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 *C. albican* ATCC 10231 and Herpes simplex virus trye1 ATCC VR-260

A. Preparation of pure compounds

Four mg of pure compounds was dissolved 100 μl with 10% DMSO in sterile distilled water (stock solution 4 mg/ml) and kept in a refrigerator at 4 ◦C for bioassay.

B. Preparation for tested bacterial

A bacterial inoculum was prepared in the same manner as described in section 3.4.2. The final inoculum was diluted with NB to obtain a cell suspension containing approximately $10^6$ CFU/ml.

C. Preparation for tested yeast

A yeast inoculum was performed in the same manner as described in section 3.4.2. The final inoculum was approximately $10^6$ CFU/ml.

D. Tested for microbial activities

Solution of pure compounds was diluted with NB and SDB for assays of antibacterial and antifungal (yeast form) activities respectively. Fifty μl of the final adjusted microbial suspension was inoculated into each well (Final inoculum size of bacterial and yeast was approximately $2.5x10^5$ and $2.5x10^4$ CFU/ml, respectively). One hundred μl of medium only was as the sterility control. A 100 μl volume of medium and microbial inoculum mixture acted as the growth control. Microbial microtiter plates were incubated at 37 ◦C for bacterial and at room temperature and yeast.

E. Reading of microtiter plates assays

Antibacterial and antifungal (yeast form) activites were determined by measuring the turbidity each well in the microtiter plates by using the Bio-Rad microplate reader before and after incubation. The lowest concentration of pure compound showing complete inhibition of growth was recorded as minimal inhibitory concentration (MIC).

F. Tested for anti-Herpes simplex virus activity and cytotoxicity within Vero cell

Compound extract was tested at a final concentration of 250 $\mu g ml^{-1}$. A 10 μl volume of compound extract in 10% DMSO was dispensed into each well of a 96-well microplate followed by the addition of 30 pfu of Herpes simplex virus ATCC VR-260. Then, Vero cells ATCC CCL-81 cultivated in minimum essential medium (MEM; HyClone) were added to a final concentration of $2x10^4$ cells $ml^{-1}$ in a volume of 200 μl. After incubation at 37 ◦C in a 5% for 48 hr, viability of Vero cells was determined by counting from inverted microscope as modified by Skehan et al.(1990). Activity against Herpes simplex virus type 1 was determined at the concentration of the compound extract that showed no toxicity to the Vero cells. Compound extract that could inhibit more than 50% of viral growth was further tested to determine the concentration inhibited 50% of viral growth ($IC_{50}$).

3.19 Cytotoxicity test

Cytotoxicity test were carried out at the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University. Bioassay of cytotoxic activity against human tumor cell culture *in vitro* was performed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric method (Carmichael et al., 1987). In Principle, the viable cell number/well was directly proportional to the production of formazan, followed by solubillization, and could be measured spectrophotometrically.

The human tumor cell line was harvested from exponential-phase maintenance cultures (T-75 $cm^2$ flask), counted by trypan blue exclusion, and dispensed into replicate 96-well culture plates in 100-μl volumes using a repeating pipette. Following a 24-h incubation at 37 ◦C, 5% $CO_2$, 100% relative humidity, 100 μl of culture medium, culture medium containing the sample was dispensed into the appropriate wells (control group, N=6; each sample treatment group, N=3). Peripheral wells of each plate (lacking cells) were utilized for sample blank (N=2) and medium/tetrazolium reagent blank (N=6) "background" determinations. Culture plates were then incubated for 4 days prior to the addition of tetrazolium reagent. MTT stock solution was prepared as follows: 5 mg MTT/ml PBS was sterilized and filtered through 0.45-μl filter units. MTT working solutions were prepared just prior to culture application by dilution of MTT stock solution 1:5 (v/v) in prewarmed standard culture medium. MTT working solution (50 μl) was added to each culture well, resulting in 50 μl MTT/250 μl total medium volumes; and cultures were incubated at 37 ◦C for 4 to 24 hr depending upon individual cell line requirements. Following incubation cell monolayers and formazan were inspected microscopically. Culture plates containing suspension lines or any detached cells were centrifuged at low speed for 5 min. All 10-20 μl of culture medium supernatant was removed from wells by slow aspiration through a blunt 18-guage needle and replaced with 150 μl of DMSO using a pipette. Following formazan solubilization, the absorbance of each well was measured using a microculture plate reader at 540 nm (single wavelength, calibration factor=1.00).

Cell line growth and growth inhibition were expressed in terms of mean (+/- 1 SD) absorbance units and / or percentage of control absorbance (+/- 1 SD %) following subtraction of mean “background” absorbance.

Samles were also tested for cytotoxic activity towards 5 cell lines, which contain of HEP-G2 (hepatoma), SW 520 (colon), Chago (lung), Kato-3 (gastric), and BT 474 (breast) following the experimental method of bioassay of cytotoxic activity.

3.20 Fungal cultivation for studies characteristic of the endophytic fungus *Fusarium proliferatum* isolated from *Piper betle* Linn. and *Fusarium proliferatum* type strain plant pathogen

The endophytic fungus *F. proliferatum* isolated from *P. betle* Linn. and *F. proliferatum* type strain plant pathogen were grown on PDA at room temperature (25-30$^{o}$C) for a week and studies characteristi c of colony and pigment were produced from *F. proliferatum,* the endophytic fungus and type strain plant pathogen.

# CHAPTER IV

# RESULTS AND DISCUSSION

## 4.1 Isolation of endophytic fungi

Thirty two endophytic fungi isolates were isolated from healthy mature leaves of *Piper betle* Linn is shown Table 4.1. Colony characteristic of some endophytic fungi isolated from *Piper betle* Linn. is shown in Figure 4.1

Table 4.1 Number and isolate of endophytic fungi from various province

| Name of province | Number of endophytic fungi Isolates | Code of Endophytic fungi isolates |
|---|---|---|
| Bangkok | 14 | PBL001-014 |
| Pathumthani | 3 | PBL015-017 |
| Rayoang | 2 | PBL018-019 |
| Nakhonsithammarat | 13 | PBL020-032 |
| total | 32 | |

## 4.2 Enumeration of test microorganisms

Viable counts of bacteria and yeast were performed for standardized inocula whose turbidity matched a 0.5 McFarland standard. The CFU/ml values are shown in Table 4.2

Table 4.2 Viable counts of bacteria and yeast

| Test microorganism | Quantity (Colony forming unit) |
|---|---|
| *Bacillus subtilis* ATCC 6633 | $3.2 \times 10^6$ |
| *Escherichia coli* ATCC25922 | $2.1 \times 10^7$ |
| *Pseudomonas aeruginosa* ATCC 27853 | $2.2 \times 10^7$ |
| *Staphylococcus aureus* ATCC 25923 | $1.8 \times 10^7$ |
| *Candida albicans* ATCC 10231 | $3.5 \times 10^5$ |

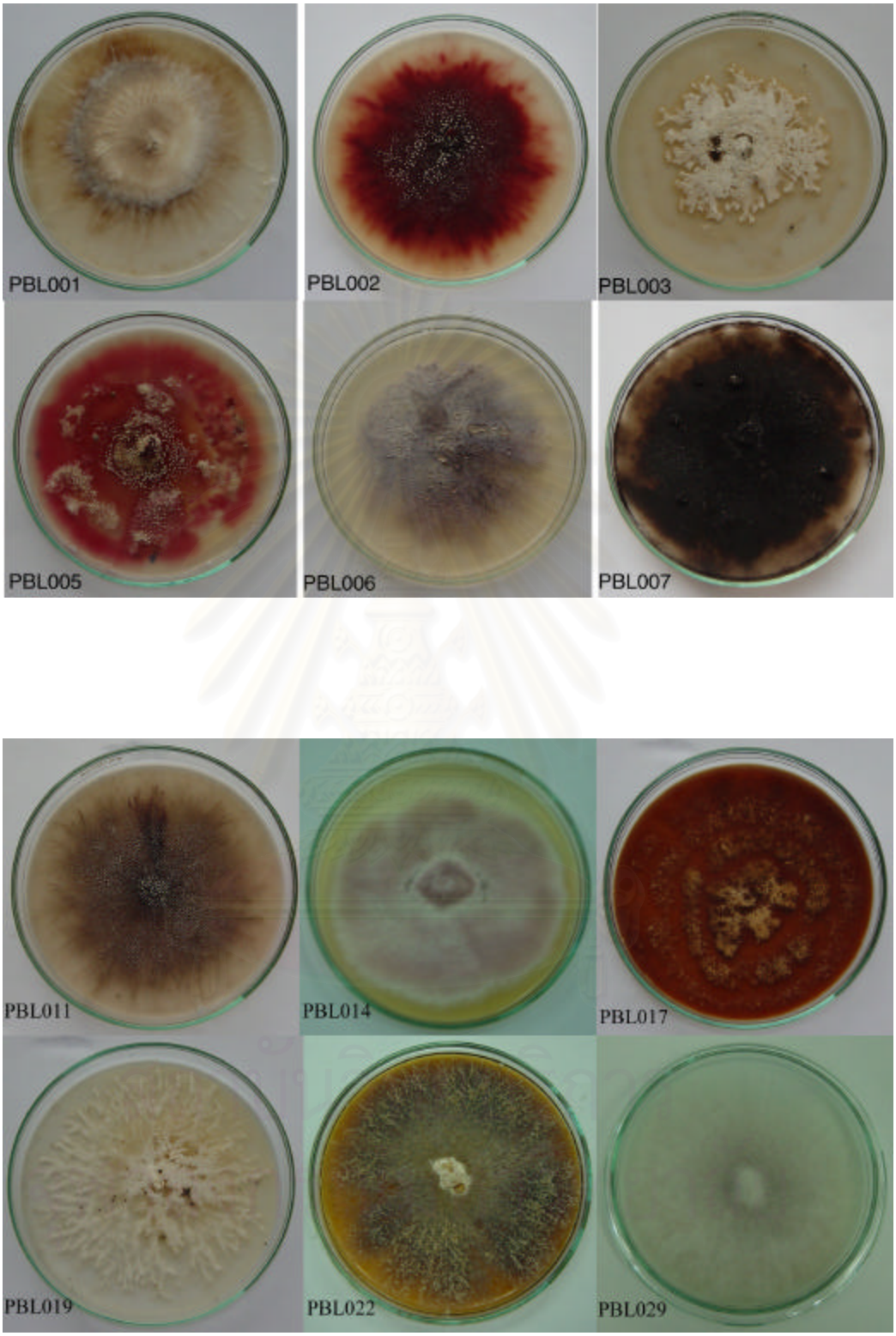



Figure 4.1 Colony characteristic of some endophytic fungi isolated on PDA after cultivation for 2 weeks at room temperature

## 4.3 Determination of antimicrobial activities

The results of antimicrobial activity assays of isolated endophytic fungi on four media (MczA, MEA, SDA and YES agar) were determined using the agar method. The antimicrobial activities of the isolates are shown in Table 4.3 ,

Table 4.3 Summarized of culture media on antimicrobial activities from fungal endophyte isolated

| Strain endophytic fungal | Media | Antimicrobial activities against | | | | |
|---|---|---|---|---|---|---|
| | | BS | EC | PA | SA | CA |
| PBL001 | MCzA | - | - | - | - | - |
| | MEA | - | - | - | - | - |
| | SDA | - | - | - | - | - |
| | YES | - | - | - | - | - |
| PBL002 | MCzA | - | - | - | - | - |
| | MEA | - | - | - | - | - |
| | SDA | - | - | - | - | - |
| | YES | - | - | - | - | - |
| PBL003 | MCzA | - | - | - | - | - |
| | MEA | - | - | - | - | - |
| | SDA | - | - | - | - | - |
| | YES | - | - | - | - | - |
| PBL004 | MCzA | ++ | ++ | - | - | ++ |
| | MEA | + | - | - | - | - |
| | SDA | + | + | - | - | - |
| | YES | ++ | - | - | - | ++ |
| PBL005 | MCzA | - | - | - | - | - |
| | MEA | - | - | - | - | - |
| | SDA | - | - | - | - | - |
| | YES | - | - | - | - | - |
| PBL006 | MCzA | - | - | - | - | - |
| | MEA | - | - | - | - | - |
| | SDA | - | - | - | - | - |
| | YES | - | - | - | - | - |
| PBL007 | MCzA | - | - | - | - | - |
| | MEA | - | - | - | - | - |
| | SDA | - | - | - | - | - |
| | YES | - | - | - | - | - |

Table 4.3 (continued)

| Strain endophytic fungal | Media | Antimicrobial activities against | | | | |
|---|---|---|---|---|---|---|
| | | BS | EC | PA | SA | CA |
| PBL 008 | MCzA | - | - | - | - | - |
| | MEA | - | - | - | - | - |
| | SDA | - | - | - | - | - |
| | YES | - | - | - | - | - |
| PBL 009 | MCzA | - | - | - | - | - |
| | MEA | - | - | - | - | - |
| | SDA | - | - | - | - | - |
| | YES | - | - | - | - | - |
| PBL 010 | MCzA | - | - | - | - | - |
| | MEA | - | - | - | - | - |
| | SDA | - | - | - | - | - |
| | YES | - | - | - | - | - |
| PBL 011 | MCzA | - | - | - | - | - |
| | MEA | - | - | - | - | - |
| | SDA | - | - | - | - | - |
| | YES | - | - | - | - | - |
| PBL 012 | MCzA | - | - | - | - | - |
| | MEA | - | - | - | - | - |
| | SDA | - | - | - | - | - |
| | YES | - | - | - | - | - |
| PBL 013 | MCzA | - | - | - | - | - |
| | MEA | - | - | - | - | - |
| | SDA | - | - | - | - | - |
| | YES | - | - | - | - | - |
| PBL 014 | MCzA | - | - | - | - | - |
| | MEA | - | - | - | - | - |
| | SDA | - | - | - | - | - |
| | YES | - | - | - | - | - |
| PBL 015 | MCzA | - | - | - | - | - |
| | MEA | - | - | - | - | - |
| | SDA | - | - | - | - | - |
| | YES | - | - | - | - | - |
| PBL 016 | MCzA | - | - | - | - | - |
| | MEA | - | - | - | - | - |
| | SDA | - | - | - | - | - |
| | YES | - | - | - | - | - |

Table 4.3 (continued)

| Strain endophytic fungal | Media | Antimicrobial activities against | | | | |
|---|---|---|---|---|---|---|
| | | BS | EC | PA | SA | CA |
| PBL 017 | MCzA | - | - | - | - | - |
| | MEA | - | - | - | - | - |
| | SDA | - | - | - | - | - |
| | YES | - | - | - | - | - |
| PBL 018 | MCzA | - | - | - | - | - |
| | MEA | - | - | - | - | - |
| | SDA | - | - | - | - | - |
| | YES | - | - | - | - | - |
| PBL 019 | MCzA | - | - | - | - | - |
| | MEA | - | - | - | - | ++ |
| | SDA | - | - | - | - | + |
| | YES | - | - | - | - | - |
| PBL 020 | MCzA | - | - | - | - | - |
| | MEA | - | - | - | - | - |
| | SDA | - | - | - | - | - |
| | YES | - | - | - | - | - |
| PBL 021 | MCzA | - | - | - | - | - |
| | MEA | - | - | - | - | - |
| | SDA | - | - | - | - | - |
| | YES | - | - | - | - | - |
| PBL 022 | MCzA | - | - | - | - | - |
| | MEA | - | - | - | - | - |
| | SDA | - | - | - | - | - |
| | YES | - | - | - | - | - |
| PBL 023 | MCzA | - | - | - | - | - |
| | MEA | - | - | - | - | + |
| | SDA | - | - | - | - | + |
| | YES | - | - | - | - | - |
| PBL 024 | MCzA | - | - | - | - | - |
| | MEA | - | - | - | - | - |
| | SDA | - | - | - | - | - |
| | YES | - | - | - | - | - |
| PBL 025 | MCzA | - | - | - | - | - |
| | MEA | - | - | - | - | - |
| | SDA | - | - | - | - | - |
| | YES | - | - | - | - | - |

