สารออกฤทธิ์ทางชีวภาพของราเอนโดไฟต์ที่แยกจากเปล้าใหญ่ *Croton oblongifolius* ที่อำเภอกุยบุรี จังหวัดประจวบคีรีขันธ์

นายบำรุงศักดิ์ ปุริโส

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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BIOACTIVE COMPOUNDS OF ENDOPHYTIC FUNGI ISOLATED FROM *Croton oblongifolius* AT KUI BURI DISTRICT PRACHUAP KHIRI KHAN PROVINCE

Mr. Bumrungsak Puriso

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Field of study	Industrial Microbiology
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นายบำรุงศักดิ์ ปุริโส: สารออกฤทธิ์ทางชีวภาพของราเอนโดไฟต์ที่แยกจากเปล้าใหญ่ *Croton oblongifolius* ที่อำเภอกุยบุรี จังหวัดประจวบคีรีขันธ์ (BIOACTIVE COMPOUNDS OF ENDOPHYTIC FUNGI ISOLATED FROM *Croton oblongifolius* AT KUI BURI DISTRICT PRACHUAP KHIRI KHAN PROVINCE) อาจารย์ที่ปรึกษา: รศ. ดร. ประกิตติ์สิน สีหนนทน์, อ.ที่ปรึกษาร่วม: ศ. ดร. โสภณ เริงสำราญ 172 หน้า. ISBN 947-17-5080-3.

งานวิจัยนี้ทำการศึกษาสารออกฤทธิ์ทางชีวภาพที่สร้างจากราเอนโดไฟต์ที่แยกจากพืชสมุนไพรเปล้าใหญ่ *Croton oblongifolius* นำส่วนของใบ กิ่งและเปลือกของตัวอย่างพืชจากอำเภอกุยบุรี จังหวัดประจวบคีรีขันธ์ มาคัดแยกราโดยผ่านวิธีการฆ่าเชื้อที่พื้นผิวนอกและวางบน malt extracts agar สามารถแยกราเอนโดไฟต์ได้ทั้งหมด 62 ไอโซเลต จากการทดสอบเบื้องต้นในการสร้างสารออกฤทธิ์ทางชีวภาพของราเอนโดไฟต์ในการยับยั้งจุลินทรีย์ทดสอบโดยวิธี Agar well diffusion method พบว่าราเอนโดไฟต์ไอโซเลต PcBr20 สามารถสร้างสารซึ่งมีฤทธิ์ทางชีวภาพในการยับยั้งจุลินทรีย์ทดสอบได้กว้าง เมื่อทำการจัดจำแนกสายพันธุ์โดยศึกษาลักษณะทางสัณฐานวิทยาและการวิเคราะห์ลำดับนิวคลีโอไทด์ในบริเวณ internal transcribed spacer ของ rDNA พบว่าราเอนโดไฟต์ไอโซเลต PcBr20 คือ *Fusarium* sp. (Anamorph) หรือ *Gibberella sacchari* (Telemorph) ตามลำดับ เมื่อทำการศึกษาเพื่อหาสารออกฤทธิ์ทางชีวภาพโดยเลี้ยงในอาหารเหลว malt extracts broth แยกสารบริสุทธิ์จากน้ำหมักด้วยวิธีทางโครมาโตกราฟีและการตกผลึก และหาสูตรโครงสร้างของสารเหล่านี้โดยอาศัยสมบัติทางกายภาพและเทคนิคทางสเปกโตสโกปี พบว่าได้ของผสม 1 ชนิด คือ fusaric acid และ dehydrofusaric acid (ของผสม BH1) และสารบริสุทธิ์ 1 ชนิด (สาร BE1) ซึ่งยังไม่สามารถบอกสูตรโครงสร้างได้ มีลักษณะเป็นของแข็งอสัณฐานสีขาว จุดหลอมเหลว 190-191^{o}C นำสารบริสุทธิ์ที่แยกได้มาทดสอบฤทธิ์ทางชีวภาพในการยับยั้งจุลินทรีย์ทดสอบพบว่าของผสม fusaric acid และ dehydrofusaric acid มีฤทธิ์ยับยั้ง *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Saccharomyces cerivisiae* TISTR 5169, *Candida albicans* ATCC 10231 และ *Trichophyton mentagrophytes* โดยมีค่า MIC เท่ากับ 0.98, 62.50, 15.62, 1x10^{3}, 62.50, 1x10^{3}, และ 1x10^{3} µg/ml, ตามลำดับ และสาร BE1 มีฤทธิ์ยับยั้ง *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922 และ *P. aeruginosa* ATCC 27853 โดยมีค่า MIC เท่ากับ 0.98, 62.50, 62.50 และ 1x10^{3} µg/ml, ตามลำดับ

ภาควิชา..จุลชีววิทยา.....ลายมือชื่อนิสิต.....

.สาขาวิชา..จุลชีววิทยาทางอุตสาหกรรม....ลายมือชื่ออาจารย์ที่ปรึกษา.....

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BUMRUNGSAK PURISO: BIOACTIVE COMPOUNDS OF ENDOPHYTIC FUNGI ISOLATED FROM *Croton oblongifolius* AT KUI BURI DISTRICT PRACHUAP KHIRI KHAN PROVINCE. THESIS ADVISOR: ASSOC. PROF. PRAKITSIN SRIHANONTH, Ph.D., THESIS COADVISOR: PROF. SOPHON ROENGSUMRAN, Ph.D. 172 pp. ISBN 947-17-5080-3.

The purpose of this research was to study bioactive compounds from endophytic fungi isolated from *Croton oblongifolius*, Thai medicinal plant. Plant samples were collected from Kui Buri District, Prachuap Khiri Khan Province. Fungal endophytes were isolated from leaves, branches, and bark sections by surface sterilization method and placed on malt extract agar. In total 62 fungal isolates were obtained and tested for the production of antimicrobial compounds. Fungal isolate PcBr20 was chosen for the further study of bioactive compounds because this isolate produced the compounds that were active against a large number of test microorganisms. Based on morphology and nucleotide sequencing of ITS regions of rDNA, isolate PcBr20 was identified as *Fusarium* sp. (Anamorph) or *Gibberella sacchari* (Telemorph), respectively. Chromatographic techniques and crystallization were used to isolate bioactive compounds from malt extract culture broth. Structure elucidation of the pure compounds were investigated using physical properties and spectroscopic techniques. Two compounds were isolated, a mixture of fusaric acid and dehydrofusaric acid (mixture BH1) and compound BE1 which was a white amorphous solid (mp. 190-191 ^{o}C). The structures of compound BE1 are still under investigation. Antimicrobial activities of pure compounds were tested. It was found that a mixture of fusaric acid and dehydrofusaric acid exhibited against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Saccharomyces cerivisiae* TISTR 5169, *Candida albicans* ATCC 10231, and *Trichophyton mentagrophyte* with MIC value of 0.98, 62.50, 15.62, 1x10^{3} , 62.50, 1x10^{3} and 1x10^{3} µg/ml, respectively and compound BE1 showed against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, and *P. aeruginosa* ATCC 27853 with MIC value of 0.98, 62.50, 62.50 and 1x10^{3}µg/ml, respectively.

Department..Microbiology……..Student's signature……..

Field of study..Industrial Microbiology..Advisor's signature……..……...

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LIST OF ABBREVIATIONS

$[\alpha]_D^{20}$	= Specific rotation at 20° and Sodium D line (589 nm)
ATCC	= American Type Culture Collection, Maryland, U.S.A.
br s	= broad singlet (for NMR spectral data)
°C	= degree Celsius
^{13}C-NMR	= carbon-13 nuclear magnetic resonance
$CDCl_3$	= deuterated chloroform
$CHCl_3$	= chloroform
CD_3OD	= deuterated methanol
cm	= centimerter
COSY	= ^{1}H-^{1}H correlation spectroscopy
δ	= 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)
ε	= molar absorptivity
EIMS	= electron impact mass spectroscopy
eq	= equatorial
EtOAc	= ethylacetate
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	= litter
µl	= microlitter
λ_{max}	= wavelength of maximum absorption

LIST OF ABBREVIATIONS (CONTINUED)

$[M+H]^+$ = protonated molecular ion

m = multiplet (for NMR spectral data)

MeOH = methanol

MIC = Minimum inhibitory concentration

mg = miligram

µg = microgram

MHz = megaheartz

MIC = minimum inhibitory concentration

ml = milliliter

mm = millimeter

ν_{max} = wave number at maximum absorption

NMR = nuclear magnetic resonance

No. = Number

ppm = part per million

s = singlet (for NMR spectral data)

SDA = sabuoraud dextrose agar

SEM = scanning electron microscope

t = triplet (for NMR spectral data)

TLC = thin layer chromatography

UV = ultraviolet

CHAPTER I

INTRODUCTION

The Fungi are everywhere and affect our lives every day. From mushrooms to industrially important products to plant symbioses to plant pathogens to human disease. Fungi affect human lives in many and varied ways, therefore it is important to know something about fungal biology in order to be able to control or exploit them for our own purpose (Carlile, Watkinson, and Gooday, 2001).

Many fungi are detrimental, inciting a large number of plant diseases which result in the loss of billions of dollars worth of economic crops each year, and an increasing number of animal diseases, including many human maladies. Fungi can cause human disease, either directly or through their toxins, including mycotoxins and mushroom poisons. They often cause rot and contamination of foods. They can destroy almost every kind of manufactured good. In this age of immunosuppression, previously innocuous fungi are causing more and more human disease (Carlile et al., 2001).

There are many ways in which people have learned to exploit fungi. There are many edible mushrooms, both cultivated and collected from the wild. Yeasts have been used in baking and brewing for many millennia. Antibiotics such as penicillin and cephalosporin are produced by fungi. The immunosuppressive anti-rejection transplant drug cyclosporin in produced by the mitosporic fungus *Tolypocladium inflatum*. Steroids and hormones, and even birth control pills are commercially produced by various fungi. For example, citric acid in cola and other soda pop products which are produced by an *Aspergillus* species. Some gourmet cheeses such as Roquefort and other blue cheeses, brie and camembert are fermented with certain *Penicillium* species. Additionally stone washed jeans are softened by *Trichoderma* species. There are undoubtedly many potential many potential uses that have not yet been explored (Carlile et al., 2001).

This research is interested in endophytic fungi. Endophytic fungi are fungi which live almost entirely within the leaves and stems of apparently healthy host plants, doing so asymptomatically, causing no visible signs of infection (Isaac, 1992). Recent interest

has focused on endophytic fungi for their pharmaceutical, medicinal, and agricultural potential. For example, the fungal endophytes *Taxomyces andreanae* and *Pestalotiopsis micorspora*, and several other fungi isolated form the bark of yew trees, are potential new sources of the anticancer drug taxol. Furthermore, the clavicipitaceous grass endophytes are known to produce indole derivatives and other products that are active as plant hormones, antifungal agents, hallucinogens, vasoconstrictors, etc. (Bacon and White, 2000).

Croton oblongifolius is a very interesting Thai medicinal plant that is studied in this research because it is believed that all parts of the plant can be used in the treatment of many ailments. For example, the leaves can be used as a tonic, the flowers are used as a teniacide, the fruits are used to treat dysmenorrhea, the seeds are used as a purgative, the bark is used to treat dyspepsia, and the roots are used to treat dysentery (เสงี่ยม พงษ์บุญรอด, 2502).

Objectives

1. To isolate and identify the endophytic fungi found within healthy young and mature leaves, branches, and barks of *Croton oblongifolius* at Kui Buri District, Prachuap Khiri Khan 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

LITERATURE REVIEW

2.1 Endophytic fungi

Endophytic fungi form inconspicuous infections within tissues of healthy plants for all or nearly all their life cycle (Siegal, Latch, and Johnson, 1987). Endophytes, in contrast to epiphytes, are contained entirely within the substrate plant (Figure 2.1) and may be either parasitic or symbiotic. Endophytic fungi are asymptomatic and may be described as mutualistic (Clay, 1991). The major features of mutualistic symbioses include the lack of destruction of most cells or tissues, nutrient or chemical cycling between the fungus and host, enhanced longevity and photosynthetic capacity of cells and tissues under the influence of infection, enhanced survival of the fungus, and a tendency toward greater host specificity than seen in nectrophic infections (Lewis, 1973). A comparison of the fitness of the host and fungus when living independently in contrast to their fitness when living in association is the major means of determining whether a specific symbiotic association is mutualistic or parasitic (Lewis, 1974).

Members of the Ascomycota, Basidiomycota, Deuteromycota, and some Oomycetes have been isolated as endophytes. Endophytic fungi have been isolated from phanerogams in alpine, temperate and tropical regions, although the plants of the Coniferae, Ericaceae and Grmineae have been most intensively sampled (Clay, 1991; Petrini, 1986; Siegal et al., 1987)

Some fungal endophyte-grass associations, such as that between the perennial rye grass *Lolium perenne* and the fungus *Epichloe* (anamorph *Acremonium*), are common and widespread in natural populations. The association has become very close and the endophyte invades the flowers, is incorporated in the seeds, and passed to the next generation of host grass. The life cycles of endophytic fungi in grasses are shown in the Figure 2.2 (Carlile et al., 2001).