Table 4.3 (continued)

| Strain endophytic fungal | Media | Antimicrobial activities against | | | | |
|---|---|---|---|---|---|---|
| | | BS | EC | PA | SA | CA |
| PBL 026 | MCzA | - | - | - | - | - |
| | MEA | - | - | - | - | - |
| | SDA | - | - | - | - | - |
| | YES | - | - | - | - | - |
| PBL 027 | MCzA | - | - | - | - | - |
| | MEA | - | - | - | - | - |
| | SDA | - | - | - | - | - |
| | YES | - | - | - | - | - |
| PBL 028 | MCzA | - | - | - | - | - |
| | MEA | - | - | - | - | - |
| | SDA | - | - | - | - | - |
| | YES | - | - | - | - | - |
| PBL 029 | MCzA | - | - | - | - | - |
| | MEA | - | - | - | - | - |
| | SDA | - | - | - | - | - |
| | YES | - | - | - | - | - |
| PBL 030 | MCzA | - | - | - | - | - |
| | MEA | - | - | - | - | - |
| | SDA | - | - | - | - | - |
| | YES | - | - | - | - | - |
| PBL 031 | MCzA | - | - | - | - | + |
| | MEA | - | - | - | - | + |
| | SDA | - | - | - | - | - |
| | YES | - | - | - | - | - |
| PBL 032 | MCzA | - | - | - | + | - |
| | MEA | - | - | - | + | - |
| | SDA | - | - | - | ++ | - |
| | YES | - | - | - | ++ | - |

Activities were classified according to the diameter of clear zone after inoculated with a small pieces of the grow fungus of isolate endophytic fungi

++ ; more than 15 mm ; + less than 15 mm ; - non inhibition

BS ; *Bacillus subtilis* ; EC ; *escherichia coli* ; PA ; *Pseudomonas aeruginosa*

SA ; *Staphylococcus aureus* ; CA; *Candida albicans*

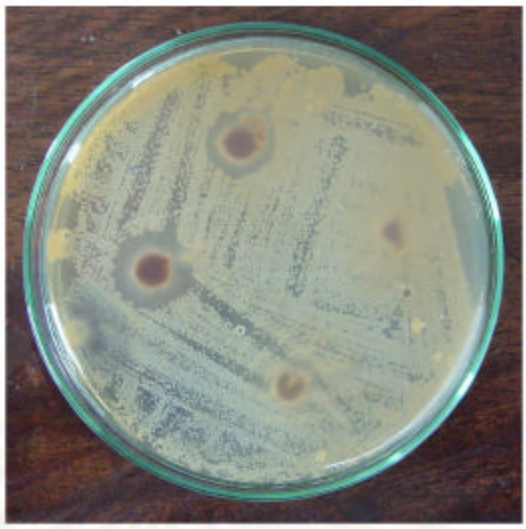


Figure 4.2 Showing zone of inhibition in plate method of small pieces of isolate PBL031 against *Candida albicans*

Results of antimicrobial activity assays against bacteria and fungi show that four isolates endophytic fungi had antimicrobial activities, isolate PBL004 was active against *B. subtilis* ATCC 6633, *E.coli* ATCC 25922 and *C. albicans* ATCC 10231, isolate PBL019 , PBL023 and PBL031 were active against *C. albicans* ATCC 10231 and isolate PBL032 was active against *S. aureus* ATCC 25923. inhibition zone in plate method of small pieces of isolate PBL031 against *Candida albicans* is chown in Figure 4.2.

Isolated PBL004 cultured on MCzA has the best activity to against *B. subtilis* ATCC 6633 , *E.coli* ATCC 25922 and *C. albicans* ATCC 10231.

4.4 Identification of fungal endophyte PBL004

Fungal isolated PBL004 was chosen for bioactive compound because PBL004 cultured on MCzA showed the best activity that active against *B. subtilis* ATCC 6633 , *E.coli* ATCC 25922 and *C. albicans* ATCC 10231 and the culture broth extracts were active against *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923 and *Candida albicans* ATCC 10231.

4.4.1 Morphology identification of endophytic fungal isolate PBL004

Fungal isolate PBL004 was identified as *Fusarium* sp. The fungus was grown on PDA for a week at room temperature ; mycelium composed of hyaline, septate, branched hyphae, simple spore and dark purple pigment. Colony morphology and slide culture of isolate PBL004 that grown on PDA is shown in Figure 4.3. Colony characteristic of isolate PBL004 had different color pigment grown on various media; pale purple in MCzA, red in MEA, pink in SDA and pale yellow in YES. Colony characteristic of isolate PBL004 grown on various media is shown in Figure 4.4 . Characteristic of mycelia and spore isolate PBL004 was branched hyphae and simple spore. Figure 4.5 is shown scanning electron microscopes of isolated PBL004.

4.4.2 Molecular identification of endophytic fungal isolate PBL004

The rDNA ITS region of isolate PBL004 was amplified with the conserved fungal primer ITS 1t and ITS4. Isolate PBL004 produced a single ITS band. The length of correnspondng fragment was 583 bp, containing a part of the 18S; ITS1, 5.8S and 28S rDNA

Sequencing of the nucleotide sequences of partial 18S. ITS region of the isolate, PBL004 resulted in a 583 bp fragment, as shown in Figure 4.6

A blast search was performed to find a similar sequence to ITS region of fungal isolate PBL004 in the Genbank DNA database. The ITS region of this isolate was similar to 99.479% identity of *Fusarium proliferatum* , as shown in Figure 4.6

Classical identification of fungi is based on observe characteristics. Assignment of morphological species can be based on colony surface texture, hyphal pigments, exudates, margin shapes, growth rates, and sporulating structures (Redlin and Carris, 1985). Fungal isolate PL004 was identified as belonging to the genera *Fusarium.*

*Fusarium* sp., mycelium composed of hyaline, septate, branched hyphae. Conidial masses typically formed in sporodochia or in ponnotes or sometimes scattered in the mycelium. Conidiophores simple or branched once or repeatedly, terminating in phialides which are sometimes formed verticillately, hyaline, septate. Phialides variable in size and shape, but mostly subulate. Conidia of two types: microconidia and

macroconidia. Microconidia usually 1-celled, variable in shape, hyaline, either produced singly at the tips of phialideds, or else abstracted in succession at the tips of the phialides to form simple shains. Macroconidia usually 3-many-septate, fusiform to falcate, dorsiventral, straight or curved variously, often with a distinct pedicellate base, with the apical part obtuse to broadly rounded to caudate or acuminate, produced singly at the tips of the phialides. Chlamydospores usually present,globose, ovoid or pear-chaped, 1-2 celled or in chain, or sometimes in cultures, terminal or intercalary, brownish in colour or becoming tinged with the colour of the stroma. Sclerotia spherical, solid, occurring singly, or in groups., or absent. Sclerotial stromata occur in many groups erumpent, hemispherical, smooth or rough and cauliflower-like, or erect, stiboid, sometimes with antler-like branching, sessile or stalked: remaining sterile or serving as a stroma for sporodochia. Conidial masses pale or brightly coloured (orange, salmon or ochre), slimy (Subramanian, 1971).

Molecular methods of identification was also performed. The nucleotide sequence of the ITS region of rDNA is conserved. It can be used to delineate species relationships and separated taxonomy from class to species (Mitchell et al., 1995). Thenucleotide sequence of the ITS region of fungal isolate PL004 was similar to 99.479% identity of *F. proliperatum* by Buzina et al., 2003.

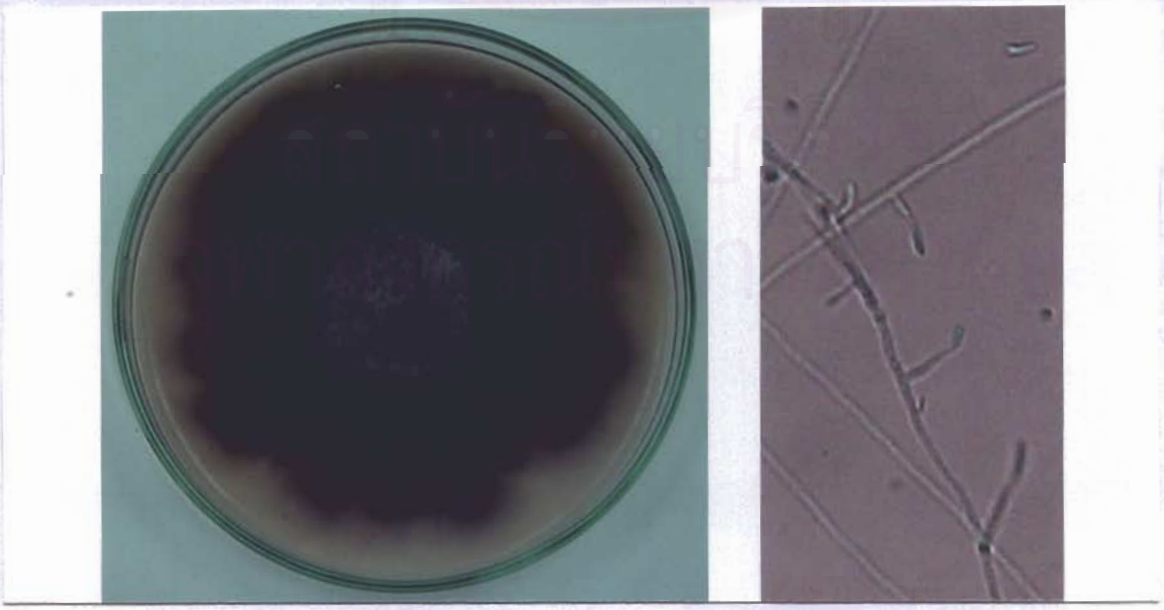


**Figure 4.3** Colony morphology and 40x microscopic structure of isolatePBL004 culture in PDA

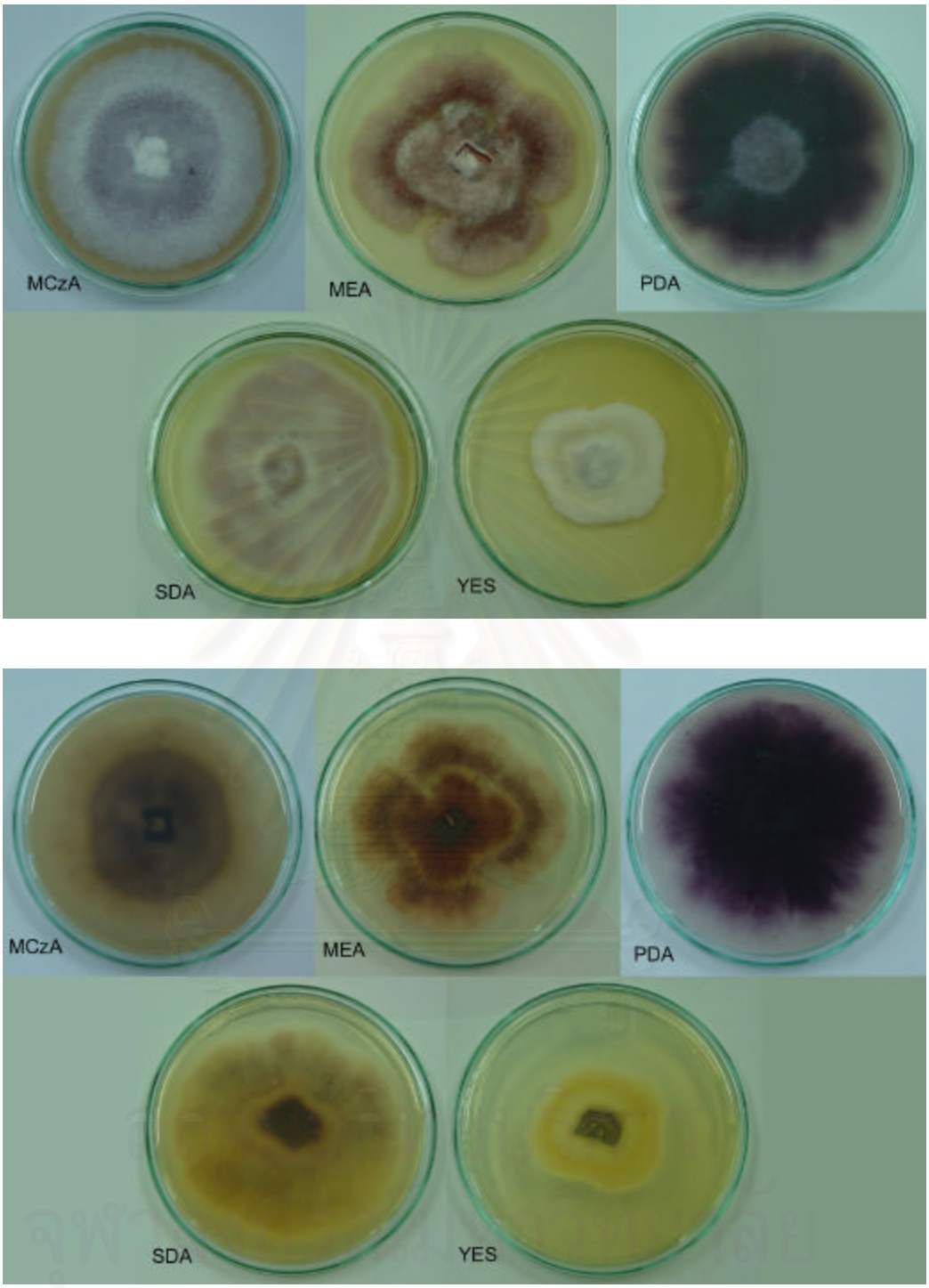



Figure 4.4 Colony characteristic of isolat e PBL004 grown various media

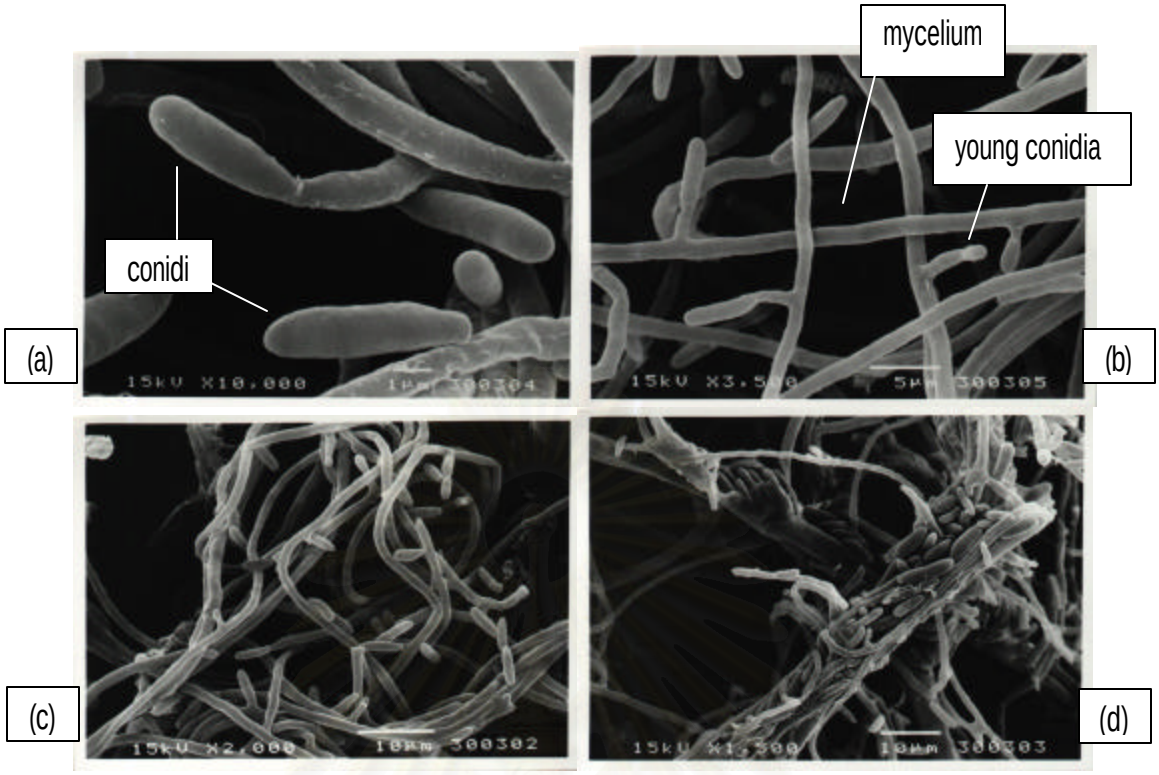



Figure 4.5 Characteristic mycelia and conidia of endophytic fungi isolated PBL004 cultured on PDA a week by scanning electron microscopes at 10,000X(a), 3,500X(b), 2,000X(c) and 1,500X(d) magnification

```
5'cttggtcatttagaggaagtaaaagtcgtaacaaggtctccgttggtgaa
 ccagcggagggatcattaccgagtttacaactcccaaaccccctgtgaaca
 taccaattgttgcctcggcggatcagcccgctcccggtaaaacgggacgg
 cccgccagaggacccctaaactctgtttctatatgtaacttctgagtaaa
 accataaataaatcaaaactttcaacaacggatctcttggttctggcatc
Agatgaagaacgcagcaaaatgcgataagtaatgtgaattgcagaattcag
 tgaatcatcgaatctttgaacgcacattgcgcccgccagtattctggcgg
 catgcctgttcgagcgtcatttcaaccctcaagcccccgggtttggtgtt
 ggggatcggcgagccctcgcggcaagccggccccgaaatctagtggcggt
 ctcgctgtagcttccattgcgtagtagtaaaaccctcgcaactggtacgc
 ggcgcggccaagccgttaaacccccaacttctgaatgttgacctcggatc
  aggtaggaatacccgctgaacttaagcatatcaataagcggagga3'
```

```
>>AF291061|AF291061.1 Fusarium proliferatum NRRL 31071 1  (596 nt)
 initn: 1750 init1: 1750 opt: 2941 Z-score: 2864.9 expect() 1.4e-151
 99.497% identity in 596 nt overlap (1-595:1-596)

               10        20        30        40        50        60
       CTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AF2910 CTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGG
               10        20        30        40        50        60
```

Figure 4.6 Nucleotide sequences of partial 18S region and alignment data of ITS region of the isolate PBL004 and reference taxa

```
              70        80        90       100       110       120
       GATCATTACCGAGTTTACAACTCCCAAACCCCTGTGAACATACCAATTGTTGCCTCGGCG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::

AF2910 GATCATTACCGAGTTTACAACTCCCAAACCCCTGTGAACATACCAATTGTTGCCTCGGCG

              70        80        90       100       110       120

              130       140       150       160       170       180
       GATCAGCCCGCTCCCGGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTCTGTTTCT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AF2910 GATCAGCCCGCTCCCGGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTCTGTTTCT
              130       140       150       160       170       180

              190       200       210       220       230       240
       ATATGTAACTTCTGAGTAAAACCATAAATAAATCAAAACTTTCAACAACGGATCTCTTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AF2910 ATATGTAACTTCTGAGTAAAACCATAAATAAATCAAAACTTTCAACAACGGATCTCTTGG
              190       200       210       220       230       240

              250       260       270       280       290       300
       TTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AF2910 TTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAG
              250       260       270       280       290       300

              310       320       330       340        350
       TGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGC-GGCATGCCTGT
       :::::::::::::::::::::::::::::::::::::::::::::::: ::::::::::::
AF2910 TGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGT
              310       320       330       340       350       360

     360       370       380       390       400       410
       TCGAGCGTCATTTCAACCCTCAAGCCCCCGGGTTTGGTGTTGGGGATCGGCGAGCCCTCG
       :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::: :
AF2910 TCGAGCGTCATTTCAACCCTCAAGCCCCCGGGTTTGGTGTTGGGGATCGGCGAGCCCTTG

           370       380       390       400       410       420

     420       430       440       450       460       470
       CGGCAAGCCGGCCCCGAAATCTAGTGGCGGTCTCGCTGTAGCTTCCATTGCGTAGTAGTA
       :::::::::::::::::::::::::::::::::::: ::::::::::::::::::::::
AF2910 CGGCAAGCCGGCCCCGAAATCTAGTGGCGGTCTCGCTGCAGCTTCCATTGCGTAGTAGTA
              430       440       450       460       470       480

     480       490       500       510       520       530
       AAACCCTCGCAACTGGTACGCGGCGCGGCCAAGCCGTTAAACCCCCAACTTCTGAATGTT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AF2910 AAACCCTCGCAACTGGTACGCGGCGCGGCCAAGCCGTTAAACCCCCAACTTCTGAATGTT
              490       500       510       520       530       540

     540       550       560       570       580       590
       GACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AF2910 GACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA
              550       560       570       580       590
```

Figure 4.6(continued)

4.5 Determination of growth profile of culture filtrate from fungal isolate PBL004

Growth profile of fungal endophyte isolate PBL004 was observed from mycelium dry weight. Figure 4.7 show the growth profile of fungal isolate PBL004 at day 1 to day 54. The raw data of mycelium dry weight are shown in Table 4.4

Cultivation of fungal isolate PBL004 was done in MCzB medium for 54 days. Fungal isolate PBL004 grow in log phase. Then the mycelium increased up to highest amount within 18 days. The growth of mycelium was in the form of stationary phase and continued to death phase in 30 days.

Table 4.4 The raw data of mycelium dry weight

| Date | Mycelium dry weight (g) |
|---|---|
| 1 | 0.00 |
| 3 | 0.30 |
| 6 | 0.62 |
| 9 | 0.78 |
| 12 | 0.79 |
| 15 | 0.85 |
| 18 | 1.05 |
| 21 | 1.01 |
| 24 | 0.99 |
| 27 | 0.98 |
| 30 | 0.95 |
| 33 | 0.95 |
| 36 | 0.86 |
| 39 | 0.84 |
| 42 | 0.83 |
| 45 | 0.85 |
| 48 | 0.82 |
| 51 | 0.81 |
| 54 | 0.81 |

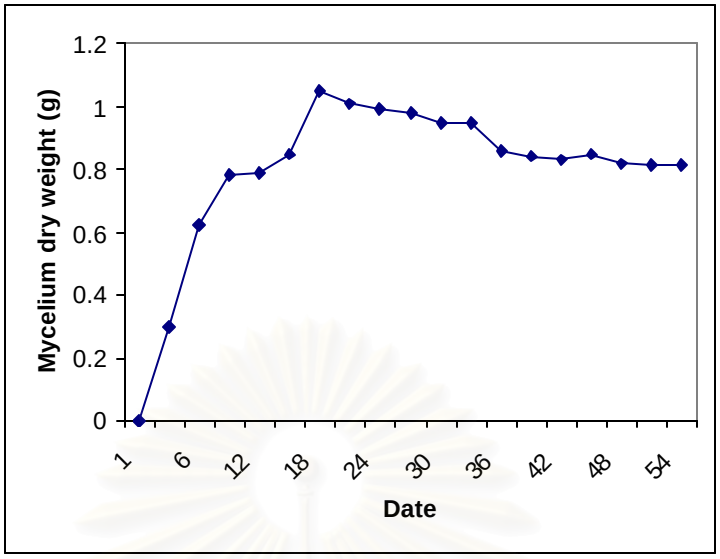



Figure 4.7 Growth profile of fungal isolate PBL004

4.6 Cultivation and extraction

Fungal isolate PBL004 was cultivated in MCzB total 10 L to yield 17.14 g crude EtOAc from mycelium extracts and 3.05 g of crude EtOAc from cultivation broth.

4.6.1 Isolation and purification of chemical compounds in crude EtOAc from mycelium extracts

Mixture M1 was isolated from mycelium extracts of the endophytic fungus isolate PBL004 that cultured on MczA. Mixture M1 ( 58.46% yield of the EtOAc extract, 1001.4 mg/L culture broth or 108.8 mg/ g dry mycelium) were obtained from fraction Mr001. Chemical structures of these mixture M1 were determined by analyzes of $^{1}$H-NMR, $^{13}$C-NMR and Gas chromatography.

4.6.1.1 Structure elucidation of mixture M1

Mixture M1 was isolated from the mycelium of the endophytic fungus isolate PBL004. Mixture M1 (58.46% yield of the EtOAc extract ) was obtained from fraction code Mr001 as a colorless viscous liquid, eluted with 100% hexane. Chemical structure of compound was determined by analyzed of spectroscopic data, FT-IR, $^{1}$H-NMR, $^{13}$C-NMR and GC as well as by comparison their spectral data with those of published values.

The IR spectrum of mixture M1 (Figure B4 in appendix B) showed important absorption bands at 3200-3600 $cm^{-1}$(O-H stretching), 2925 and 2854 $cm^{-1}$(C-H stretching), 1743 $cm^{-1}$(C-=O stretching) and 1245 $cm^{-1}$(C-O stretching). The IR data certainly supported the assignment of mixture M1 as shown in Table 4.5.

Table 4.5 The IR absorption band assignments of mixture M1

| Wave number ($cm^{-1}$) | Peak intensity | Tentative assignment |
|---|---|---|
| 3600-3200 | Broad | O-H stretching |
| 2925, 2854 | Strong | C-H stretching |
| 1743 | Strong | C=O stretching |
| 1465 | Medium | C-H bending |
| 1245 | Weak | C-O stretching |
| 757 | Weak | C-H rocking mode of $-(CH_2)_n-$ |

The $^{1}$H-NMR spectrum ( Figure B2 in appendix B) indicated that it possesses a methyl proton at $\delta$ 0.91 ppm, a methylene proton attached to a carbonyl proton (-C$H_2$-COOH) at $\delta$ 2.32 ppm and four olefinic protons at $\delta$ 4.14, 4.29, 5.26 and 5.34 ppm.

The $^{13}$C-NMR spectrum ( Figure B3 in appendix B) indicated the NMR spectroscopic pattern of long chain carboxylic acid; one methyl carbon signal at $\delta$ 14.1 ppm, eighteen methylene carbon signals at $\delta$ 22.6, 24.9, 27.2, 27.3, 29.1, 29.2, 29.3, 29.4, 29.4, 29.5, 29.6, 29.7, 29.7, 29.7, 29.8, 32.0, 34.1 and 34.2 ppm, two olefinic carbon signals at $\delta$129.7 and 130.0 ppm, and the carbon signal at 173.2 and 172.7 ppm should be the carbonyl group of carboxylic acid.

The structure of mixture M1 was established on base of spectroscopic analysis and based on retention time of standard methyl ester of fatty acid by gas chromatography. The relative percentages of fatty acids were determined by the area of the peaks in the chromatograms. Chromatogram of mixture M1 is shown in Figure B1 in appendix B and comparison their spectral data with those of published values. Results is shown in Table 4.6 and 4.7.

Table 4.6 Results of diglyceride was identification by GC

| Fatty acid | Structure | Name of fatty acid | % Fatty acid | Source |
|---|---|---|---|---|
| C14:0 | CH3(CH2)12COOH | n-tetradecanoic (myristic acid) | 0.40 | Coconut |
| C16:0 | CH3(CH2)14COOH | n-hexadecanoic (palmitic acid) | 27.21 | Lipid from animal and plant |
| C18:0 | CH3(CH2)16COOH | n-octadecanoic (stearic acid) | 16.42 | Lipid from animal and plant |
| C18:1 | CH3(CH2)7CH=CH(CH2)7COOH | Oleic acid | 37.49 | Lipid from animal and plant |
| C18:2 | CH3(CH2)4CH=CHCH2=CH2(CH2)7COOH | Linoleic acid | 17.26 | Corn ,peanut, soybean |
| - | Other | - | 1.22 | - |

Table 4.7 Results of diglyceride was identification by GC (weight (mg)/ L culture broth And weight (mg)/ g dry mycelium)

| Fatty acid | Weight (mg)/ L culture broth | Weight (mg)/ g dry weight mycelium |
|---|---|---|
| C14:0 | 4.0 | 0.43 |
| C16:0 | 272.5 | 29.60 |
| C18:0 | 164.4 | 17.87 |
| C18:1 | 375.4 | 40.79 |
| C18:2 | 172.8 | 18.78 |

4.7 Result of cultural condition on diglyceride production with four substrate in czapek broth and potato dextrose broth

Result of cultural condition on diglyceride production with four substrate in czapek broth and potato dextrose broth is shown in Table 4.8. Rice bran substrate in czapek broth wæs produced diglyceride more than other substrates in czapek broth and PDB but less than MCzB.

Table 4.8 Result of cultural condition on diglyceride production modified in czapek broth with various substrates and potato dextrose broth

| Medium | Dry weight (g)/L culture broth | Diglyceride (mg)/ g dry weight mycelium | Diglyceride (mg)/ L culture broth |
|---|---|---|---|
| substrate in czapek broth | | | |
| ◆ Betel leaves blended | 3.55 | 6.63 | 23.53 |
| ◆ ME and YE ,1:1 ratio | 18.62 | 2.05 | 38.2 |
| ◆ Rice bran | 11.17 | 16.67 | 195 |
| ◆ Yeast extract | 19.28 | 3.37 | 36.2 |
| Potato dextrose broth | 1.98 | 1.4 | 2.92 |

4.8 Isolation and purification of bioactive compounds in crude EtOAc from broth culture of endophytic fungal isolate PBL004

Chemical compound was isolated from the culture broth of the endophytic fungus isolatePBL004. Mixture CB1 (220 mg, 7.21% yield of the EtOAc extract ) was obtained from fraction B008 as a round white solid. Chemical structure of compound was determined by analyzed of spectroscopic data, FT-IR, UV, $^{1}$H-NMR, $^{13}$C-NMR and EI-MS, as well as by comparison their spectral data with those of published values.

4.8.1 Structure elucidation of mixture CB1

Mixture CB1 was round white solid, m.p.158-159 $^{0}$C and optical rotatory power ($[\alpha]_D^{20}$ ) was – 14 at concentration 0.2 g / 100 ml

The structure of mixture CB1 was elucidated by using spectroscopic techniques.

The FT-IR spectra of mixture CB1 is shown in appendix B, Figure B10 and the absorption peaks were assigned as summarized in Table 4.9. Its FT-IR spectrum indicated important absorption bands at 3100-3500 $cm^{-1}$ (O-H stretching vibration), 2925 and 2855 $cm^{-1}$ (C-H stretching vibration), 1614 and 1446 $cm^{-1}$(C=C stretching vibration), 1264 and 1123 $cm^{-1}$ (C-O stretching vibration).

Table 4.9 The FT-IR absorption band assignment of mixture CB1

| Wave number ($cm^{-1}$) | Intensity | Vibration |
|---|---|---|
| 3100-3500 | Broad | O-H stretching |
| 2925 | Medium | C-H stretching |
| 2855 | Weak | C-H stretching |
| 1614 | Strong | C=C stretching |
| 1446 | Medium | C=C stretching |
| 1264, 1123 | Medium | C-O stretching |

The $^{1}$H-NMR spectrum (Figure B5 in Appendix B) of Mixture CB1 indicated that it possesses two methyl groups at 0.96 and 1.67 ppm, four aliphatic methylene protons at 1.42, 1.55. 1.94 and 2.23 ppm, a methine proton attached with oxygen (C*H*-OH) at 4.54 ppm, two benzylic protons at 5.44 and 5.48 ppm, and an aromatic proton at 6.25 ppm.