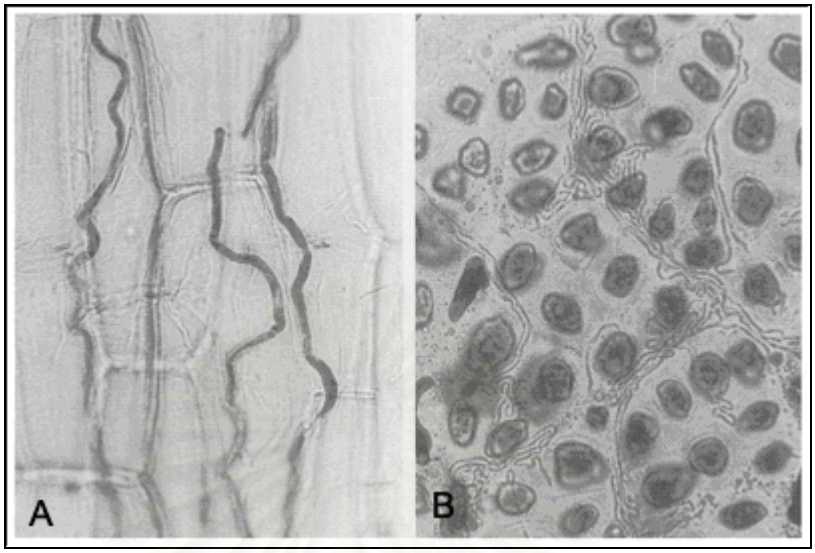


Figure 2.1 Vegetative growth in endophytic fungi of grasses. (A) Endophytic hyphae of *Acremonium coenophialum* in *Festuca arundinacea* (tall fescue grass) leaf tissue. (B) Hyphae of *Acremonium lolii* in the aleurone layer of seed from *Lolium perenne* (perennial rye grass) (Isaac, 1992).

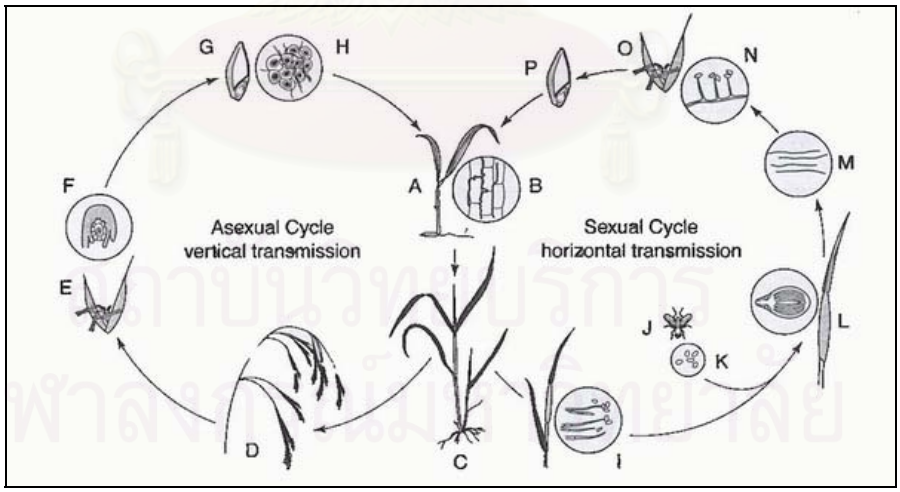


Figure 2.2 The life cycles of endophytic fungi in grasses. Left: in some fungus-endophyte associations the fungus is transmitted from parent to progeny by vertical transmission down the generations. The fungus does not sporulate at all on the surface of the plant (D), but its mycelium is passed to the next

generation exclusively through infection of the ovule (E,F) and seeds (G,H) by vegetative mycelium (B) present in the tissues of the host grass. This is the sole mode of transmission in some grass-endophyte associations, for example *Neotyphodium lolii* in the perennial rye grass *Lolium perenne.* It results in the inheritance of clones of the endophyte, unchanged by sexual genetic recombination. Molecular phylogenetic studies comparing ribosomal or ribosomal or nuclear gene sequences in grasses and their endophytes show that the fungus and grass species in such associations have often evolved in parallel. Right: The fungus is transmitted from one grass plant to another by means of spores produced on the fungal reproductive structure (stroma). A stroma forms (I) on which the fungus's sexual structures, receptive hyphae and spermatia are produced. Insects (j) carry spermatia (K) from one stroma to another, effecting cross-fertilization which is followed by the production of ascospores in perithecia (L) developing in the stroma on the grass. The thread-like ascospores (M) are wind dispersed to grass flowers, where they germinate and produce secondary conidia (N) which may infect ovules (O) and developing seeds (P) of a new host after entering through stigmata. As a result the next generation of plants is infected by new strains of the endophyte. A typical example of such an association is *Epichloe typhina* infecting the cocksfoot grass *Dactylis glomerata.* Several grass endophyte associations, for example *Epichloe festucae* on red fescue, *Festuca rubra*, are capable of both seed and ascospore transmission, the balance depending on the environmental conditions or genotypes involved (Carlile et al., 2001).

By definition, endophytic colonization or infection cannot be considered as causing disease, since a plant disease is an interaction between the host, parasite, vector and the environment over time which results in the production of disease signs and/or symptoms. The distinction between an endophyte and a pathogen is not always clear. A mutation at a single genetic locus can change a pathogen to nonpathogenic

endophytic organism with no effect on host specificity (Freeman and Rogriguez, 1993). Many pathogens undergo an extensive phase of asymtomatic growth corresponding to colonization and then latent infection before symptoms appear. Additonally many pathogens of economically important crops may be endophytic or latent in weeds (Cerkauskas, 1988; Cerkauskas et al., 1983; Hartman et al., 1986; Hepperly et al., 1980; Kulik, 1984; McLean and Roy, 1988; Raid and Pennypacker, 1987) . Alternately, nonpathogenic endophytic organisms may play a role as biocontrol agents (Freeman and Rogriguez, 1993). Both endophytic and latent infection fungi can infect plant tissues and become established after penetration. However, infection does not imply the production of visible disease symptoms (Redin and Carris, 1996).

2.2 Host specificity

The degree of host specificity which operates in endophytic fungi is not yet clear. Some species are commonly occurring and may be isolated from various host plant species and from different locations with differing environmental conditions. In general terms, the geographical occurrence of endophytes is related to the distribution of host species. In some cases almost all individuals in a plant population may be infected by endophytes. *Cladosporium* spp., *Nodulisporium* spp. and *Pleospora* spp. are common. Some endophytes, however, do not show such a wide species range and are often isolated from plants of the same family or closely related families. Other species are only rarely detected (Isaac, 1992).

The degree to which endophytes are tissue or organ specific is also not yet clear. Some species are most commonly isolated from similar tissues, particularly the endophytes of conifer needles (Carroll and Petrini, 1983). In other cases the occurrence is less distinct. However, only limited surveys have been carried out to date (Isaac, 1992).

2.3 Endophytic mutualism

2.3.1 Effects of endophyte infections

Many endophytes live almost entirely within the host plant tissues, often without causing any visible signs of infection. Fungal hyphae penetrate between plant cells or may also grow intracellularly and must obtain nutrient materials through this intimate contact with the host. The occurrence of specialised feeding structures has not been reported in these fungi. Hyphae are sometimes quite wide in diameter (10-15μm) when in association with plant cells and may be distorted, irregular or bulbous in form. In some instances considerable amounts of fungal biomass are supported in host plant tissues. In physiological terms relatively little is known about the endophytic interactions between host and fungus and it is not easy to see how a host plant may benefit from such a relationship. Endophytic associations do not lead to the development of disease symptoms but do result in some morphological and physiological changes in host tissues which increase the survival and vigour of the plants concerned. Such physiological enhancement would be likely to increase the capacity of a plant to resist disease. It has also been suggested that endophyte-infected plants are more tolerant of water stress and recover more quickly than uninfected individuals (Belesky et al., 1987), although it is not clear quite how photosynthetic rates are affected by the presence of endophytes (Clay, 1989). There are suggestions that endophytes may produce plant growth regulators, which may alter the normal developmental pattern of the host plant (Porter et al., 1985). Reports of secondary metabolite production by endophytes, e.g. alkaloids and antibiotics, which affect a range of herbivores, have attracted a great deal of attention. It has also been suggested that endophytes provide the plant with a chemical defense mechanism (Isaac, 1992).

2.3.2 Effects on insects

One of the main reasons for the recent increase in interest in endophytic fungi has been the realization that endophyte infections have a marked effect on the grazing of insect pests. The presence of endophytes makes plant tissues unacceptable and

unpalatable to insects such that infected tissues are avoided. Additionally, infected tissues may give rise to toxic effects on the insects causing poor larval growth and development, reduction in reproduction capacity or death of individuals. Therefore having more far-reaching effects on insect populations (Isaac, 1992).

A range of species of insects has been reported to be negatively affected by endophyte-infected grasses, including crickets, aphids, armyworms and flour beetles. Much of this information has led to the suggestion that endophytic fungi may be suitable biocontrol agents for the protection of grass species (Clay, 1989). A range of field and laboratory feeding experiments with endophyte-infected grasses saw that these were toxic to insects and led to a reduction in the rate of growth and development (Isaac, 1992).

An interesting example of the effects of endophytes on insects is provided by the interaction between elm bark beetles and elm trees. The beetles attack elm trees by burrowing into the inner bark tissues and in doing so infection the trees with spores of *Ceratocystis ulmi*, which causes Dutch Elm disease which is responsible for the death of many elm trees. A correlation has been demonstrated between the presence of the endophyte *Phomopsis oblonga* and the demise of the bark beetles. Feeding on endophyte-infected wood led to a reduction in the reproductive capacity and a decline in the beetle populations (Webber, 1981).

A number of endophytic fungi isolated from needles of Canadian fir trees were shown to produce secondary metabolites in culture. These metabolites were toxic to spruce budworms, both decreasing growth rate and increasing mortality of larvae (Miller, 1986). It is not clear to what extent such toxins are produced *in vivo* or the degree of protection which is afforded to the conifers from this source. The presence of the endophytic fungus *Rhabdocline parkeri* in Douglas-fir needles (Carroll, 1986, 1988) incresed the mortality of gall-forming midge larvae (*Contarinia*). Toxic metabolites from the fungus were implicated as the responsible agents (Isaac, 1992).

2.3.3 Effects on other herbivores

Although toxicoses induced in domestic herbivores have been know and related to grazing fodder for many years, it is only recently that the observed effects have been correlated with the presence of fungal endophytes in pasture grass populations. Endophytes in grasses, responsible for poisoning mammalian stock have now been positively identified. Major examples of such problems have been encountered in grazing areas. In the southern USA the effects of fescue toxicosis has been known for many years. Cattle grazing on infected tall fescue (*Festuca arunginacea*) can develop symptoms including poor weight gain, increased body temperature, lameness, gangrene and limb loss. The effects develop particularly in the summer during hot, dry conditions when the livestock are under most physiological stress. A further example is the condition known as ryegrass staggers, typified by muscular spasms and, in severe cases, an inability to even stand. These symptoms have developed in sheep in New Zealand, and have been correlated with grazing on endophyte-infected *Lolium perenne*. These effects incur substantial economic losses annually (Isaac, 1992).

Although such conditions have been attributed to the presence of fungal endophytes the exact causes are not entirely clear. The endophytes of grasses are Clavicipitaceous fungi and are therefore likely to produce alkaloids, indeed the symptoms of the induced toxicoses are related to those of ergot poisoning. It has now been shown that alkaloids are present in the tissues of these host plants. However, alkaloids have been reported to occur in healthy plant tissues only extremely rarely. Fungi belonging to *Balansia* species do produce alkaloids in culture. It is likely that these are the cause of the toxic effect in mammals (Isaac, 1992).

2.3.4 Plant resistance to microbial pathogens

An increase in host plant vigour will probably enhance the plants inherent ability to resist disease. It has also been suggested that the presence of endophytic fungal infections may protect a host plant from some potentially virulent pathogens (Carroll, 1988). Infected perennial ryegrass plants were apparently protected, to some degree, from *Puccinia coronata* since the development of rust pustules was less, per unit leaf

area, than for uninfected plants (Clay, 1989). It has also been suggested that some endophytic species may be antagonistic towards other fungal species. Such antagonism has been demonstrated in culture, and culture filtrates from the grass endophyte *Acremonium coenophialum*, have been shown to have inhibitory effects on the growth of potential grass pathogens (White and Cole, 1985).

2.3.5 Endophytic strategies

Although investigations concerning endophytic associations are limited, the available evidence suggests that endophytes have evolved from plant pathogenic fungi. Many of the endophytes which have been described are very closely related to virulent pathogens and occur on the same, or related, host plants also. For example, on grasses *Acremonium coenophialum* is related to the pathogen *Epichloe typhina* and occurs on a similar range of host plants. *Rhabdocline parkeri* is a frequently isolated endophyte of conifers and has been shown to infect Douglas fir (Sherwood-Pike et al., 1986; Stone, 1987, 1988). In autumn, germinating conidia become attached to the needle surface by mucilage and produce penetration pegs by which the plant tissues are invaded. Hyphae form intracellularly within individual epidermal cells and remain there for a long time (2-5 years), not proliferating further until needle senescence. As the host tissues age, the fungus then invades surrounding tissues, forming structures somewhat akin to haustoria. Sporulation occurs after needle abscission. *Rhabdocline parkeri* is related to the plant pathogens *R. wierii* and *R. pseudotsugae*, and, in fact all these species may infect the same tree simultaneously. However the distinctions between endophytic and pathogenic relationships are often not clear. Some pathogenic species may show a long period of apparently endophytic growth in a host plant before symptom development occurs. Additionally, the growth requirements of endophytic species indicate the utilisation of a limited range of materials as substrates, a characteristic which is often associated with pathogenic species (Isaac, 1992).

Two strategies of endophytic mutualism have been described (Carroll, 1986, 1988). In grasses, many fungal species have been identified which do not leave the host plants at reproduction and do not produce any external fruiting bodies. Spread of the

fungus is achieved by vegetative growth of hyphae into the ovules of the host so that dispersed seed is already infected by these fungi. This has been termed constitutive mutualism (Carroll, 1986). Such endophytes infect aerial parts of the plant systemically, commonly developing a large biomass of fungal mycelium in host tissues. These species do not appear to harm the host plant but often produce toxins which may have important deterrent effects on grazing herbivores and may therefore provide protection to the plant. In some instances the presence of these endophytes within ovule tissues results in host plant sterilisation and therefore the cost of this association to the plant in high. However, endophyte-induced sterility may result in more vigorous vegetative growth. Grasses may produce more vegetative tillers enhancing competitive ability in some ecological situations. It is very likely that the metabolic and physiological drains on host plant reserves and energy supplies are also high, since a large fungal biomass is supported by the plant (Isaac, 1992).

An alternative strategy, adopted by some endophytic species, is inducible mutualism, in which a looser relationship is formed with the host plant (Carroll, 1986, 1988). In these associations the distribution of the endophyte in plant tissues is patchy and probably affected by ageing in the host. These endophytes normally inhabit senescent host tissues and only penetrate metabolically active regions when the plant is stressed. Herbivore wounding of tissues leads to endophyte invasion of active regions. The toxins produced by endophyte activity then give rise to destruction in the herbivore population. There may be of little benefit to the host plant directly, although it has been suggested that the host population may benefit, albeit often in the long term, as a result of the reduction in herbivory (Carroll, 1988). Here, the metabolic cost to the plant is relatively small (Isaac, 1992).

2.4 Plant sample

Croton (or "Plao") (ลัดดาวัลย์ บุญรัตนกรกิจ, 2535) belongs to the family Euphorbiaceae, which have many species widely distributed in Thailand. Many species are commonly used in folk medicine. For example, Plao Nam Ngoen (*C. cascarilloides* Raeusch.) can be used as an antifebrile, Plao Lueat (*C. robutus* Kurz.) can be used as an antianemic agent, and Plao Noi (*C. sublyratus* Kurz.) can be used as antiulceric agent (โรงเรียนแพทย์แผนโบราณ วัดพระเชตุพน, สมาคม. 2521).

2.4.1 Botanical aspects of *Croton oblongifolius*

Croton oblongifolius is a medium sized deciduous tree in the Euphorbiaceae family. There are about 700 species in this family. In Thailand, it is commonly called Plao Yai (central) or Plao Luang (Northern). It is distributed throughout forests or shrubland below 700 meters above sea level. Its calyx and ovary are clothed with minute orbicular silvery scales. Leaves are 5.6-12.0 by 13.0-24.0 cm in size. The shape of the leaf blade is oblong-lanceolate. Its flowers are pale yellowish green and solitary in the axials of minute bracts on long erect racemes. The male flowers are located in the upper part of the raceme and the females in the lower part. The male flowers are slender and have pedicels of 4.0 mm in length. The calyx is more than 6.0 mm long and segments are woolly. The twelve stamens are inflexed in bud and the length of the filaments is 3.0 mm. In female flowers, the pedicels are short and stout. Its sepals are more acute than in the male with densely ciliated margins. The diameter of the fruit is less than 1.3 cm, slightly 3-lobed and clothed with small orbicular scales and quite smooth on the back (เต็ม สมิติ นันท์, 2523; ลีนา ผู้พัฒนพงศ์, 2530). The leaf, flower, and stem bark of *Croton oblongifolius* are shown in Figure 2.3.

Figure 2.3 Leaf (A), flower (B) and stem bark (C) of *Croton oblongifolius*

2.5 Study of secondary metabolites from endophytic fungi

The fungi provide us with an enormous variety of strange and wonderful 'secondary metabolites', some of which have profound biological activities that we can exploit. Secondary metabolites are those that are not essential for vegetative growth in pure culture. Secondary metabolism occurs as growth rate declines and during the stationary phase, and often is associated with differentiation and sporulation (Carlile et al., 2001).

Recently, fungal endophyte research has focused on screening of secondary metabolites that exhibit interesting biologically activitie compounds such as antibacterial, antifungal, antiviral, algicidal, herbicidal, insecticidal, antifeedant, antioxidant and anticancer drugs. For example, three new cytochalasins were discovered in *Hypoxylon fragiforme* because one of them, L-696,474 (18-dehydroxy cytochalasin H), was a potent competitive inhibitor of HIV-1 viral protease (Dombrowski et al., 1992; Ondeyka et al., 1992). Echinocandin analogues, potent lipopeptide inhibitors of fungal β-1,3 glucan synthase, have been isolated from *Cryptosposiopsis* spp. (Noble et al., 1991; Tscherter and Dreyfuss, 1982). Furthermore, a novel lactone with potential antiherpes and antimicrobial properties was discovered in an endophytic *Microsphaeropsis* strain (Tscherter et al., 1988). Additionally, a new cyclodepsipeptide antihelminthic was produced by sterile fungus isolated from *Camellia japonica* L. (Sasaki et al., 1992). The chemical compounds, sources, biological activities of secondary metabolites of endophytic fungi were summarized in Table 2.1 and Figure 2.4.

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 4	Lactones 1893 A 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 8	7-Butyl-6,8-dihydroxy-3 (*R*)-pent-11-enylisochroman-1-one 7-Butyl-15-enyl-6,8-dihydroxy-3(*R*)-pent-11-enylisochroman-1-one 7-Butyl-6,8-dihydroxy-3 (*R*)-pentylisochroman-1-one	*Geotrichum* sp.	*Crassocephalum crepidioides*	Antimalarial, antituberculous and antifungal	Kongsaeree et al., 2003

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 12 13	Preaustinoid A Preaustinoid B 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 16	Jesterone hydrosy-jesterone	*Pestalotiopsis jesteri*	*Fragraea bodenii*	Antioomycete	Li et al., 2001
17 18 19	Preussomerin G Preussomerin H Preussomerin I	Mycelia sterilia	*Atropa belladonna*	Antibacterial, antifungal and antialgal	Krohn et al., 2001

Table 2.1 (continued)

No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
20 21 22	Preussomerin J Preussomerin K Preussomerin L	Mycelia sterilia	*Atropa belladonna*	Antibacterial, antifungal and antialgal	Krohn et al., 2001
23 24 25	Dicerandrols A Dicerandrols B Dicerandrols C	*Phomopsis longicolla*	*Dicerandra frutescens*	Antibiotic and cytotoxic	Wagenaar and Clardy, 2001
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 40 41 42	Cytochalasin 1 Cytochalasin 2 Cytochalasin 3 Cytochalasin E	*Rhinocladiella* sp.	*Tripterygium wilfordii*	Cytotoxic	Wagenaar et al., 2000
43	Cryptocandin	*Cryptosporiopsis* cf. *quercina*	*Tripterigeum wilfordii*	Antimycotic	Strobel et al., 1999
44 45	Geniculol Cytochalasin F	*Geniculosporium* sp.	*Teucrium scorodania*	Antialgal	Konig et al., 1999
46 47	Sequoiatones A Sequoiatones B	*Aspergillus parasiticus*	*Sequoia sempervirens*	Antitumor	Stierle et al., 1999
48	Terpendole M	*Neotyphodium lolii*	*Lolium perenne*	neurotoxins	Gatenby et al., 1999
49 50 51	Tricin (1) 7-O-(B-D-glucopyranosyl) tricin Isoorientin (3)	*Neotyphodium typhnium*	*Poa ampla*	Insecticidal	Ju et al., 1998

Table 2.1 (continued)

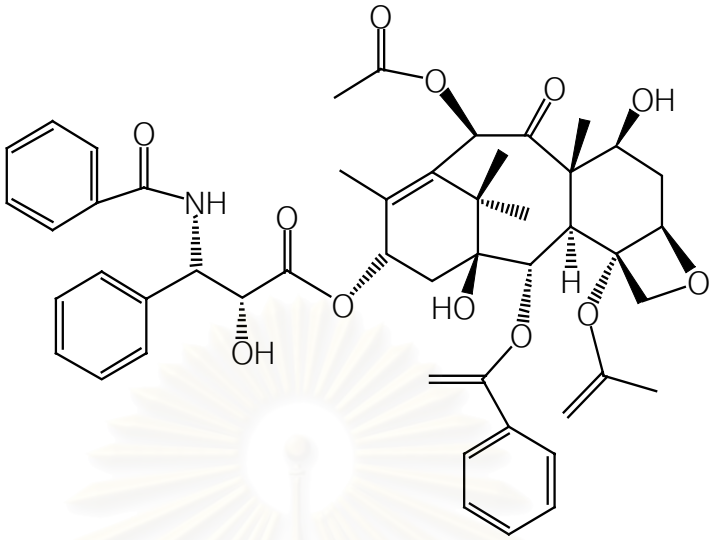
No.	Compounds	Endophytic fungi	Host plants	Biological activities	References
52	7-o-[α-L-Rhamnopyranosyl(1-6)-β-D-glucopy-ranosyl]tricin	*Neotyphodium typhnium*	*Poa ampla*	Insecticidal	Ju et al., 1998
53	Lolitrem B	*Acremonium lolii*	*Lolium perenne*	Neurotoxic	Berny et al., 1997
54	Leucinostatin A	*Acremoium* sp.	*Taxus baccata*	Antifungal and anticancer	Strobel et al., 1997
55 56 57 58	Oreganic acid (1) Trimethyester (2) Desulfated analog (3) Desulfated analog (4)	Endophytic fungus (MF 6046)	*Berberis oregana*	Anticancer	Jayasuriya et al., 1996
59 60	Pestalotiopsins A Pestalotiopsins B	*Pestalotiopsis* sp.	*Taxus brevifolia*	-	Pulici et al., 1996
61 62	(R)-mellein (-)-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				

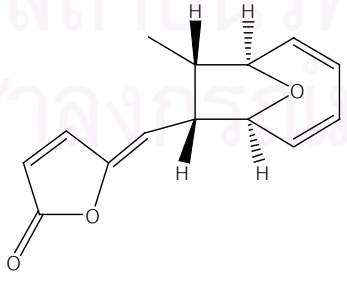
Table 2.1 (continued)

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)				

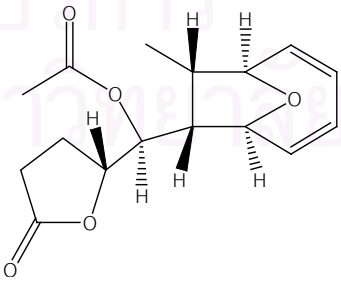


[1] Taxol

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

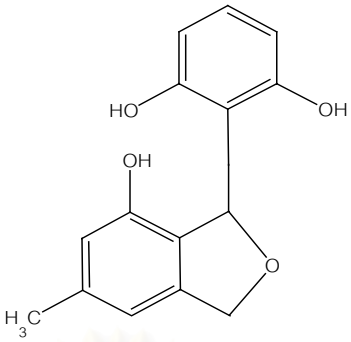


[3] Lactones 1893 A

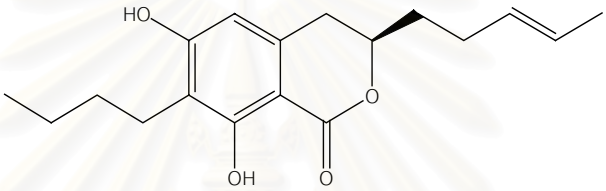


[4] Lactones 1893 B

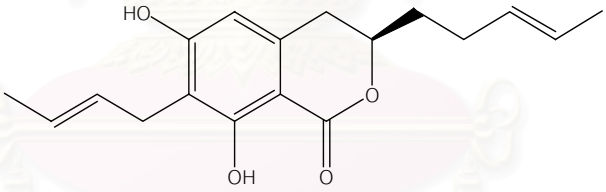
Figure 2.4 Structure of secondary metabolites of endophytic fungi