The $^{13}$C-NMR spectrum (Figure B6 in Appendix B) of Mixture CB1 showed 20 signals, which the carbonyl group of ester corresponded to the signal at 170.4 ppm.

DEPT 135 experiment (Figure B7 in Appendix B) of Mixture CB1 showed six methylene carbons at 22.3, 22.8, 27.8, 30.9, 32.9 and 34.7 ppm, and two methyl carbons at 14.0 and 17.9 ppm, which indicated that the carbon signals at 101.7, 114.8, 138.2,160.0 and 162.3 ppm were quaternary.

DEPT 90 experiments (Figure B7 in Appendix B) indicated the presence of four sp carbons at 78.5, 106.5, 126.4 and 129.5 ppm.

The EI mass spectrum (Figure B12 in Appendix B) showed the molecular ion at *m/z* 304. It is assumed that this compound contains carbons, protons, and oxygens, then the molecular formula of $C_{18}H_{24}O_4$. The molecular formula, $C_{18}H_{24}O_4$ of this compound indicated six degree of unsaturation; therefore, mixture CB1 must consist of one aromatic ring and one carbonyl group in addition to one double bone

The information from 2D-NMR techniques: HSQC correlation (Figure B9 in Appendix B) HMBC correlation (Table 4.10, Figure 4.10, and Figure B5 in Appendix B), and COSY correlation (Table 4.10 Figure 4.9, and Figure B6 in Appendix B) were used to assist the interpretation of mixture CB1 structure

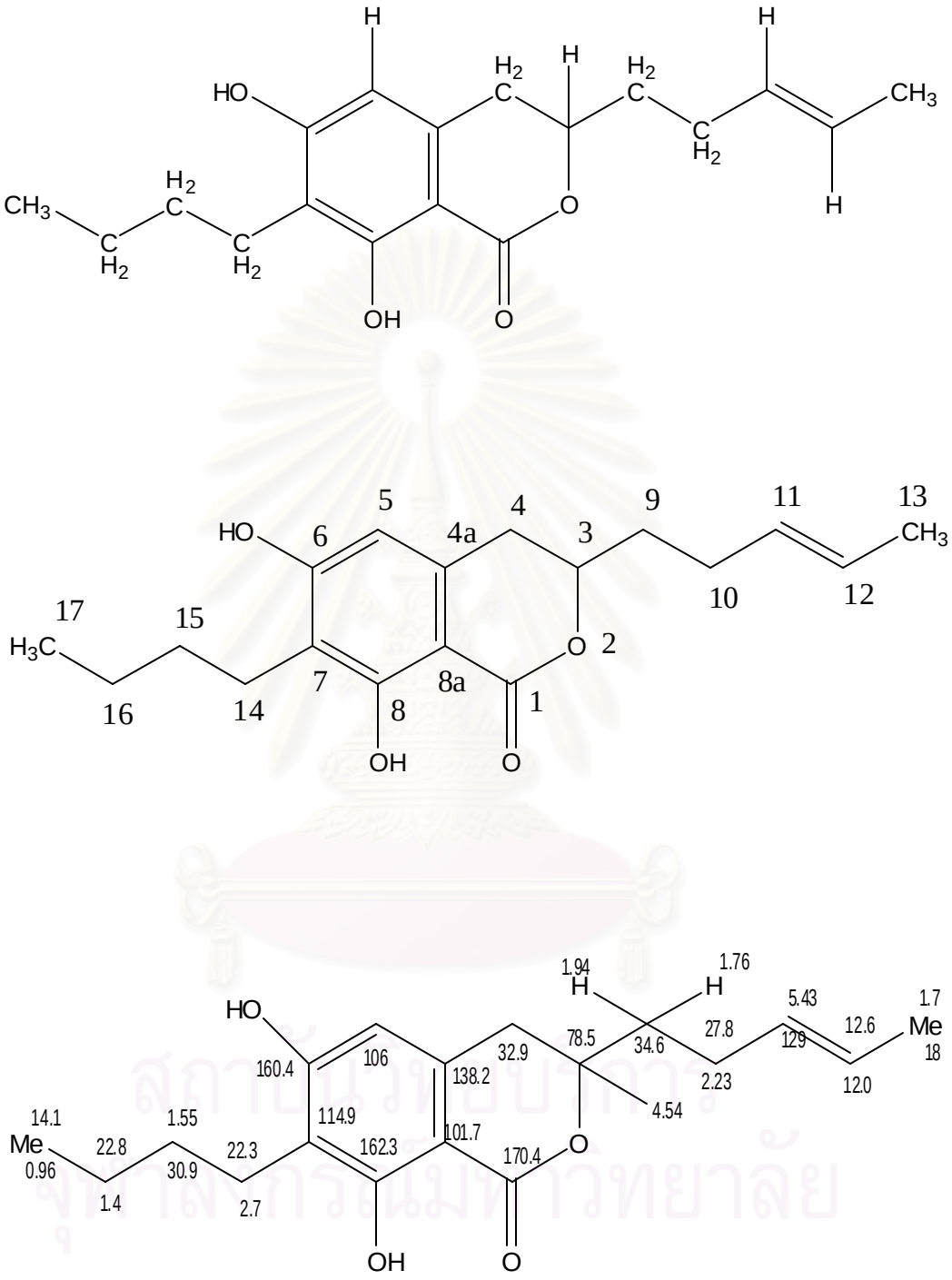



Figure 4.8 Major chemical structure of Mixture CB1

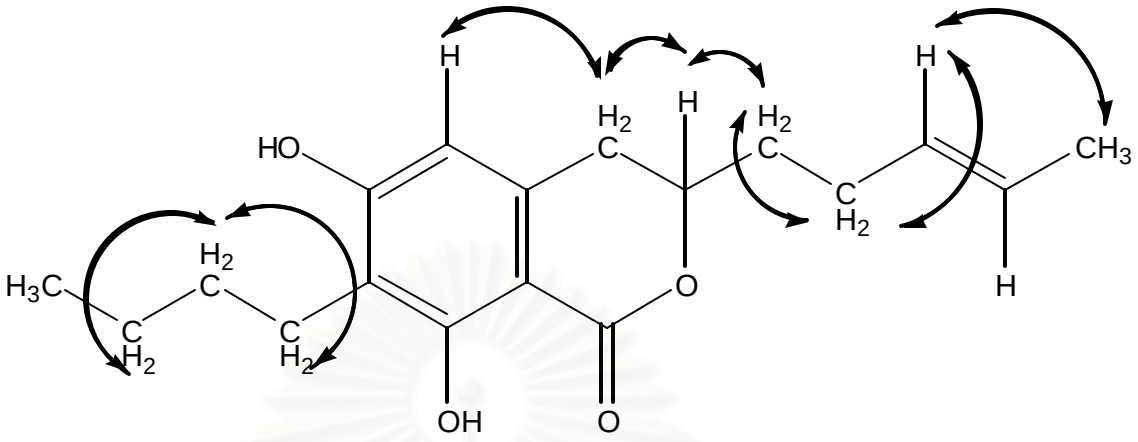



Figure 4.9 COSY correlation of Mixture CB1

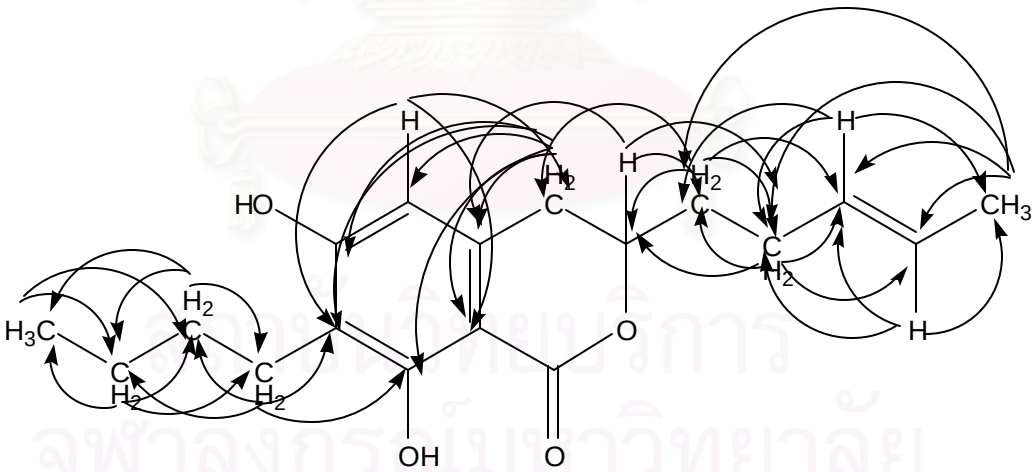



Figure 4.10 HMBC correlation of Mixture CB1

Table 4.10 HSQC, HMBC, and COSY spectral data of mixture CB1

| Position | δC | δH | HMBC (H to C) | COSY |
|---|---|---|---|---|
| 1 | 170.4(s) | - | - | |
| 3 | 78.5(d) | 4.54 (1H,m) | C-4a, C-9, C-10, | H-4(2.84), H-9(1.94) |
| 4 | 32.9(t) | 2.84 (2H, ddd, *J*=3.6, 16.4) | C-3, C-4a, C-5, C-6, C-7, C-8, C-8a, C-9 | H-3(4.54), H-5(6.25) |
| 4a | 138.2(s) | - | - | |
| 5 | 106.5(s) | 6.25(1H,s) | C-4, C-7, C-8a | H-4(2.84) |
| 6 | 160.0(s) | - | - | |
| 7 | 114.8(s) | - | - | |
| 8 | 162.3(s) | - | - | |
| 8a | 101.7(s) | - | - | |
| 9 | 34.7(t) | 1.94(2H,m) | C-3, C-4, C-10, C-11 | H-3(4.54), H-10(2.23) |
| 10 | 27.8(t) | 2.23(2H,m) | C-3, C-9, C-11, C-12 | H-9(1.94), H-11(5.44) |
| 11 | 129.5(d) | 5.44(1H,m) | C-9, C-10, C-13 | H-10(2.23) |
| 12 | 126.4(d) | 5.48(1H,m) | C-10, C-11, C-13 | H-13(1.67) |
| 13 | 17.9(q) | 1.67 (3H, d, *J*=6.0) | C-9, C-10, C-11, C-12 | H-12(5.48) |
| 14 | 22.3(t) | 2.66 (2H, t, *J*=7.6) | C-6, C-7, C-8, C-15, C-16 | |
| 15 | 30.9(t) | 1.55 (2H, q, *J*=7.6) | C-14, C-16, C-17 | H-14(2.66), H-16(1.42) |
| 16 | 22.8(t) | 1.42 (2H, qn, *J*=7.0) | C-14, C-15, C-17 | H-15(1.55),H-17(0.96) |
| 17 | 14.0(q) | 0.96 (3H, t, *J*=7.2) | C-15, C-16 | H-16(1.42) |
| 8-OH | | 11.42(1H,s) | | |

It could be concluded that mixture CB1 exhibited the $^{1}$H-NMR and $^{13}$C-NMR chemical shifts similar to 7-butyl-6,8-dihydroxy-3(*R*)-pent-11-enylisochroman-1-one. The spectroscopic and physical data of mixture CB1 were identical to those reported for 7-butyl-6,8-dihydroxy-3(*R*)-pent-11-enylisochroman-1-one obtained from *Geotrichum sp.*, an Endophytic fungus of *Crassacephalum crepidioides* was found to be active against *Plasmodium falciparum* and against *Candida albicans.* ( Kongsaeree *et al*., 2003)

The $^{1}$H-NMR and $^{13}$C-NMR chemical shifts of compound 1 and 7-butyl -6,8-dihydroxy -3(*R*)-pent-11-enylisochroman-1-one were compared as shown in Table 4.11 and 4.12, respectively.

Table 4.11 $^{1}$H-NMR spectra of mixture CB1 and 7-butyl-6,8-dihydroxy-3(*R*)-pent-11-enylisochroman-1-one

| Position | Chemical shifts (ppm) | |
|---|---|---|
| | CB1 | 7-butyl -6,8-dihydroxy -3(R)-pent-11-enylisochroman-1-one |
| 3 | 4.54 (1H,m) | 4.53 (1H,m) |
| 4 | 2.84(2H, ddd, *J*=3.6, 16.4) | 2.84 (2H, ddd, *J*=3.6, 16.4) |
| 5<br>8-OH | 6.25(1H,s)<br>11.42(1H,S) | 6.22(1H, s)<br>11.42(1H,S) |
| 9 | 1.94(2H,m) | 1.94 (2H, m) |
| 10 | 2.23(2H,m) | 2.22(2H,m) |
| 11 | 5.44(1H,m) | 5.45(1H,m) |
| 12 | 5.48(1H,m) | 5.50(1H,m) |
| 13 | 1.67(3H, d, *J*=6.0) | 1.66(3H, d, *J*=6.0) |
| 14 | 2.66(2H, t, *J*=7.6) | 2.65(2H, t, *J*=7.6) |
| 15 | 1.55(2H, q, *J*=7.6) | 1.54(2H, q, *J*=7.6) |
| 16<br>17 | 1.42(2H, qn, *J*=7.0)<br>0.96(3H, t, *J*=7.2) | 1.42(2H, qn, *J*=7.0)<br>0.95(3H, t, *J*=7.2) |

Table 4.12 $^{13}$C-NMR spectra of Mixture CB1 and 7-butyl-6,8-dihydroxy-3(*R*)-pent-11-enylisochroman-1-one

| Position | Chemical shifts (ppm) | |
|---|---|---|
| | CB1 | 7-butyl -6,8-dihydroxy -3(R)-pent-11-enylisochroman-1-one |
| 1 | 170.4(s) | 170.5(s) |
| 3 | 78.5(d) | 78.5(d) |
| 4 | 32.9(t) | 32.9(t) |
| 4a | 138.2(s) | 138.2(s) |
| 5 | 106.5(s) | 106.0(s) |
| 6 | 160.0(s) | 160.1(s) |
| 7 | 114.8(s) | 114.8(s) |
| 8 | 162.3(s) | 162.2(s) |
| 8a | 101.7(s) | 101.6(s) |
| 9 | 34.7(t) | 34.5(t) |
| 10 | 27.8(t) | 27.7(t) |
| 11 | 129.5(d) | 129.5(d) |
| 12 | 126.4(d) | 126.4(d) |
| 13 | 17.9(q) | 17.9(q) |
| 14 | 22.3(t) | 22.3(t) |
| 15 | 30.9(t) | 30.9(t) |
| 16 | 22.8(t) | 22.8(t) |
| 17 | 14.0(q) | 14.0(q) |
| 8-OH | | |

4.8.2. Antimicrobial and cytotoxic activities of mixture CB1

The antimicrobial activity of mixture CB1s was evaluated by the antimicrobial susceptibility test, broth microdilution method. The mixture CB1was examined at a concentration of 3.81-1,000 μg/ml (two-fold dilution). Antimicrobial activity tests were performed agains *B. subtilis* ATCC 6633, *C. albicans* ATCC 10231 and HSV-1 ATCC VR-260. The characteristic of Vero cell was infected by HSV-1 ATCC VR-260 is shown in Figure 4.11. The lowest concentration of mixture CB1 showing complete inhibition of growth is recorded as the minimal inhibition concentration (MIC) of antimicrobial activity and cytotoxic activity against 5 cell lines of mixture CB1 is shown in Table 4.13

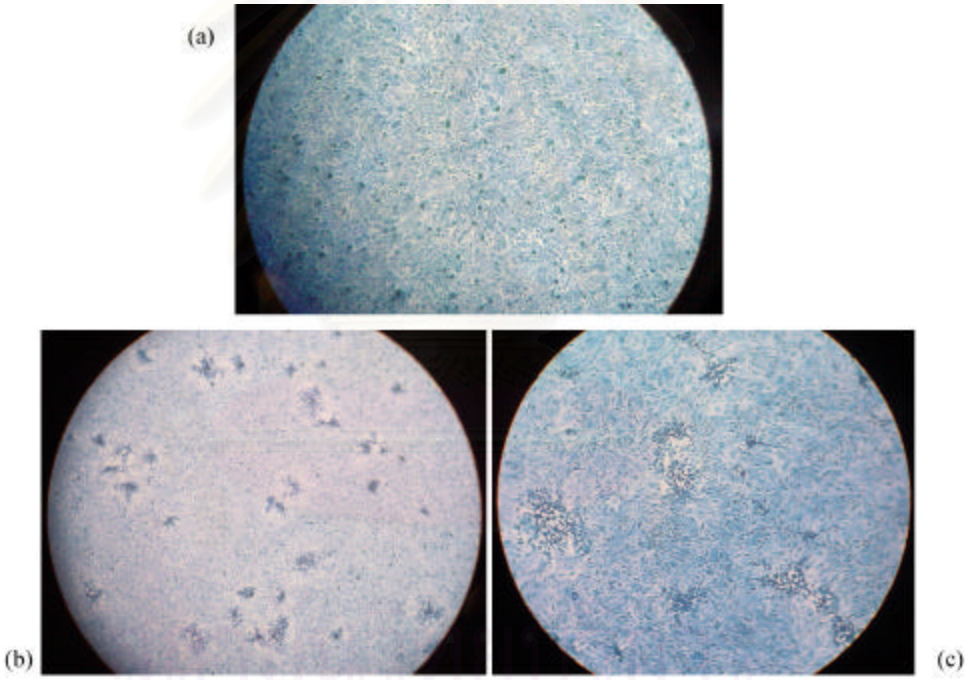



Figure 4.11 (a) normal Vero cell (b) and (c) herpes simplex virus type I infected in Vero cell at10X and 40X from inverted microscope

Table 4.13 Antimicrobial and cytotoxic activity of mixture CB1

| Test microorganism and cell | Results of activity (μg/ml) | |
|---|---|---|
| | MIC | $IC_{50}$ |
| Bacteria | | |
| ◆ *B. subtilis* ATCC 6633 | 3.81 | - |
| (Tetracycline HCl; MIC 31.25 μg/ml) | - | - |
| ◆ *E. coli* ATCC 25922 | - | - |
| ◆ *P. aeruginosa* ATCC 27853 | - | - |
| ◆ *S. aureus* ATCC 25923 | | |
| Fungi | | |
| ◆ *C. albicans* ATCC 10231 | 1000 | - |
| Virus | | |
| Herpes simplex virus type I ATCC VR -260 | - | 6.25 |
| Cytotoxic activity | | |
| Normal cell | | |
| ◆ Vero cell ATCC CCL-81 | - | 25 |
| Tumer cell | | |
| ◆ HEP-G2 (hepatoma) | - | 6.8 |
| ◆ SW620 (colon) | - | 5.5 |
| ◆ CHAGO (lung) | - | >10 |
| ◆ KATO-3 (gastric) | - | 7.4 |
| ◆ BT474 (breast) | - | 6.8 |

4.9 The characteristic of the endophytic fungus *Fusarium proliferatum* isolated from *Piper betle* Linn. and *Fusarium proliferatum* type strain plant pathogen

The characteristic of colony and pigment were difference color ; endophytic fungus *F. proliferatum* was produced dark purple but *F. proliferatum* type strain plant pathogen was produced pule purple on PDA. The colony characteristic of endophytic fungus *F. proliferatum* was produced dark purple but *F. proliferatum* type strain plant pathogen is shown in Figure 4.12

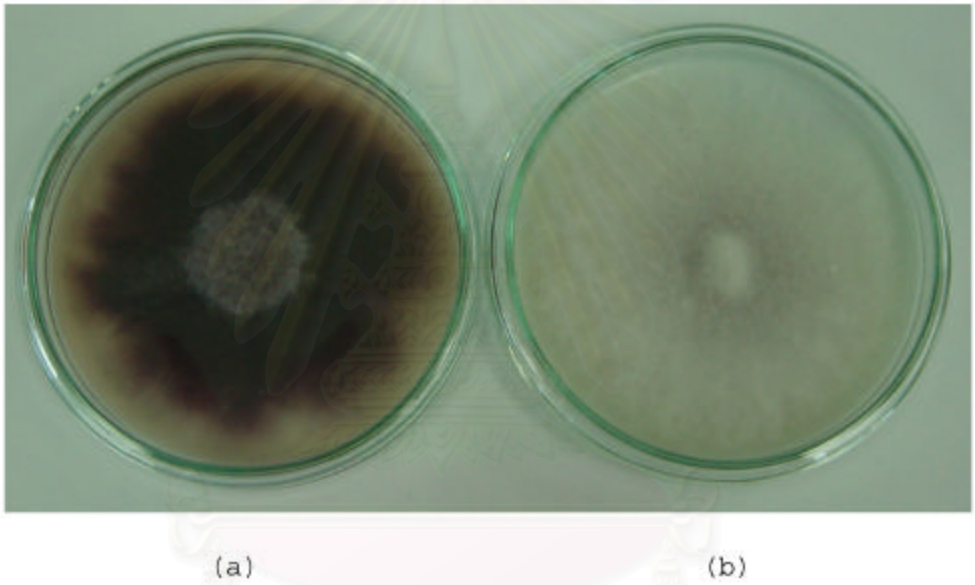





Figure 4.12 The colony characteristic of endophytic fungus *F. proliferatum* was produced dark purple (a) and *F. proliferatum* type strain plant pathogen *proliferatum* was produced pule purple (b)

The major fatty acids in all of the total lipid extracts from fungi were 16:0, 16:1, 18:0, 18:1, 18:2 and 18:3 (Cecil, 1966). The present study shows *Fusarium proliferatum* cultured in MCzB that produced the fatty acid were 14:0, 16:0, 18:0,18:1 and 18:2. The diglyceride were extracted 10.88 % from biomass. *Mucorales* was found that lipid content of fungi grown on glucose as the sole carbon source varied from 5-30% (Certik et al, 1993). In total 48 *Mucorales* fungi grown on media with sunflower oil accumulated 32.7-65.8% lipid in biomass (Certik et al, 1997).

The endphytic fungus isolate PBL004 cultured in various media for bioactive compounds produced by culturing were difference Csource, N- source and mineral, the fungus produced difference bioactive compounds.

In research, *F. proliferatum* produced bioactive compound was 7-butyl-6,8-dihydroxy-3(R)-pent-11-enylisochroman-1-one was a round white solid, m.p.158-159 $^{0}$C active against *B. subtilis* ATCC 6633, *C. albican* ATCC 10231, HSV-1 ATCC VR260 and tumor cells: HFP-G2(hepatoma), SW620(colon), KATO-3(gastric) and BT474(breast). Compound 7-butyl-6,8-dihydroxy-3(R)-pent-11-enylisochroman-1-one was found from endophytic fungi *Geotrichum* sp. isolated from stems of *Crassocephalum crepidiodes* S. Moore and was found to be active against *Plasmodium falciparum* and *C. albicans* (antimalarial and antifungal) (Kongsaeree et al., 2003).

*F. proliferatum* is found in a soid and plants produced mycotoxin: moniformin, fumonisins B1 and beauvericin. *F. proliferatum* was plant pathogen with corn, panicle and leaf sheath blight in rice or rots in orchid (Joseph et al, 2002, Rose et al, 1990, Ronald et al, 1994). Mycotoxin from *F. proliferatum* was case of equine leukoencephalomalacia (ELEM) and porcine pulmonary edema syndrome (PPE) with swine because contaminated in feed. *F. proliferatum* was reported as the agent of fatal disseniated infection in a child with lymphoblastic leukemia (Richard et al, 1988, Ronald et al, 1994).

The fungus *F. proliferatum* ; endophytic fungus and type strain plant pathogen were produced difference color pigment therefore *F. proliferatum* endophytic fungus and strain plant pathogen were difference strain.

# CHAPTER V
# CONCLUSION

Thirty-two isolates endophytic fungi from leaves of *Piper belte* Linn. were collected from Bangkok, Pathumthani, Rayong and Nakornsithammarat Province.

Endophytic fungi were isolated by using the surface sterilization method. The antimicrobial activities of these endophytes were investigated by agar diffusion method.

Fungal isolated PBL004 was chosen for further study because the this isolate PBL004 has the highest antimicrobial activities agent such as *B. subtilis* ATCC 6633, *E. coli* ATCC 25922 and *C. albicans* ATCC 10231. Fungal isolate PBL004 was identified as *Fusarium* sp. Based on morphological features. Moreover, this *Fusarium* sp. suggested on nucleotide sequencing of ITS region is *F. proliferatum*.

Chromatographic and crystallization techniques were used to isolate 7-butyl-6,8-dihydroxy-3(R)-pent-11-enylisochroman-1-one(220 mg, 7.21% yild of the EtOAc extract) from culture broth extract and mycelium extracts of endophytic fungi isolate PBL004. Mixture CB1, the major structures is 7-butyl-6,8-dihydroxy-3(*R*)-pent-11-enylisochroman-1-one that elucidated by using their physical properties and spectroscopic techniques. Mixture CB1 was isolated from broth extracts and diglyceride was isolated from mycelium extracts that value of 1001.4 mg/L culture broth. Diglyceride consist of five fatty acids ; myristic acid (0.40%), palmitic acid (27.21%), stearic acid (16.42%), oleic acid (37.49%) and linoleic acid (17.26%) with weight value of 0.4, 27.25, 16.44, 37.54 and 17.28 mg/ml, respectively.

Antimicrobial activity of isolate PBL004 from culture broth extract were active against *B. subtilis* ATCC 6633, *E. coli* ATCC25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923 and *C. albicans* ATCC 10231. The major structure of mixture CB1 is 7-butyl-6,8-dihydroxy-3(R)-pent-11-enylisochroman-1-one. This compound is round white solid (melting point 158-159 °C) that same as compound was found from endophytic fungi, *Geotrichum sp.* (Endomycetaceae), which isolated from stems of *Crassocephalum crepidiodes* S. Moore and found to be active against *Plasmodium*

*falciparum* (Kongsaeree et al., 2003). Antimicrobial activities and cytotoxicity of the mixture CB1 was tested. mixture CB1 was active against *B. subtilis* ATCC 6633 , *C. albicans* ATCC 10231 at the concentration 3.81, 1,000 respectively and HSV-1 ATCC VR-260 active against at $IC_{50}$ 6.25 μg/ml and activity on cytotoxicity against to tumer cells; HEP-G2, SW620, KATO-3 and BT474 with $IC_{50}$ 6.8, 5.5, 7.4 and 6.8 μg/ml, respectively.

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# APPENDICES

# APPENDIX A

## MEDIA

The media were prepared by sterilization in the autoclave at 121$^{0}$C for 15 minutes.

1. Media

1.1 Malt Czapek Agar (MCzA)

| | | |
|---|---|---|
| Czapek stock solution A | 50 | ml |
| Czapek stock solution B | 50 | ml |
| Sucrose | 30 | g |
| Malt Extract | 40 | g |
| Agar | 20 | g |
| Distilled water | 1 | L |

Czapek stock solution A

| | | |
|---|---|---|
| $NaNO_3$ | 4 | g |
| KCl | 1 | g |
| $MgSO_4 7H_2O$ | 1 | g |
| $FeSO_4 7H_2O$ | 0.02 | g |
| Dissolved in distillation water | 100 | ml |

Czapek stock solution B

| | | |
|---|---|---|
| $K_2HPO_4$ | 2 | g |
| Dissolved in distillation water | 100 | ml |

1.2 Malt Extract Agar (MEA)

| | | |
|---|---|---|
| Malt extract (powdered) | 20 | g |
| Peptone, bacteriological | 1 | g |
| Glucose | 20 | g |
| Agar | 15 | g |
| Distilled water | 1 | L |

1.3 Potato Dextrose Agar (PDA)

| | | |
|---|---|---|
| Potatoes, peeled and diced | 200 | g |
| Glucos e | 20 | g |
| Agar | 15 | g |
| Distilled water | 1 | L |

Boil 200 g of peels, diced potatoes for 1 hr in 1 L of distilled water. Filter, and make up the filtrate to 1 L. Add the glucose and agar and dissolve by steaming and sterilize by autoclaving at 121 ◦C for 15 min.

1.4 Dabouraud' s Dextrose Agar (SDA)

| | | |
|---|---|---|
| Peptone | 10 | g |
| Dextrose | 40 | g |
| Agar | 15 | g |
| Distilled water | 1 | L |

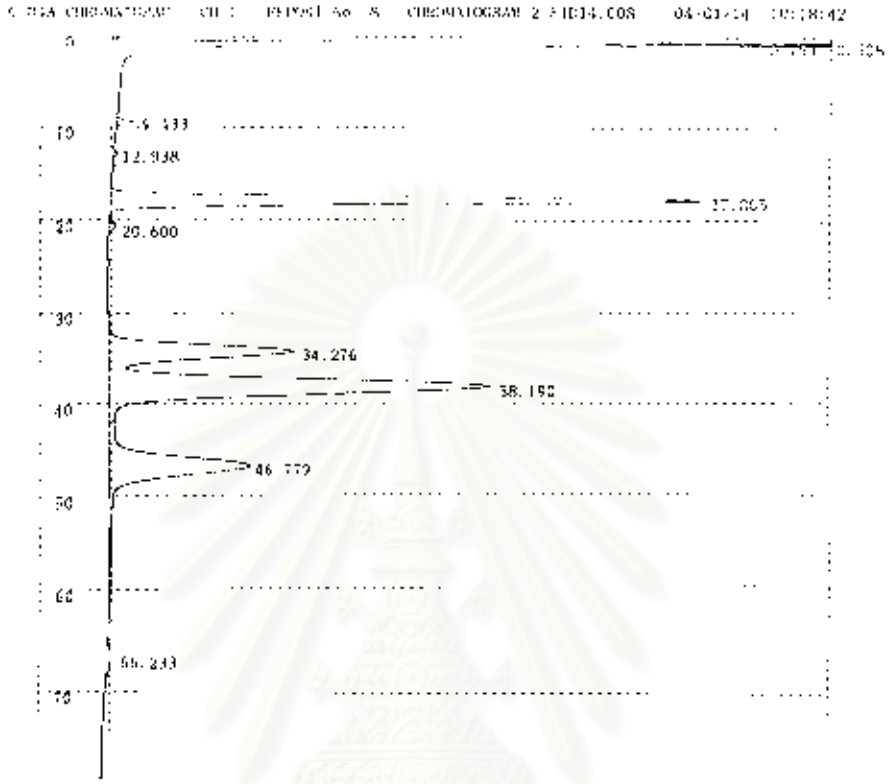
1.5 Yeast Extract Sucrose Medium (YES)

| | | |
|---|---|---|
| Yeast extract | 20 | g |
| Sucrose | 150 | g |
| Distilled water | 1 | L |

1.6 Nutrient Agar (NA)

| | | |
|---|---|---|
| Peptone | 5 | g |
| Beef extract | 3 | g |
| Agar | 15 | g |
| Distilled water | 1 | L |