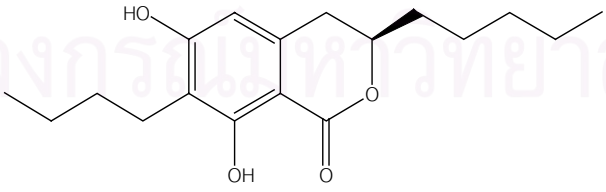
[5] Pestacin



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



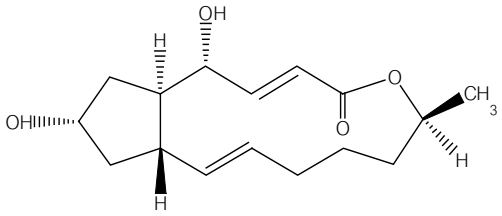
[7] 7-Butyl-15-enyl-6, 8-dihydroxy-3(R)-pent-11-enylisochroman-1-one



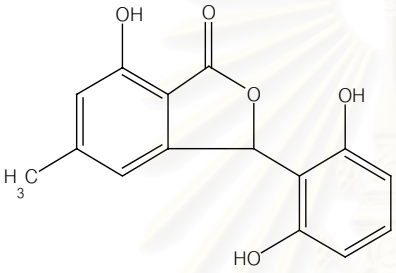
[8] 7-Butyl-6, 8-dihydroxy-3(R)-pentylisochroman-1-one

Dihydroisocumarins [6-8]

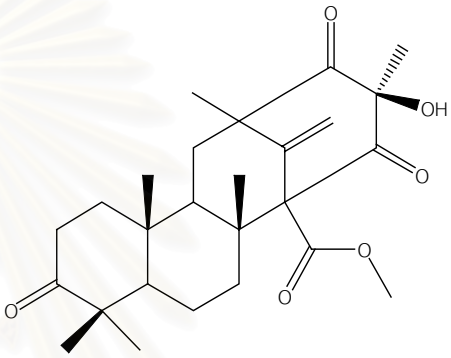
Figure 2.4 (continued)



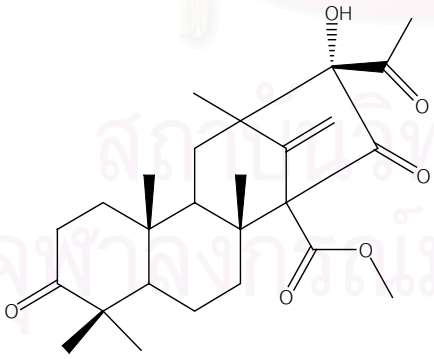
[9] Brefeldin A



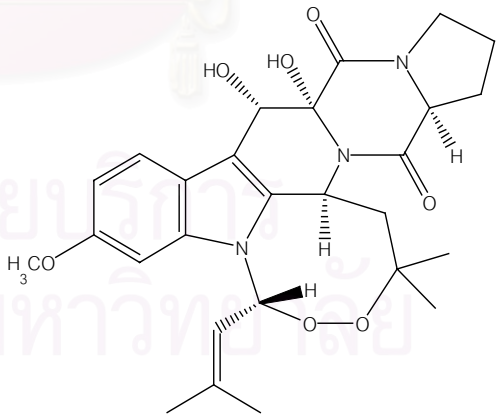
[10] Isopestacin



[11] Preaustinoid A

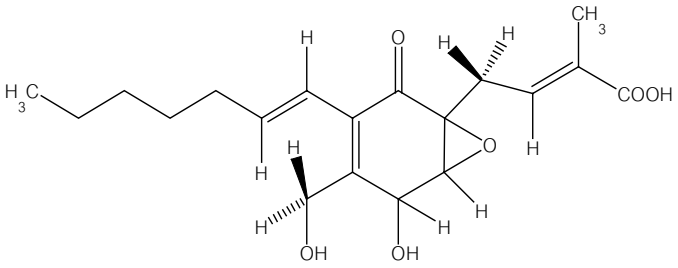


[12] Preaustinoid B

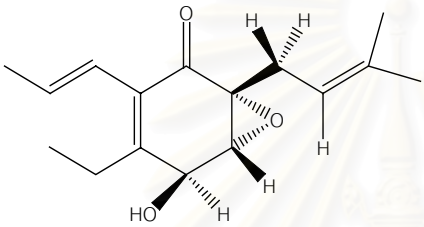


[13] Alkaloid verruculogen

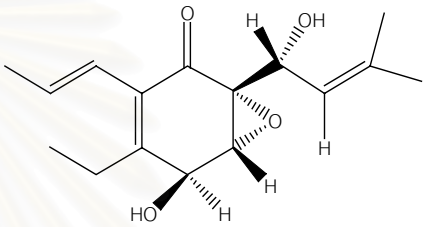
Figure 2.4 (continued)



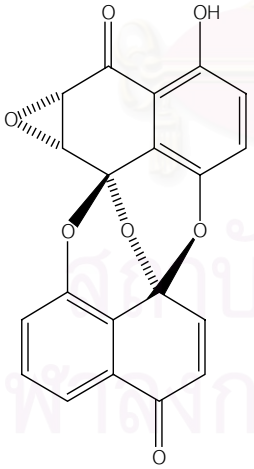
[14] Ambuic acid



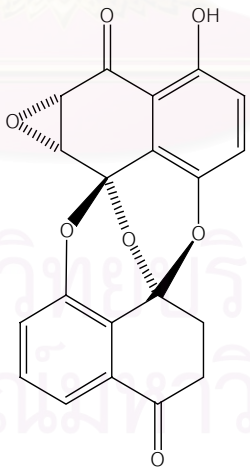
[15] Jesterone



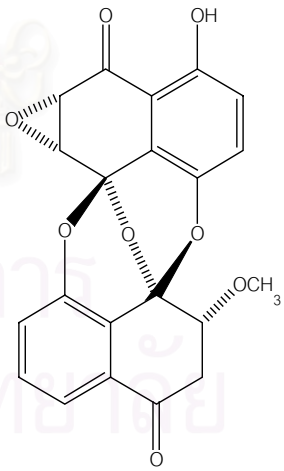
[16] Hydroxy-jesterone



[17] Preussomerin G

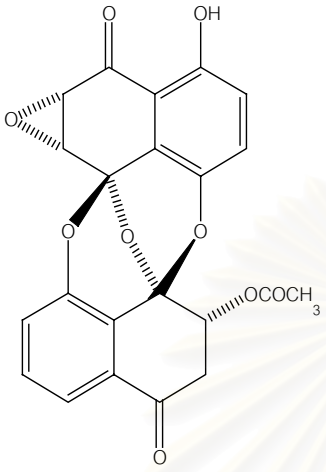


[18] Preussomerin H

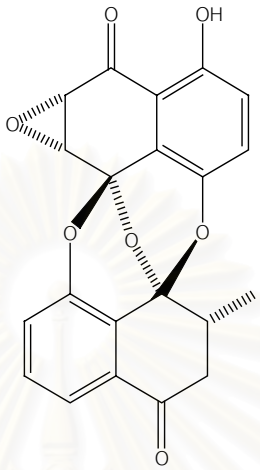


[19] Preussomerin I

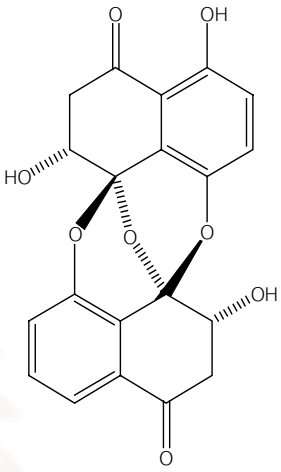
Figure 2.4 (continued)



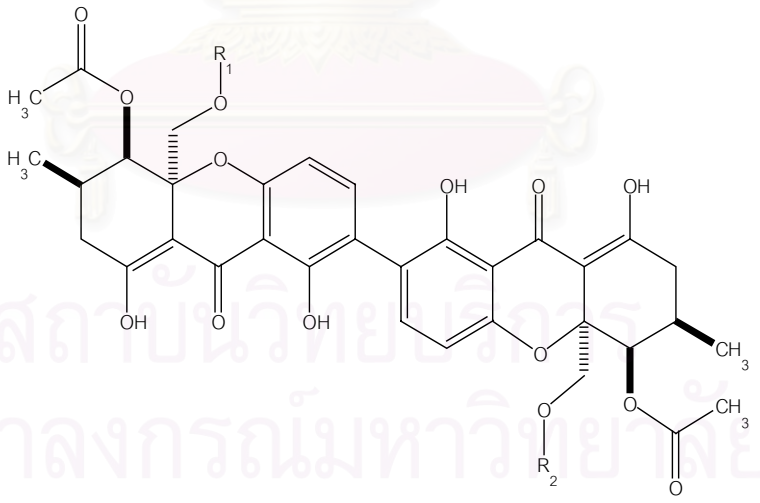
[20] Preussomerin J



[21] Preussomerin K



[22] Preussomerin L

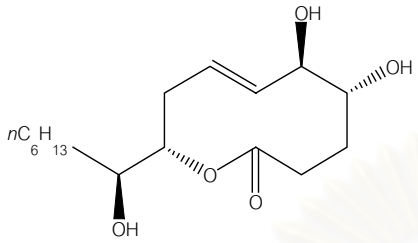


[23] Dicerandrols A, R1=R2=H

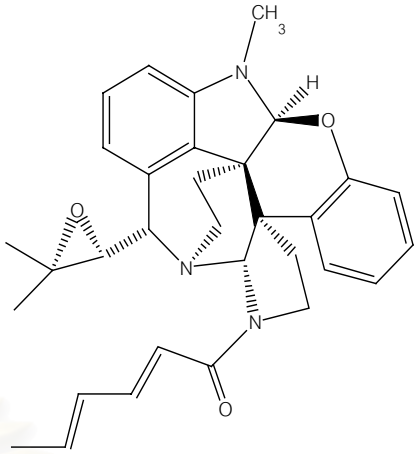
[24] Dicerandrols B, R1=Ac, R2=H

[25] Dicerandrols C, R1=R2=Ac

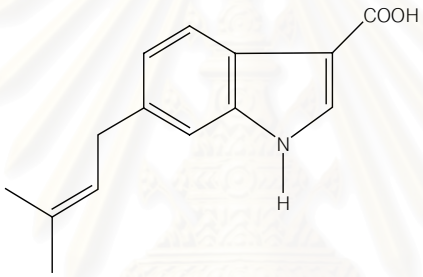
Figure 2.4 (continued)



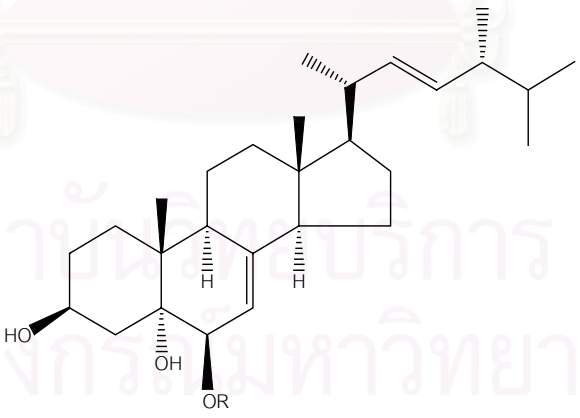
[26] Microcarpalide



[27] Nomofungin



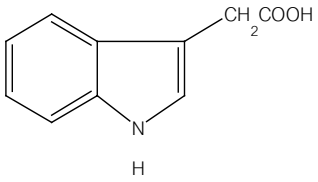
[28] Isoprenylindole-3-carboxylic acid



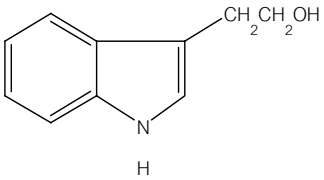
[29] 3beta, 5alpha-Dihydroxy-6beta-acetoxy-ergosta-7, 22-diene, R=$COCH_3$

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

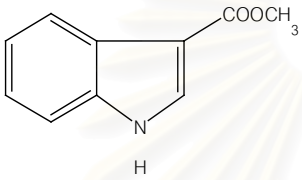
Figure 2.4 (continued)



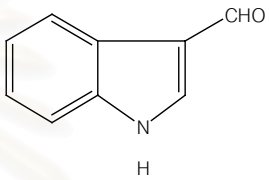
[31] Indole-3-acetic acid (IAA)



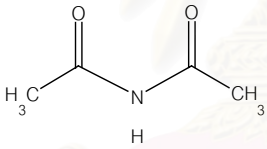
[32] Indole-3-ethanol (IEtOH)



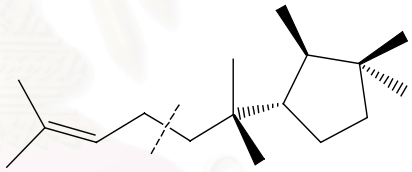
[33] Methylindole-3-carboxylate



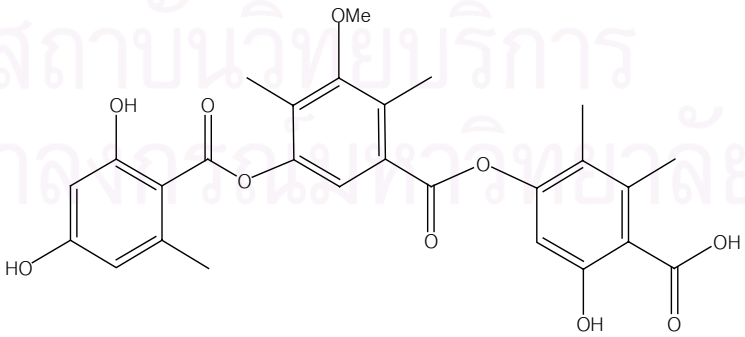
[34] Indole-3-carboxaldehyde



[35] Diacetamide

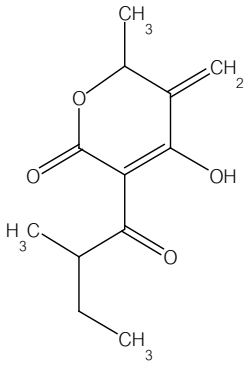


[36] Cyclonerodiol

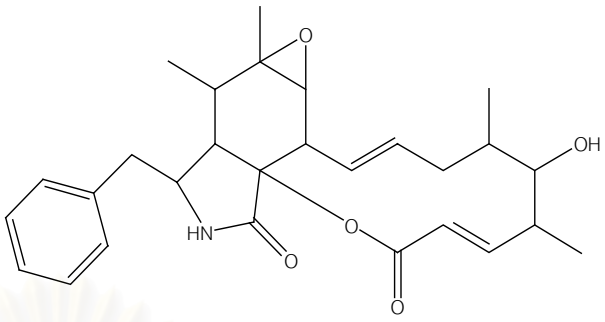


[37] Colletotric acid

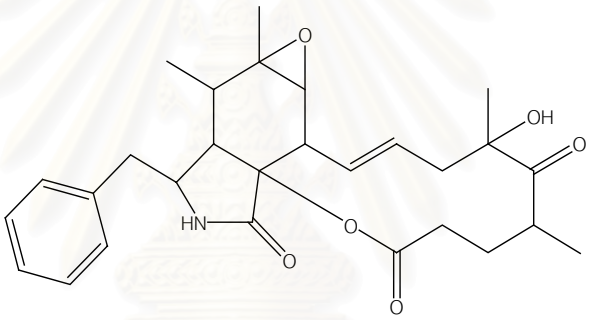
Figure 2.4 (continued)



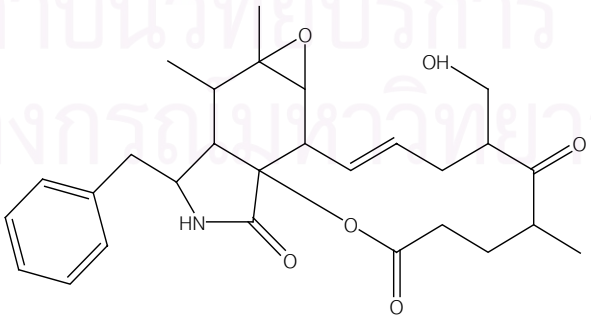
[38] CR377, pentaketide



[39] Cytochalasin 1

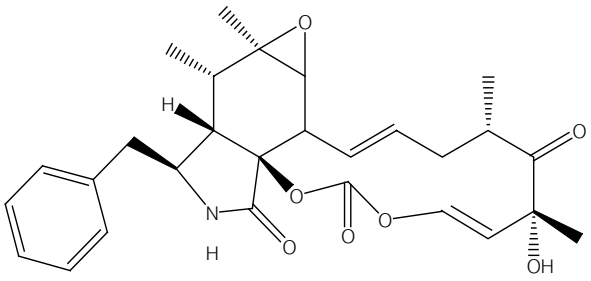


[40] Cytochalasin 2

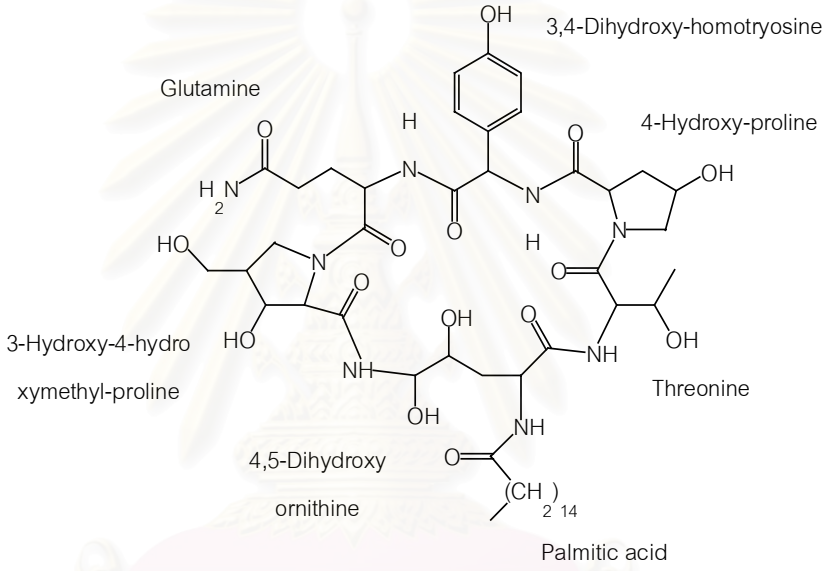


[41] Cytochalasin 3

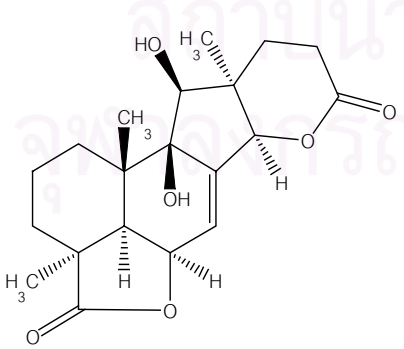
Figure 2.4 (continued)



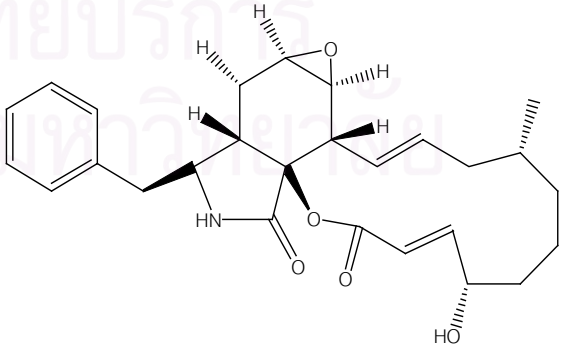
[42] Cytochanlasin E



[43] Cryptocandin

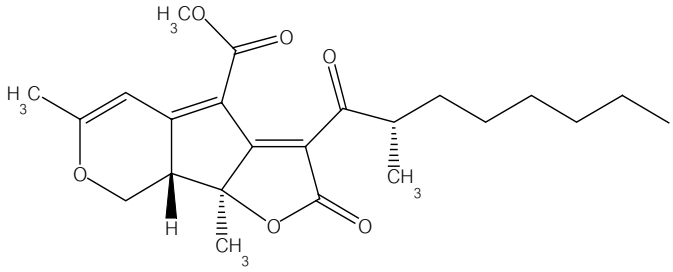


[44] Geniculol

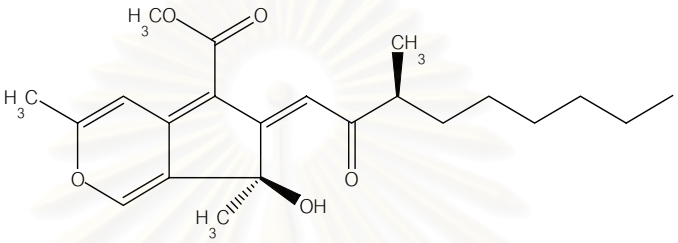


[45] Cytochalasin F

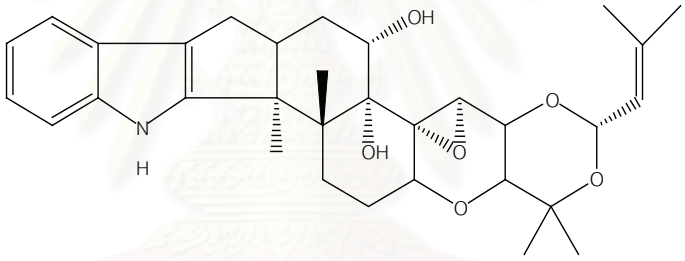
Figure 2.4 (continued)



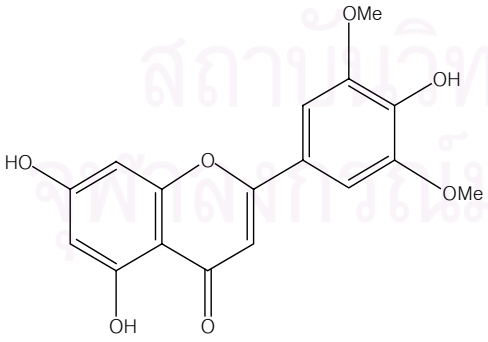
[46] Sequoiatones A



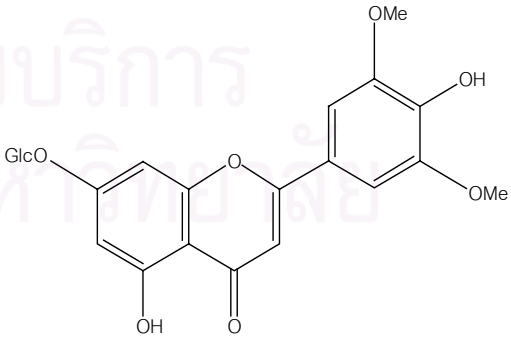
[47] Sequoiatones B



[48] Terpendole M

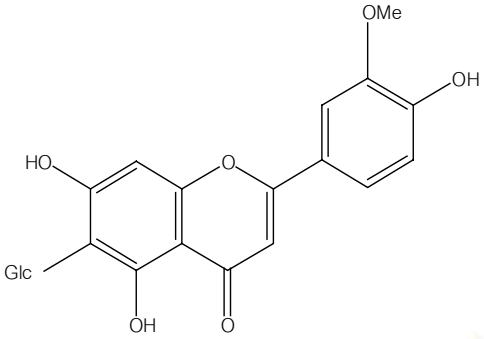


[49] Tricin

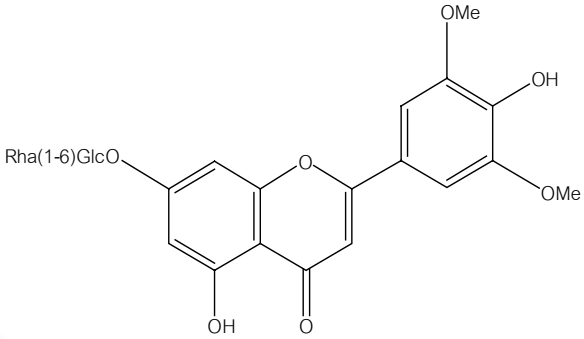


[50] 7-O-(B-D-glucopyranosyl)-tricin

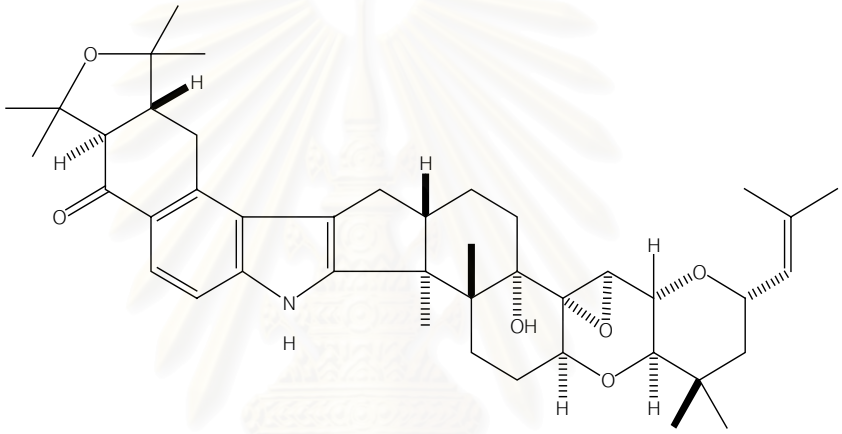
Figure 2.4 (continued)