# APPENDIX B




** CALCULATION REPORT **

| PK | PKNO | TIME | AREA | HEIGHT | MK | IDNO | CONC | NAME |
|---|---|---|---|---|---|---|---|---|
| I | 2 | 0.508 | 845D1D1 | 1202497 | SVE | | 89.5289 | |
| | 3 | 0.754 | 107125 | 23726 | T | | 1.0843 | |
| | 5 | 9.433 | 5052 | 184 | | | 0.0511 | |
| | 6 | 12.938 | 1248 | 36 | | | 0.0126 | |
| | 8 | 17.865 | 348674 | 6573 | | | 3.5322 | |
| | 9 | 20.6 | 4453 | 77 | | | 0.0451 | |
| | 10 | 34.276 | 217216 | 2058 | | | 2.1986 | |
| | 11 | 38.19 | 493089 | 4275 | V | | 4.9909 | |
| | 12 | 46.779 | 243711 | 1566 | V | | 2.4668 | |
| | 13 | 56.233 | 8845 | 49 | | | 0.0895 | |
| | | TOTAL | 9879810 | 1241041 | | | 100 | |



Figure B1 Result of diglyceride detected by GC

Figure B2 $^{1}$H-NMR spectrum of mixture M1

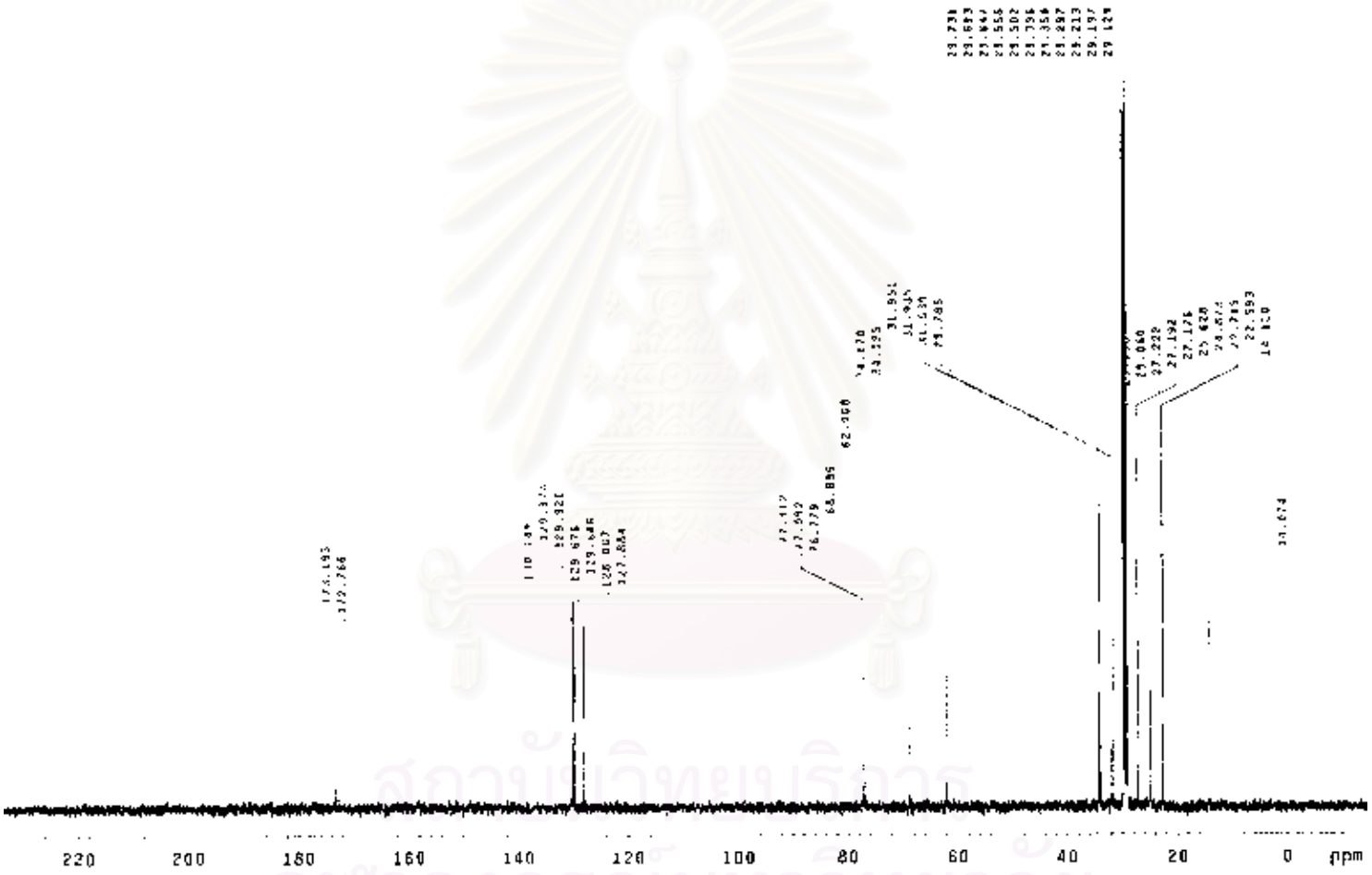



Figure B3 $^{13}$C-NMR spectrum of mixture M1

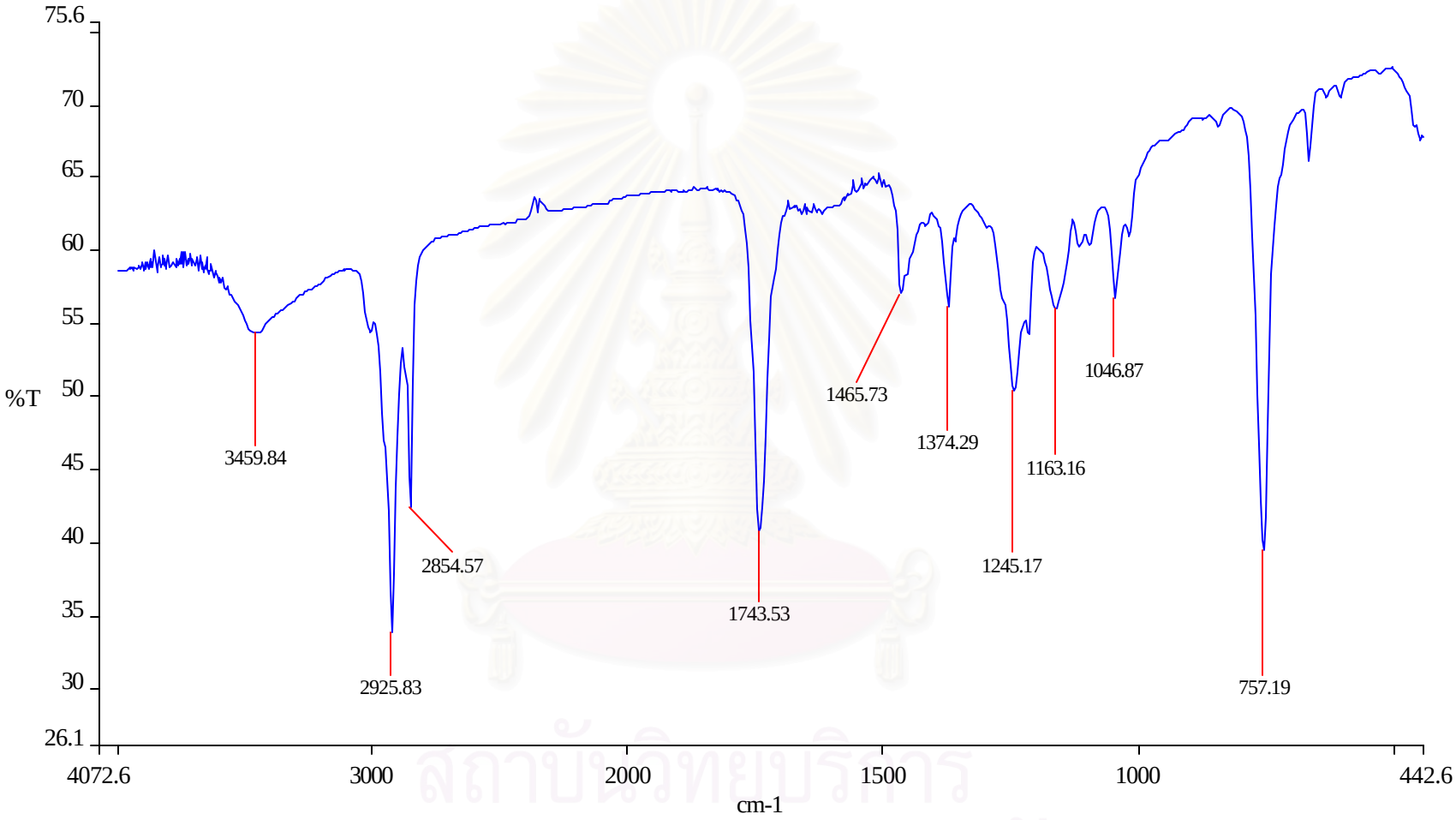



Figure B 4 IR spectrum of mixture M1

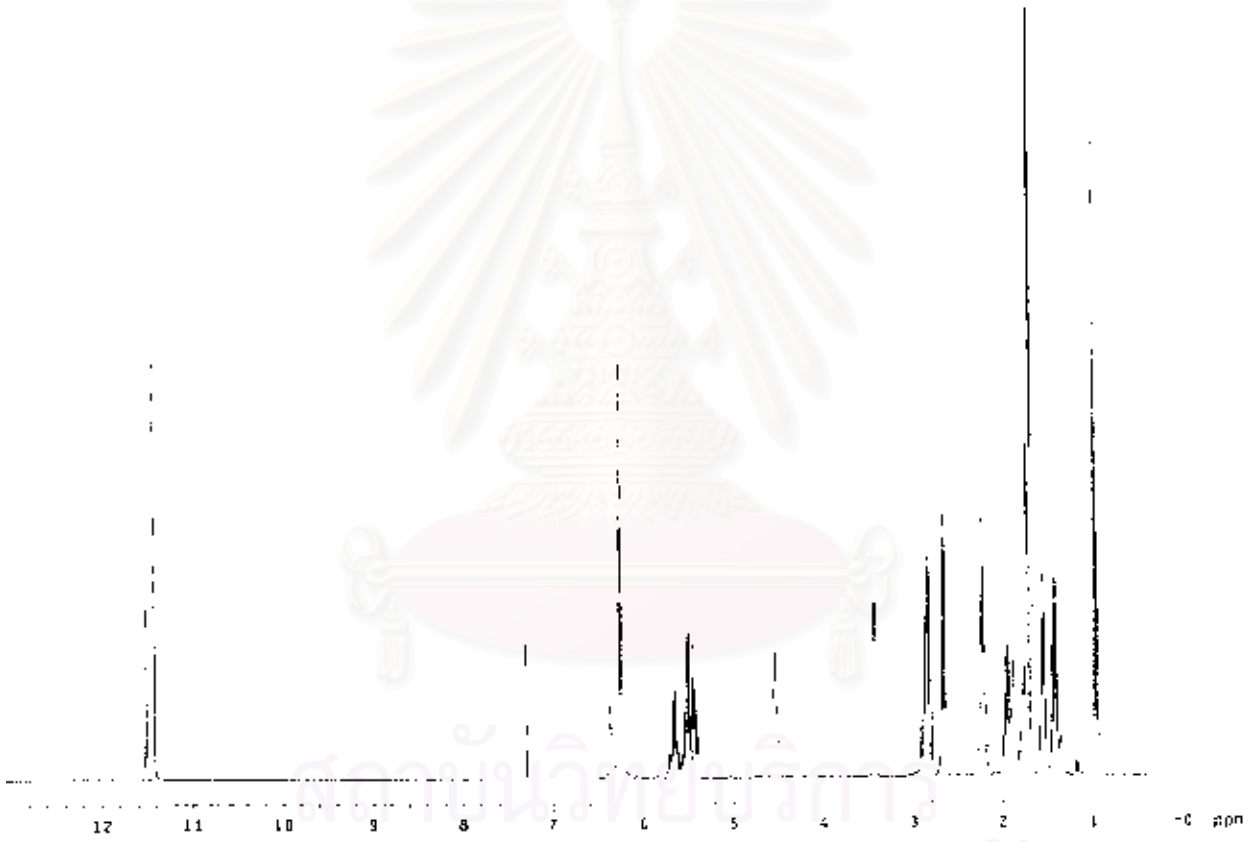



Figure B5 $^{1}$H-NMR spectrum of mixture CB1

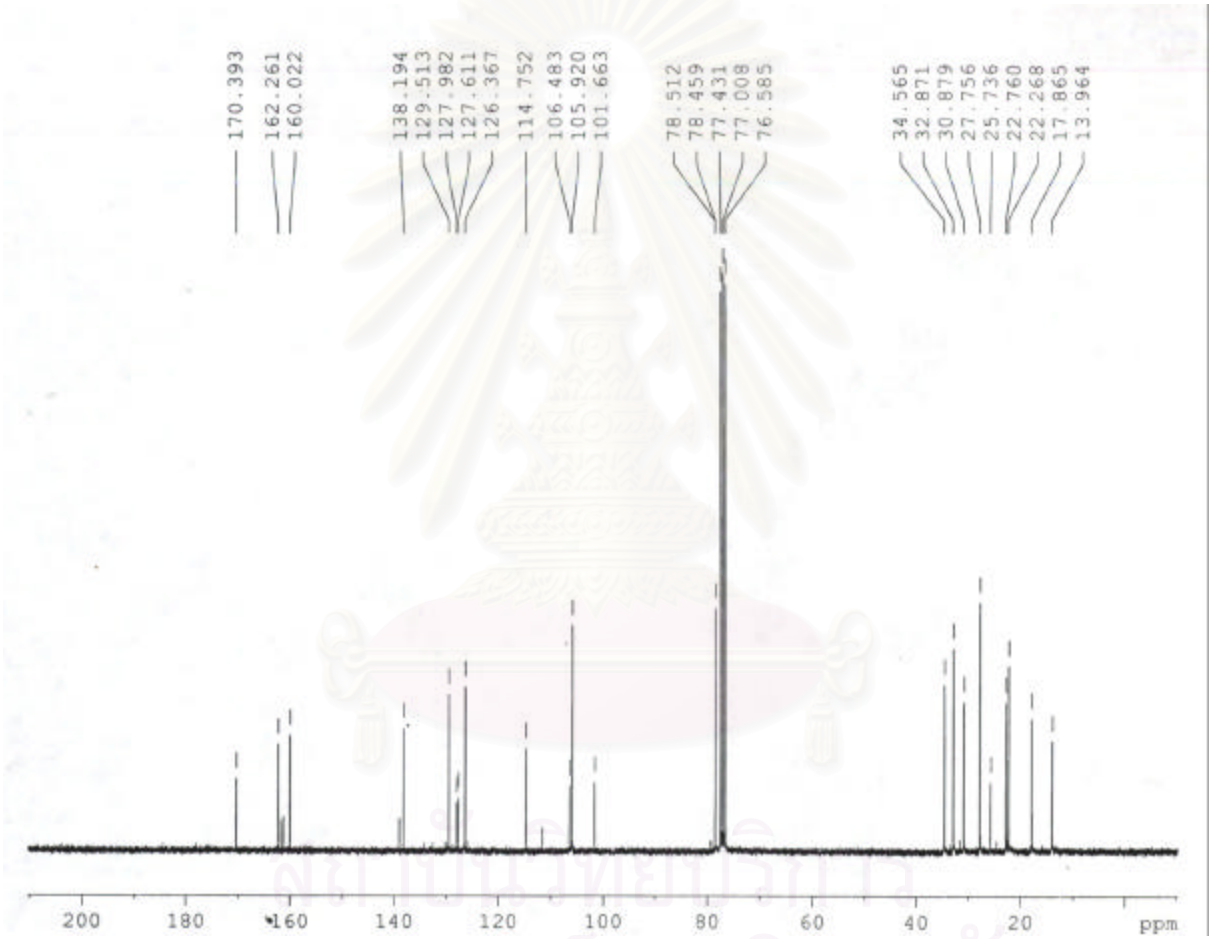



Figure B 6 $^{13}$C-NMR spectrum of mixture CB1

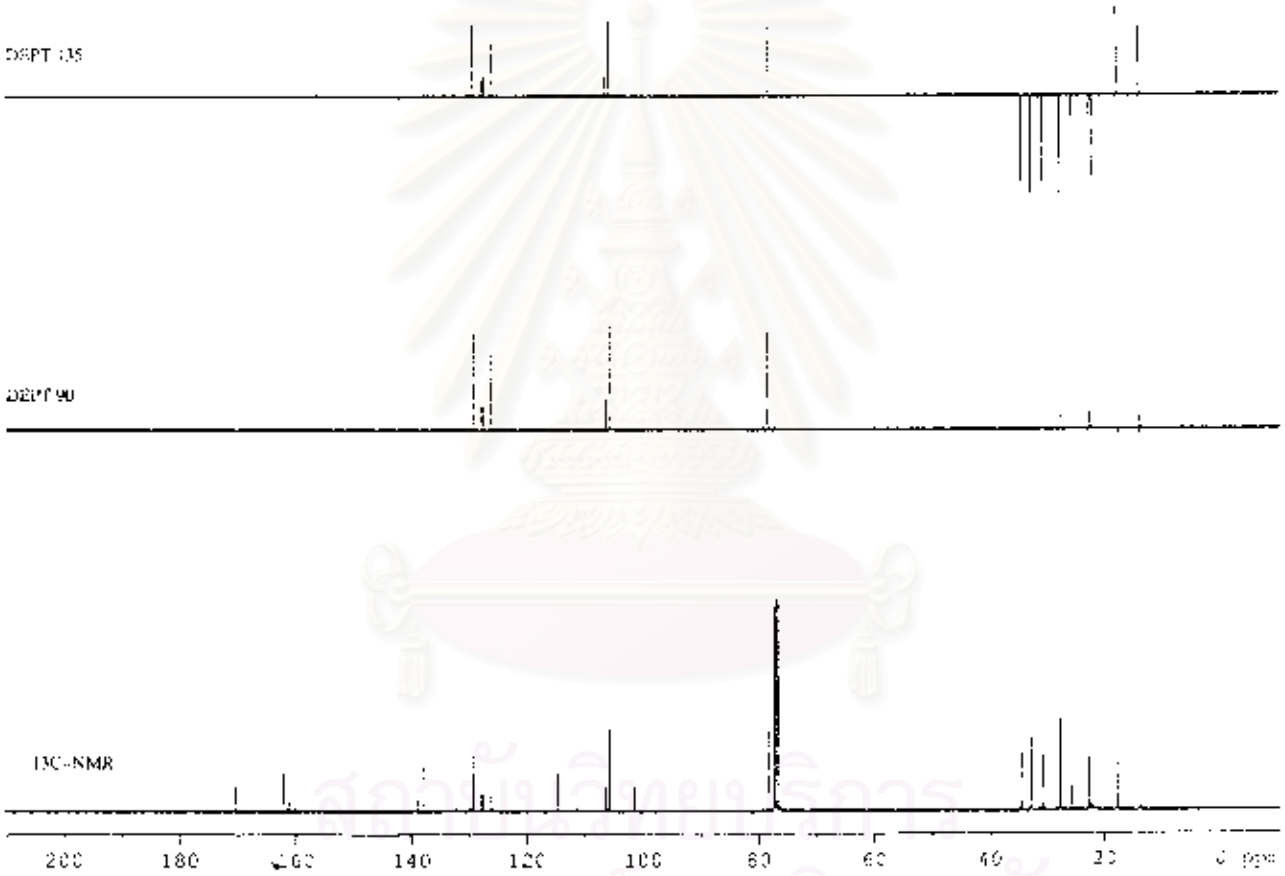



Figure B 7 DEPT spectrum of mixture CB1

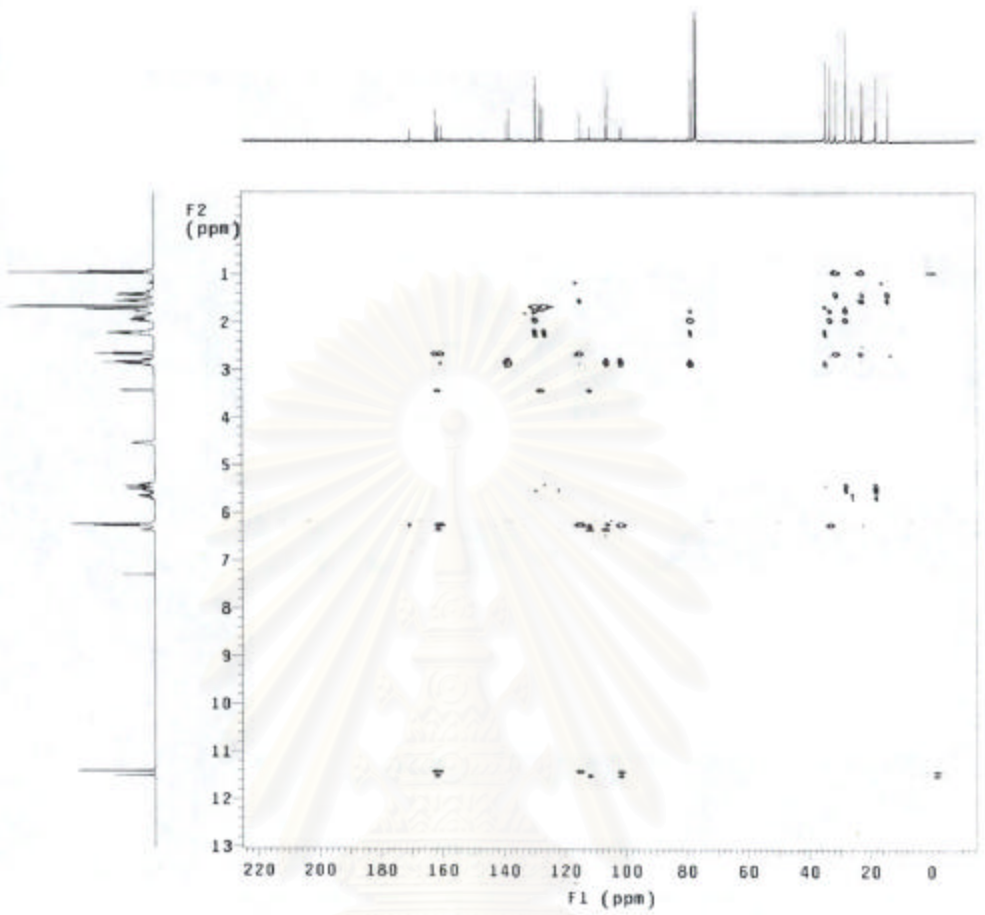



Figure B8 gHMBC spectrum of  mixture CB1

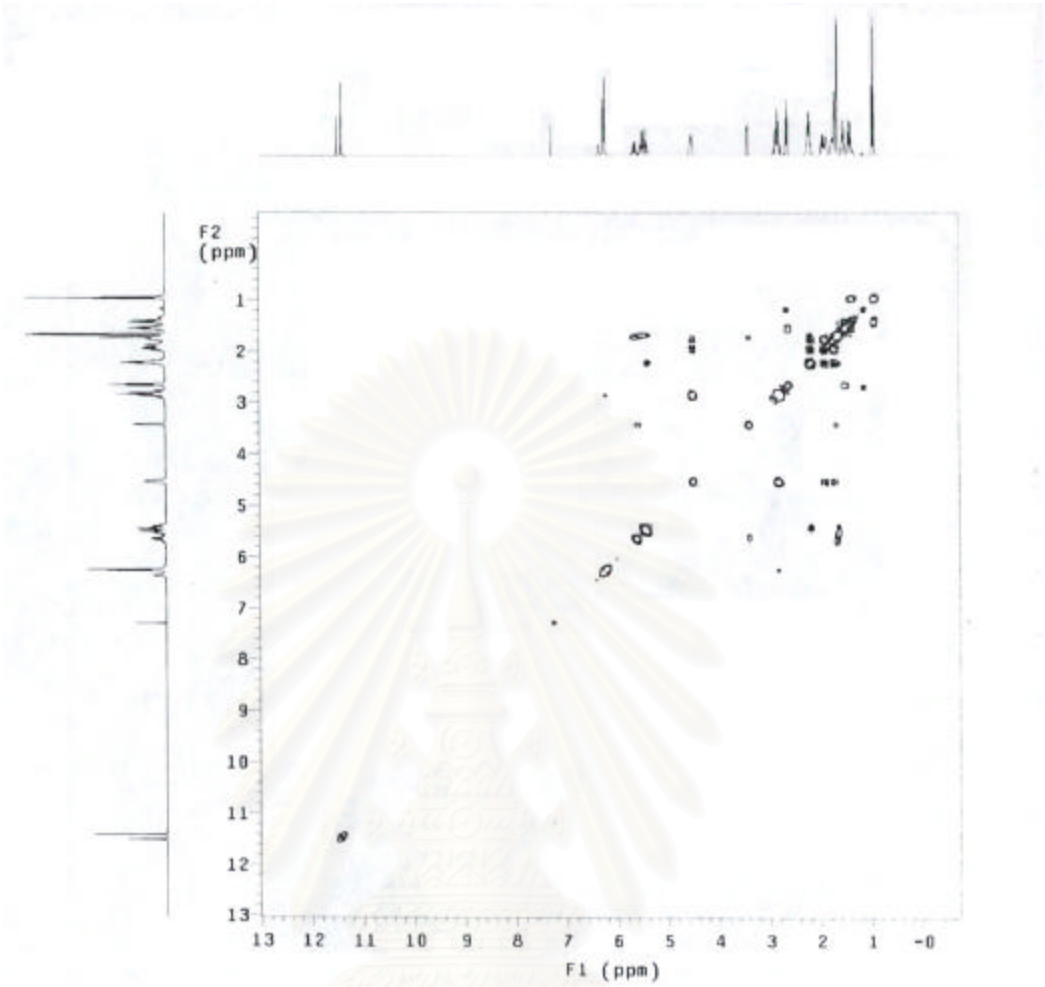



Figure B 9 COSY spectrum of mixture CB1

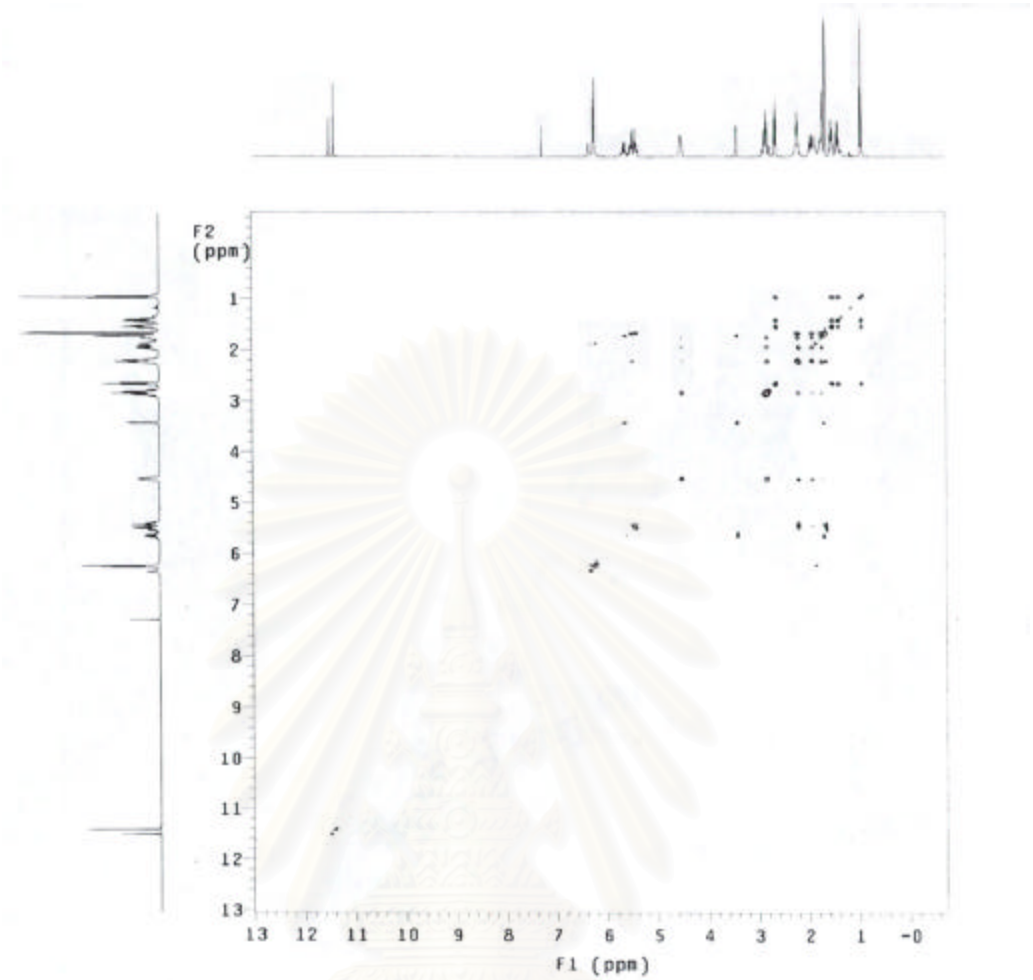



Figure B 10 TOCSY spectrum of mixture CB1

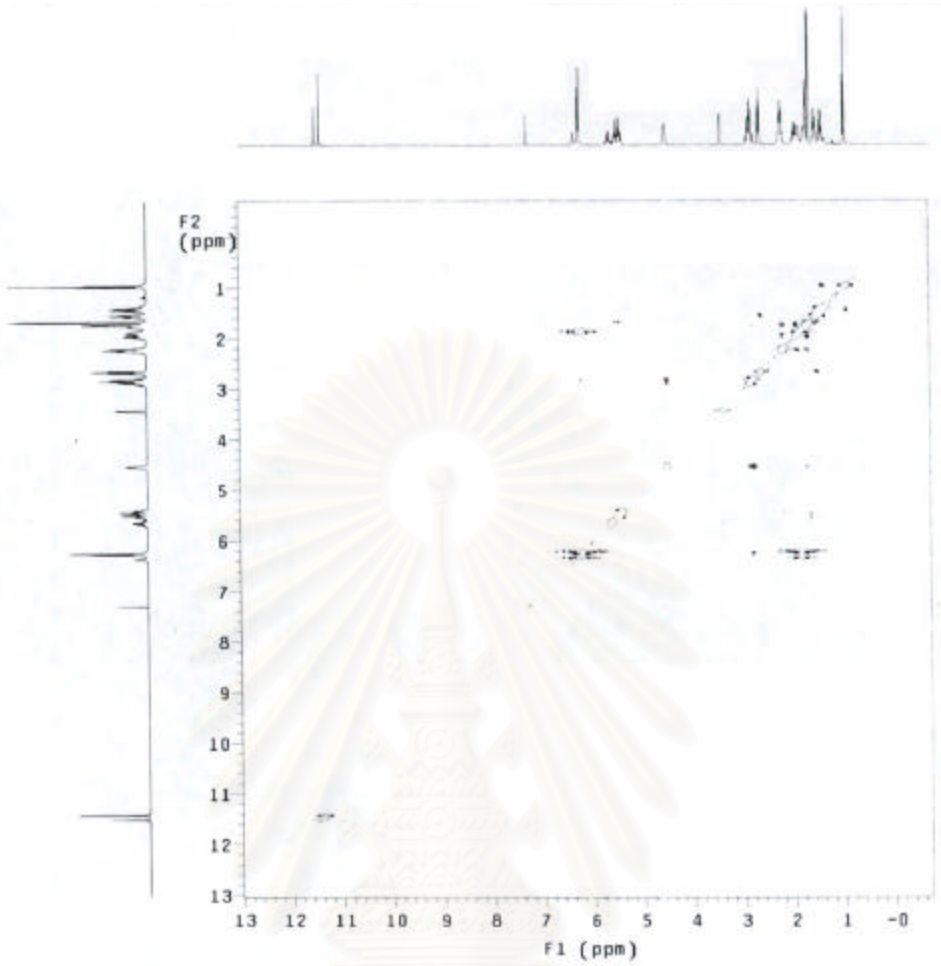