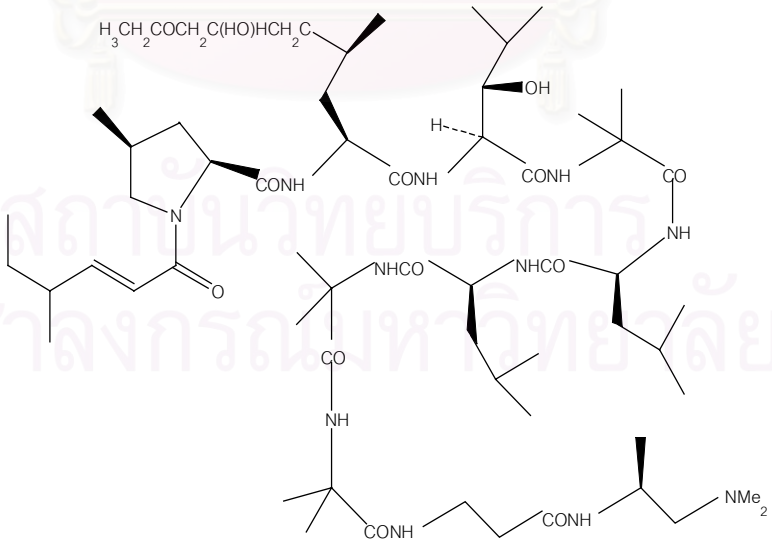
[51] Isoorientin



[52] 7-**o**-[**α**-L-Rhamnopyranosyl(1-6)-**β**-D-glucopy-ranosyl]tricin

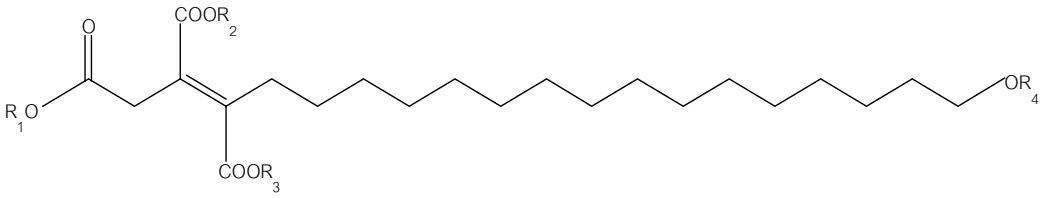


[53] Lolitrem B



[54] Leucinostatin A

Figure 2.4 (continued)

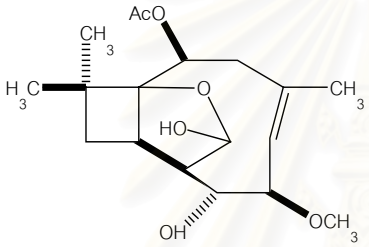


[55] 1: Oreganic acid, R1=R2=R3=H, R4=SO3H

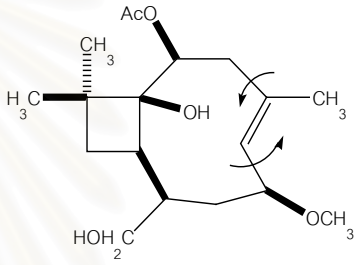
[56] 2: Trimethyester, R1=R2=R3=CH3, R4=SO3H

[57] 3: Desulfated analog, R1=R2=R3=CH3, R4=H

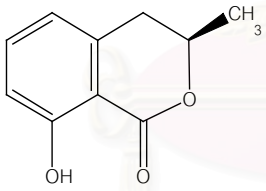
[58] 4: Desulfated analog, R1=R2=R3=R4=H



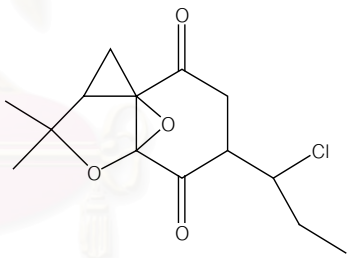
[59] Pestalotiopsins A



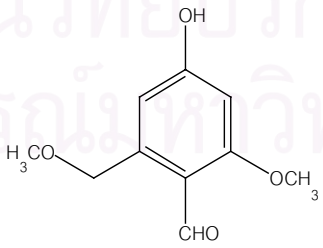
[60] Pestalotiopsins B



[61] (R)-mellein

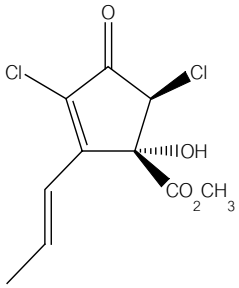


[62] (-)-mycorrhizin A

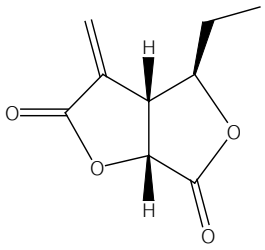


[63] 2-methoxy-4-hydroxy-6-methoxymethyl-benzaldehyde

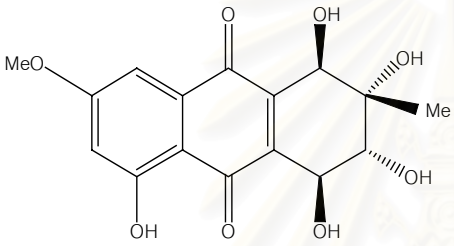
Figure 2.4 (continued)



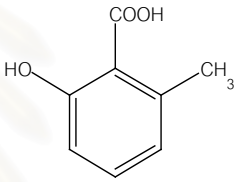
[64] (+)-cryptosporiopsin



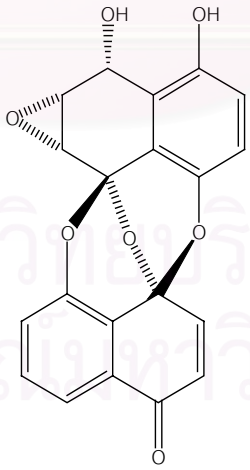
[65] 4-epi-ethiosolide



[66] Altersolanol A

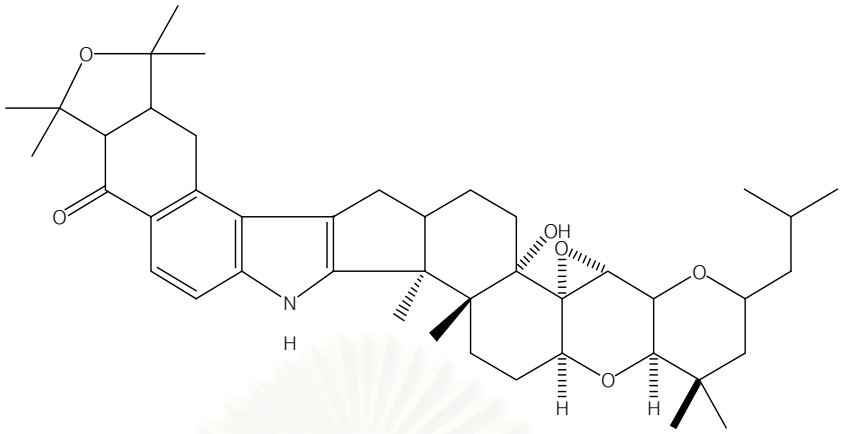


[67] 2-hydroxy-6-methyl benzoic acid

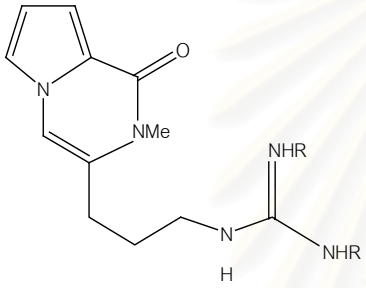


[68] Preussomerin D

Figure 2.4 (continued)

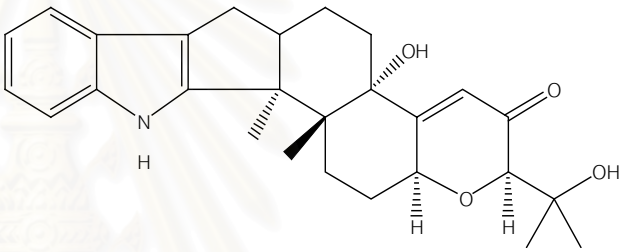


[69] Lolitrem C

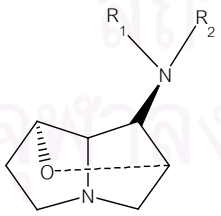


[70] Peramine R=H

[71] Diacetylperamine R=Ac

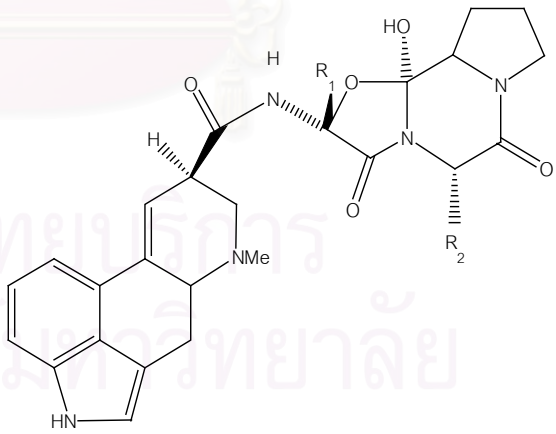


[72] Paxilline



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

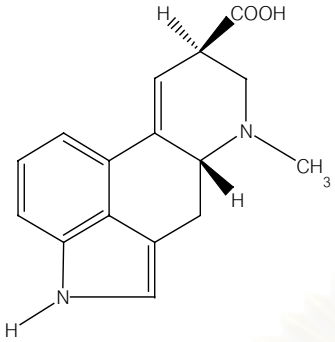
R_2= H, HCO, Ac



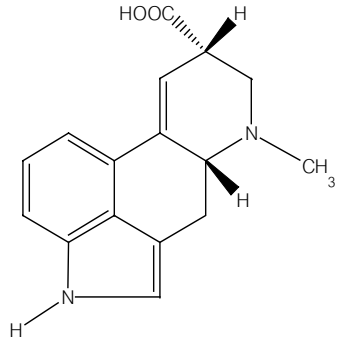
[74] Ergopeptine alkaloids

Ergovaline R_1=Me, R_2=i-Pr

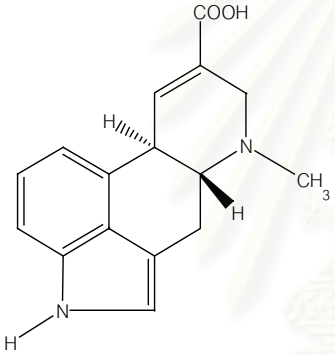
Figure 2.4 (continued)



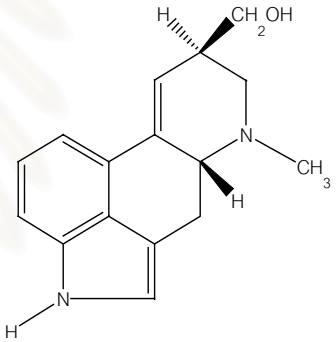
[75] Lysergic acid



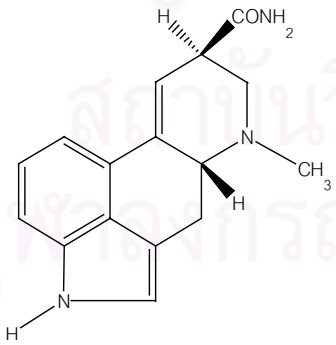
[76] Isolysergic acid



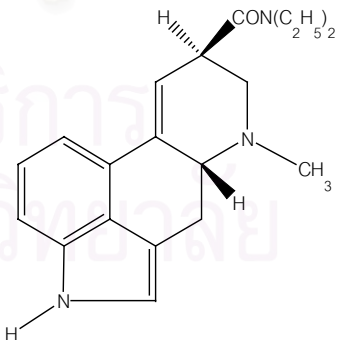
[77] Paspalic acid



[78] Lysergol

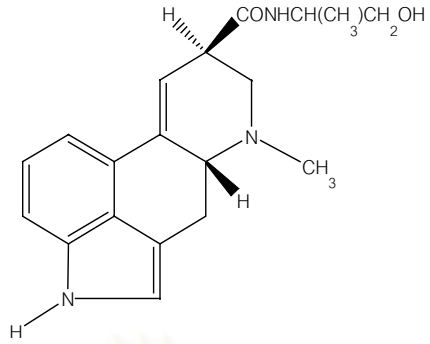


[79] Lysergic acid amide



[80] Lysergic acid diethyl amide

Figure 2.4 (continued)



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

Figure 2.4 (continued)

CHAPTER III

MATERIALS AND METHODS

3.1 Plant samples collection

Healthy young and mature leaf, branch, and bark samples of *Croton oblongifolius* were collected from Kui Buri District, Prachuap Khiri Khan Province, Thailand, on November 14, 2001. Plant samples were kept in a plastic bag. Fresh specimens were processed in 24 hours after collection.

3.2 Culture media

Culture medium used for isolation, cultivation, and morphology observation of endophytic fungi was malt extract medium (agar and broth).

The medium for growing bacteria was nutrient medium (agar and broth). Yeast-malt extract medium (agar and broth) was used for growing yeasts. Sabouraud's dextrose agar (SDA) and V8 agar were used for growing dermatophytic fungi and plant phatogenic fungi, respectively.

The formula for media is shown in Appendix A.

3.3 Fungal isolation and culture methods

Endophytic fungi were isolated by using the surface sterilization method. Plant samples were cleaned with tap water and dried in laminar air flow.

The leaf sections (1x1 cm) were cut from the middle, rib, and lamina and were immersed in 95% ethanol for 1 minute, followed with Clorox® (5% available chlorine) for 5 minutes and then were transferred to 95% ethanol for 30 seconds before rinsing twice with sterile distilled water. Surface sterilization method was modified from the method described by Blodgett et al., (2000).

The branch and bark sections (1x1x0.5 cm) were cut from the plant samples, and were immersed in saturated calcium hyperchorite (20% available chlorine) for 5 minutes, and were rinsed twice with sterile distilled water. Surface sterilization method was modified from the method described by White, Drake, and Martin (1996).

The surface sterilized pieces of plant samples were allowed to dry on sterile filter papers in sterile petri dishes and were placed on malt extract agar (MEA). All petri dishes were incubated at room temperature (25-30 ^{o}C) and examined every day under a stereomicroscope for fungal growth. Fungal endophytes germinating from the plate tissues were transferred to MEA by hyphal tip transfer. They were incubated for 30 days at room temperature and purity was determined by colony morphology. Fungal isolates were kept for further study.

3.4 Identification and classification of endophytic fungi

3.4.1 Morphological identification

A. Microscopical features

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

B. Macroscopical features

Specimens characteristic such as shape, size, color, margin, pigment, and others were studied using stereomicroscope on a Leica MZ6.

3.4.2 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 (pH8.0), 20 mM EDTA (pH8.0), 1.4M NaCl and 0.5% 2-mercaptoethanol) at 65 ^{o}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 pricipitated with isopropanol and centrifuged at 8000 rpm for 5 min. Fungal DNA was dissolved in 10 µl TE buffer (10mM Tris-HCl (pH8.0) and 1mM EDTA) and kept at -30 ^{o}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; Perkin 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 ^{o}C for 9 min, followed by 38 cycles of a denaturing step at 94 ^{o}C for 1 min, an annealing step at 51 ^{o}C for 1 min, and an extension step at 72 ^{o}C for 1 min, and ended with an additional 5-min extension step at 72 ^{o}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 and ITS2 sequence of rRNA gene.

ITS1f CTTGGTCATTTAGAGGAAGTAA

ITS4 TCCTCCGCTTATTGATATGC

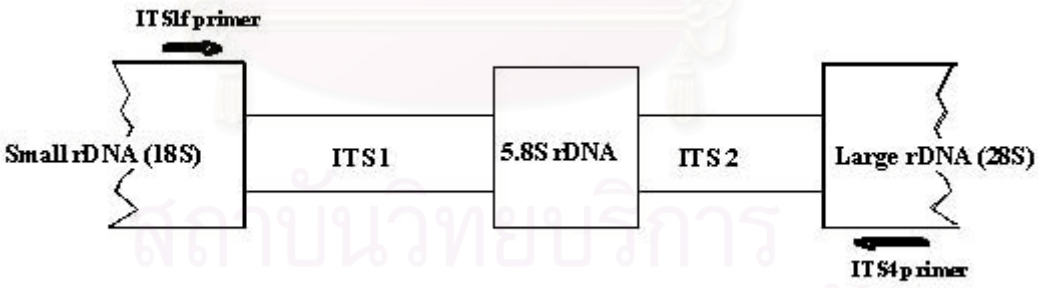


Figure 3.1 ITS regions of rDNA (Kanchanaprayudh et al., 2003).

3.5 Preservation of endophytic fungi

A. Storage under 15% glycerol

Fungal endophyte isolates were grown on MEA agar slants at room temperature (25-30 °C) for 1-2 weeks depending on the individual fungal growth rate. The mature cultures were then covered up to 10-mm height with sterile 15% glycerol and kept at 4 °C. The 15% glycerol was steriled by autoclaving twice at 121 °C for 15 mintutes.

B. Storage in 15% glycerol

Seven-mm pieces were cut from the growing edge of fungal colonies grown on MEA. Five to ten pieces of agar blocks were put in a glass vials containing 5-ml sterile 15% glycerol and kept at -20 °C. The 15% glycerol was steriled by autoclaving twice at 121 °C for 15 mintutes.

3.6 Fungal cultivation and metabolite extraction

3.6.1 Fungal cultivation

Each fungal endophyte isolate was grown on MEA at room temperature (25-30 °C) for 1-2 weeks depending on the individual fungal growth rate. The agar cultures were then cut into 7 mm diameter disks by a flamed cork hole borer. One disk was inoculated into 250 ml Erlenmeyer flasks containing 100 ml of malt extract broth each. All cultures were incubated for 8 weeks at room temperature (25-30 °C) under static condition.

3.6.2 Metabolite extraction

The culture broths were filtered twice through a filter paper (Whatman No. 4) and evaporated by using a rotary evaporator (Eyela, Type N-N series, Japan) at 35 °C and freeze-dried by using a freeze dryer (Labconco, Model 77520, USA). The culture broths were extracted with MeOH and dried and kept in a refrigerator at 4 °C for bioassays.

The fungal mycelia were extracted with methanol (100 ml methanol per 1 sample). The flasks of fungal mycelium were extracted on rotary shaker (200 rpm) at room temperature for overnight. 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 ^{o}C, and then transferred to a glass vial. The mycelium extracts were kept in the refrigerator at 4 ^{o}C for bioassays.

3.7 Determination of antimicrobial activity

Antimicrobial activities of culture broth extracts and mycelium extracts against test microorganisms were determined by the agar well diffusion method which was modified from the method described by Weaver, Angel, and Botlomley (1994) and Joseph, Dave, and Shah (1998).

3.7.1 Test microorganisms

The test microorganisms are listed in Table 3.1 (below).

Table 3.1 Test microorganisms for assay.

Type of microorganisms	Reference strains
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
Fungus, yeast form	*Saccharomyces cerevisiae* TISTR 5169
Fungus, yeast form	*Candida albicans* ATCC 10231
Fungus, filamentous form	*Microsporum gypseam* (clinical isolate)
Fungus, filamentous form	*Trichophyton mentagrophytes* (clinical isolate)

3.7.2 Procedures

A. Preparation of samples

Ten mg each of culture broth extract and mycelium extracts was dissolved in 1 ml of 10% DMSO in sterile distilled water. All samples were kept in a refrigerator at 4 ^{o}C for bioassay.

B. Preparation of bacterial inoculum

Bacteria were grown on Nutrient agar (NA) for 24 h at 37 ^{o}C. Selected fresh single colonies (4-5) were inoculated into 5 ml of Nutrient broth (NB) and incubated at 37 ^{o}C for 2-6 h, 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 0.1 at 625 nm).

C. Preparation of yeast inoculum

Yeasts were grown on Yeast-malt extract agar (YMA) for 24 h at room temperature (25-30 ^{o}C). Selected fresh single colonies (4-5) were inoculated into 5 ml of Yeast-malt extract broth (YMB) and incubated at room temperature for 2-3 h, depending on the growth rate. The turbidity of the yeast suspension was adjusted with YMB to match the turbidity of a 0.5 McFarland (OD 0.1 at 625 nm).

D. Preparation of filamentous fungal inoculum

Filamentous fungi were grown on Saborouad dextrose agar (SDA) for 1-2 weeks at room temperature (25-30 ^{o}C) until good conidia production was obtained. Conidia of filamentous fungi were harvested by flooding the slant culture with 5 ml of sterile 0.05% Tween 80 in normal saline solution. The conidial solution was filtrated through a spores filter. The final inoculum was diluted with sterile 0.05% Tween 80 in normal saline solution to obtain a conidial suspension containing approximately 1X10^{7} conidial/ml.

E. Inoculation of the test plate

Sterile cotton applicators were immersed in the inoculum suspension and pressed lightly against the tube wall to remove excess moisture. The agar was

inoculated by streaking the swab 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.

F. Application of culture broth extracts and mycelium extracts

Wells were made in the agar by removing disks cut (7 mm diameter) cut with a flamed cork hole borer. One hundred µl of culture broth extracts and mycelium extracts was pipetted into the agar wells. This was absorbed by the media surrounding the wells. Bacteria and yeast plates were incubated at 37 ^{o}C and room temperature, respectively for 24 h. and filamentous fungi plates were incubated at room temperature for 5 days. Inhibition zones around the wells were measured in mm with a ruler.

3.8 Determination of metabolites profile of the extracts from endophytic fungi

A few milligrams of the culture broth extracts and mycelium extracts in the section 3.6.2 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 ^{o}C)
Detection	: 1. Visual detection under ultraviolet light at wavelengths 254 and 365 nm
	2. Visual detection in iodine vapour

3.9 Determination of growth profile, pH, and antimicrobial activity of culture filtrate from fungal isolate PcBr20

Fungal endophyte PcBr20 was grown on MEA 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. Three disks were inoculated into 250 ml Erlenmeyer flasks containing 100 ml of MEB medium. The cultures were incubated at room temperature (25-30 °C) under shake condition, 200 rmp for 14 days.

Mycelium dry weights were obtained by harvesting the mycelium on pre-activated (at 80 °C for 24 hr), and pre-weighted Whatmann 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.

The filtrate was measured pH by using pH meter (Cyberscan 2000, Singapore). Antimicrobial activities of culture filtrate against test microorganisms were determined by the agar well diffusion method in the same manner as described in section 3.7

3.10 Fungal cultivation for study metabolites of bioactive compounds

Endophytic fungal isolate PcBr20 was chosen for the study of metabolites and cultivated on MEA 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. Three pieces of agar culture were inoculated into 250 ml Erlenmeyer flasks containing 100 ml of malt extract broth. The flasks were incubated on a rotary shaker (200 rpm) at room temperature for 14 days. Several flasks of culture were prepared to obtain 17 l of MEB.

3.11 Chromatographic techniques

3.11.1 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 ^{o}C)

Detection : 1. Visual detection under ultraviolet light at wavelengths 254 and 365 nm.

2. Visual detection in iodine vapour.

3. Visual detection under daylight after spraying with vanillin reagent (Dissolve 1 g vanillin in 95 ml ethanol and add 4 ml concentrated sulfuric acid) or anisaldehyde reagent (0.5% anisaldehyde reagent, 5% sulfuric acid, and 10% glacial acetic acid in MeOH) and heating untill the colors developed.

3.11.2 Column chromatography

Flash column chromatography

Adsorbent : Silica gel 60 (No. 7734) particle size 63-200 μm (70-230 Mesh ASTM) (E. Merck).

Packing method : Wet packing, the adsorbent was suspended in an eluent and then poured into a column, set it tight by using air pump before used.

Loading method : Dry loading: The sample was mixed in a small amount of a silica gel and then applied gently on the top of the column.

Detection : Fractions were examined by TLC technique in the same manner as described in section 3.10.1.

3.12 Crystallization

The mixture BH1 was crystallized from a mixture of hexane and $CHCl_3$. It was dissolved in $CHCl_3$ until saturation, and then hexane was added. The solution was left standing at room temperature.

3.13 Spectroscopy

3.13.1 UV-VIS spectrometer

UV-VIS spectra were recorded on a Perkin Elmer Lambda 25 UV/VIS Spectrometer in MeOH.

3.13.2 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.

3.13.3 Mass spectra

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

3.13.4 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* ($CDCl_3$) and methanol-*d*4 (CD_3OD) were used in NMR experiments. Reference signals were the signals of residual protonated solvents

at δ 7.24 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.

3.14 Optical rotation

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

3.15 Melting point

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

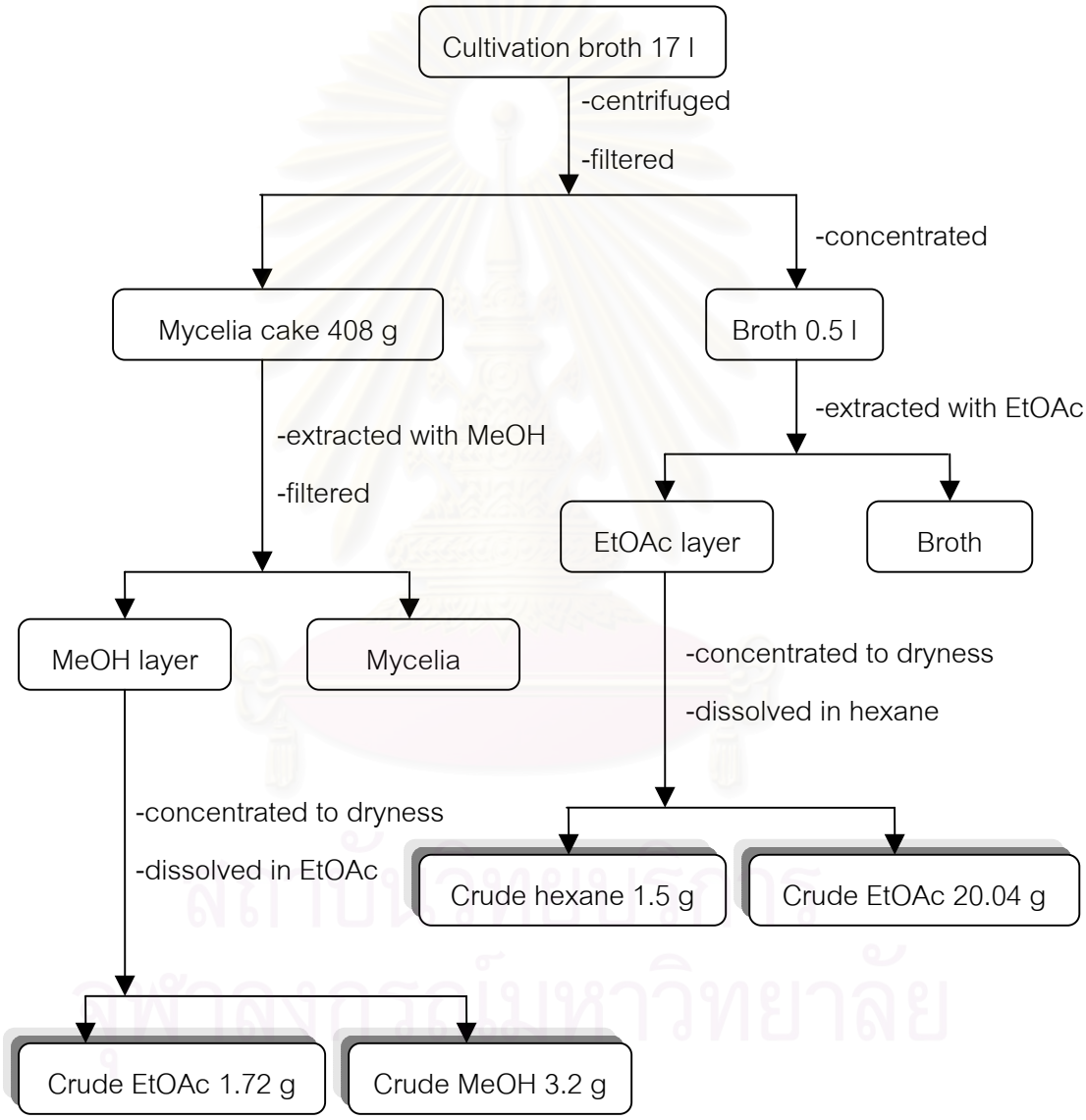
3.16 Solvent

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. The reagent grade solvents were used for recrystallization.