Figure B 11 NOESY spectrum of mixture CB1

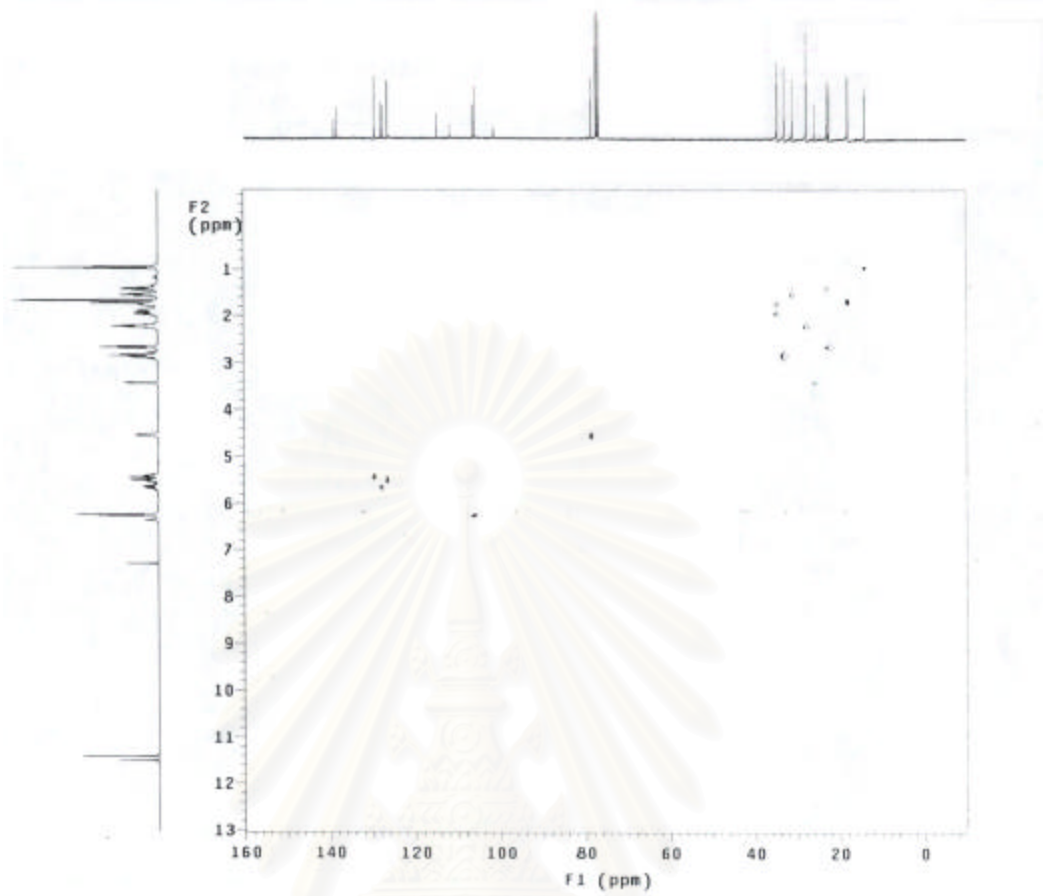



Figure B 12 gHSQC spectrum of mixture CB1

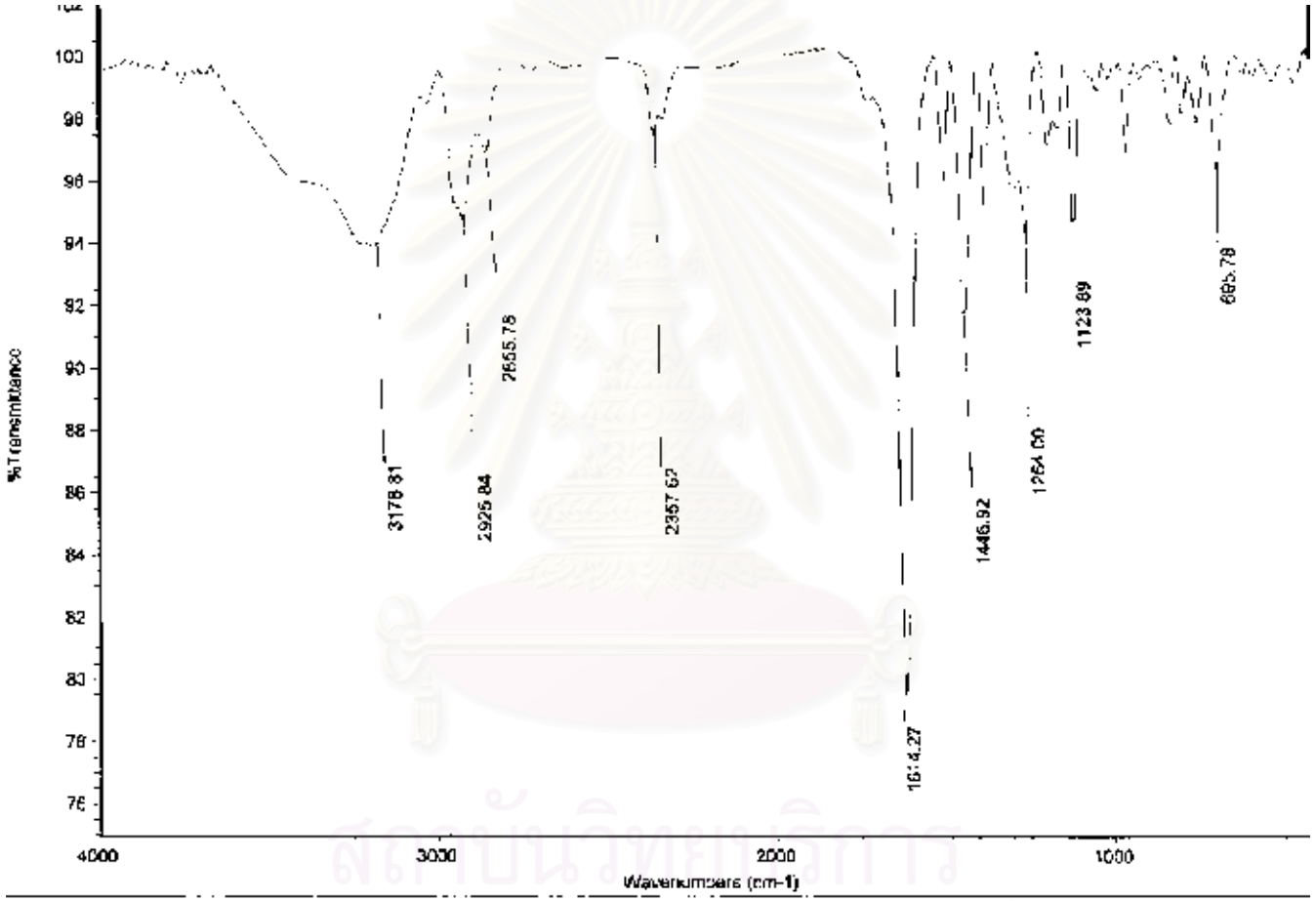



Figure B13 IR spectrum of mixture CB1

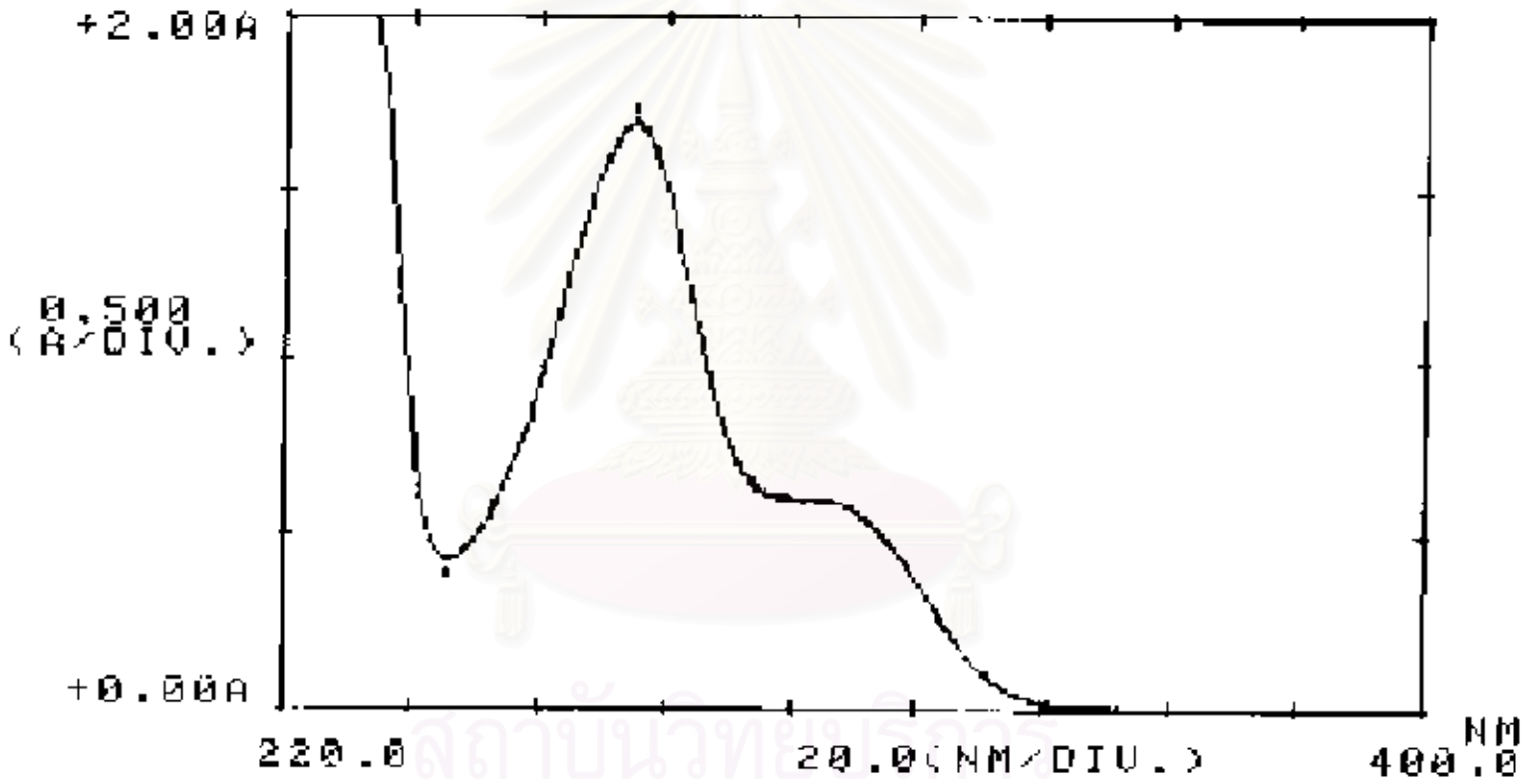



Figure B14 UV spectrum of mixture CB1

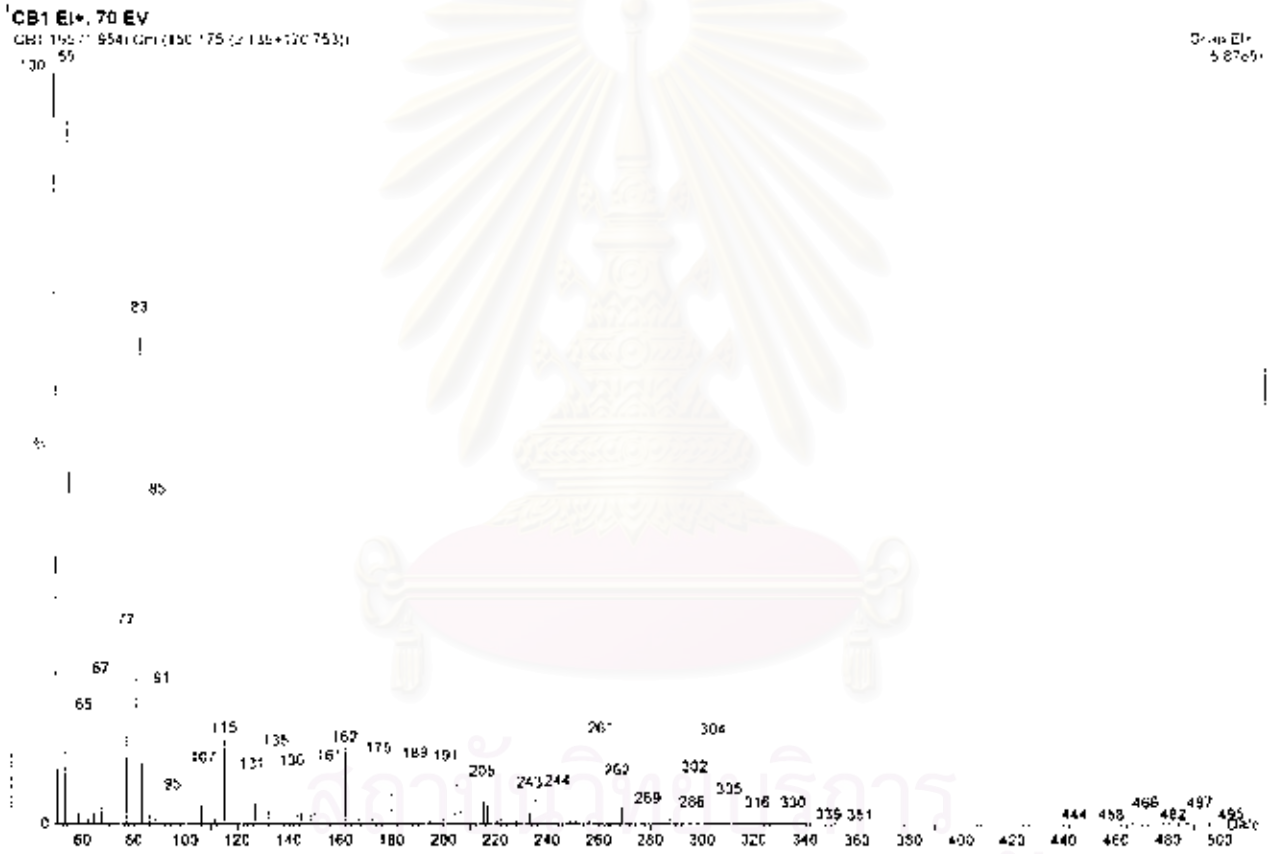



Figure B15 EI-MS spectrum of mixture CB1

# BIOGRAPHY

Mrs. Anuree Khanbun was born on May 16, 1974 in Nakornsithammarat province, Thailand. She graduated with a Bachelor Degree of Science in Microbiology from the faculty of Science, Prince of Songkhla University , Thailand in 1996. She has been studying for a Mastes Degree of Science in Biotechnology, Faculty of Science, Chulalongkorn University, Thailand since 2002.