3.17 Extraction

The cultivation broth of Isolate PcBr20 (17 l) was centrifuged at 10,000 rpm for 15 min and filtered through a filter paper (Whatman No. 4). The filtrate was evaporated and partitioned with an equal volume of EtOAc 20 times. The EtOAc layer was collected and concentrated to dryness under reduced pressure at 35 ^{o}C and extracted again with 100 ml of hexane 3 times. The crude hexane extract was obtained as a mixture of white solid and yellow oil (1.5 g). The crude EtOAc extract was obtained as a mixture of brown solid and dark brown viscous liquid (20.04 g). Mycelia cake 408 g was extracted with 2 liters MeOH and filtered through filter paper (Whatman No. 4). The MeOH layer was collected and concentrated to dryness under reduced pressure at 35 ^{o}C. The dry crude MeOH extract was extracted again with EtOAc. The crude EtOAc extract of mycelia cake was obtained as a dark brown viscous liquid (1.72 g) and the crude MeOH extract of mycelia cake was obtained as a dark brown viscous liquid (3.2 g). The extraction of

the cultivation broth and mycelia of the endophytic fungus isolate PcBr20 is shown in Scheme 3.1.



Scheme 3.1 Extraction of culture broth and mycelia of endophytic fungus isolate PcBr20

3.18 Isolation of bioactive compounds of endophytic fungus isolate PcBr20

3.18.1 Isolation of bioactive compounds in crude EtOAc from mycelium extract

The crude EtOAc from endophytic fungus isolate PcBr20 mycelia extract (1.72 g) was subjected to column chromatography (silica gel, 100 g), using wet packing and dry loading method. Eluents of increasing polarity from hexane to MeOH were used. Gradient elution with hexane, hexane, and $CHCl_3$ mixtures, $CHCl_3$, $CHCl_3$ and MeOH mixtures, and MeOH were used. Fractions (25 ml each) were collected and examined. Fraction combination was by TLC on Silica gel plates with hexane, hexane and $CHCl_3$ mixtures, $CHCl_3$, $CHCl_3$ 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.

Table 3.2 The results from separation of EtOAc crude from mycelia extract

Fraction code	Fraction No.	Eluents	Appearance	Weight (mg)
ME01	1-8	100% Hexane	Yellow viscous liquid	5.8
ME02	9-15	5% $CHCl_3$ in Hexane	Yellow viscous liquid	4.8
ME03	16-20	5% $CHCl_3$ in Hexane	Yellow viscous liquid	1
ME04	21-29	5% $CHCl_3$ in Hexane	Yellow viscous liquid	3.1
ME05	30-40	5% $CHCl_3$ in Hexane	Yellow viscous liquid	1
ME06	41-62	10% $CHCl_3$ in Hexane	Yellow viscous liquid	22.4
ME07	63-66	15% $CHCl_3$ in Hexane	Yellow viscous liquid	1
ME08	67-70	15% $CHCl_3$ in Hexane	Yellow viscous liquid	6.1
ME09	71-78	20% $CHCl_3$ in Hexane	Yellow viscous liquid	13.2
ME10	79-85	25-30% $CHCl_3$ in Hexane	White solid and yellow viscous liquid	114.8
ME11	86-100	30-35% $CHCl_3$ in Hexane	Yellow viscous liquid	170.8
ME12	101-112	35-40% $CHCl_3$ in Hexane	Yellow brown viscous liquid	262.7
ME13	113-119	40% $CHCl_3$ in Hexane	Yellow brown viscous liquid	40
ME14	120-128	45% $CHCl_3$ in Hexane	Yellow brown viscous liquid	18.6
ME15	129-138	50% $CHCl_3$ in Hexane	brown viscous liquid	11.1
ME16	139-149	60% $CHCl_3$ in Hexane	brown viscous liquid	28.4
ME17	150-165	70-80% $CHCl_3$ in Hexane	brown viscous liquid	131.3

Table 3.2 (continued)

Fraction code	Fraction No.	Eluents	Appearance	Weight (mg)
ME18	166-172	80% $CHCl_3$ in Hexane	brown viscous liquid	37.2
ME19	173-178	90% $CHCl_3$ in Hexane	brown viscous liquid	36.6
ME20	179-184	90% $CHCl_3$ in Hexane	brown viscous liquid	34.1
ME21	185-193	100% $CHCl_3$	brown viscous liquid	32.1
ME22	194-206	100% $CHCl_3$	brown viscous liquid	77.5
ME23	207-221	5% MeOH in $CHCl_3$	brown viscous liquid	132.6
ME24	222-235	5% MeOH in $CHCl_3$	brown viscous liquid	65.6
ME25	236-280	10% MeOH in $CHCl_3$ to 100% MeOH	brown viscous liquid	540

3.18.1.1 Isolation of EtOAc crude from mycelia extracts of fraction code ME11

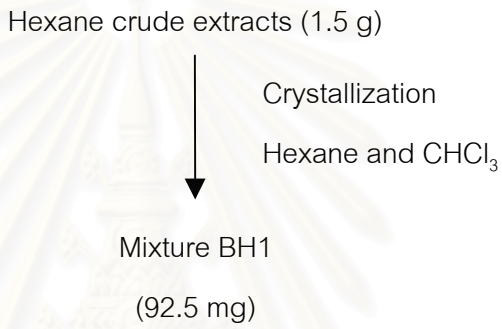
The EtOAc crude of mycelia extracts of fraction code ME25 (540 mg) was separated by column chromatography (silica gel, 35 g) using eluents of increasing polarity from $CHCl_3$ to MeOH and acetic acid mixture. The results from the separation of EtOAc crude of mycelia extracts (fraction code ME25) were presented in Table 3.3.

Table 3.3 The results from isolation of EtOAc crude from mycelia extract (Recolumn of fraction code ME25)

Fraction code	Fraction No.	Eluents	Appearance	Weight (mg)
ME2501	1-14	100% $CHCl_3$	Yellow viscous liquid	5.2
ME2502	15-22	100% $CHCl_3$	Yellow viscous liquid	3.7
ME2503	23-31	100% $CHCl_3$	Yellow viscous liquid	2.6
ME2504	32-85	0-2.5% MeOH in $CHCl_3$	Yellow brown viscous liquid	19.8
ME2505	86-190	2.5-12.5% MeOH in $CHCl_3$	Yellow brown viscous liquid	114.3
ME2506	191-203	2.5-12.5% MeOH in $CHCl_3$	Yellow brown viscous liquid	9.1
ME2507	204-232	12.5-20% MeOH in $CHCl_3$	Green viscous liquid	34.9
ME2508	233-287	20-90% MeOH in $CHCl_3$	Yellow brown viscous liquid	161.3
ME2509	288-391	0-1%Acetic acid in MeOH	Yellow brown viscous liquid	258.3

3.18.2 Isolation of bioactive compounds in crude hexane from broth culture extract

The crude hexane from endophytic fungus isolate PcBr20 culture broth extract (1.5 g) was purified by crystallization with a mixture of hexane and $CHCl_3$. It was dissolved in $CHCl_3$ until saturation, and then hexane was added. The solution was left standing at room temperature. The procedure for separation of mixture BH1 from hexane crude extract of culture broth extract is shown in scheme 3.2. The biological activity was examined and described in Chapter 4.



Scheme 3.2 Isolation of mixture BH1 from hexane crude of culture broth extract

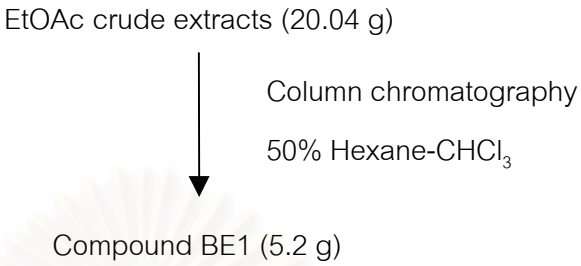
3.18.3 Isolation of bioactive compounds in crude EtOAc from broth culture extract

The crude EtOAc from endophytic fungus isolate PcBr20 culture broth extract (20.04 g) was subjected to column chromatography (silica gel, 360 g), using wet packing and dry loading method. Eluents of increasing polarity from hexane and $CHCl_3$ mixtures to MeOH were used. Gradient elution with hexane and $CHCl_3$ mixtures, $CHCl_3$, $CHCl_3$ 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 $CHCl_3$ mixtures, $CHCl_3$, $CHCl_3$ 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 biological activity of each pool fraction was examined and described in Chapter 4.

Table 3.4 The results from isolation of EtOAc crude from cultivation broth extract

Fraction code	Fraction No.	Eluents	Appearance	Weight (mg)
BE01	1-15	50% $CHCl_3$ in Hexane	Yellow viscous liquid	80.7
BE02	16-17	50% $CHCl_3$ in Hexane	Yellow viscous liquid	32.1
BE03	18-21	50% $CHCl_3$ in Hexane	Yellow viscous liquid	113.6
BE04	22-24	50% $CHCl_3$ in Hexane	Yellow viscous liquid	48.2
BE05	25-27	50% $CHCl_3$ in Hexane	Red brown viscous liquid	59.9
BE06	29-30	50% $CHCl_3$ in Hexane	Red brown viscous liquid	63.7
BE07	31-36	50% $CHCl_3$ in Hexane	Yellow brown viscous liquid	103.6
BE08	37	50% $CHCl_3$ in Hexane	Amorphous white solid in Red brown viscous liquid	60.1
BE09	28-42	50% $CHCl_3$ in Hexane	Amorphous white solid in red brown viscous liquid	1278.5
BE10	43-54	50% $CHCl_3$ in Hexane	Amorphous white solid in red brown viscous liquid	4198.5
BE11	55-75	50% $CHCl_3$ in Hexane	Amorphous white solid in red brown viscous liquid	1574.7
BE12	76-84	50% $CHCl_3$ in Hexane	Red brown viscous liquid	444.4
BE13	85-96	50% $CHCl_3$ in Hexane	Red brown viscous liquid	502.6
BE14	97-138	50% $CHCl_3$ in Hexane	Black brown viscous liquid	757.5
BE15	139-159	50% $CHCl_3$ in Hexane	Red brown viscous liquid	495.0
BE16	160-180	50% $CHCl_3$ in Hexane	Red brown viscous liquid Red brown	457.3
BE17	181-201	50% $CHCl_3$ in Hexane	viscous liquid	283.7
BE18	202-222	55% $CHCl_3$ in Hexane	Red brown viscous liquid	302.3
BE19	223-243	60% $CHCl_3$ in Hexane	Red brown viscous liquid	333.6
BE20	244-259	60% $CHCl_3$ in Hexane	brown viscous liquid	321.1
BE21	260-270	70% $CHCl_3$ in Hexane	Red brown viscous liquid	897.4
BE22	271-296	70% $CHCl_3$ in Hexane	Red brown viscous liquid	938.3
BE23	297-346	75% $CHCl_3$ in Hexane	Red brown viscous liquid	442.3
BE24	347-366	75% $CHCl_3$ in Hexane	Red brown viscous liquid	267.5
BE25	367-370	80% $CHCl_3$ in Hexane	Red brown viscous liquid	573.0
BE26	371-497	80-95% $CHCl_3$ in Hexane	Orange brown viscous liquid	309.7
BE27	498-597	95% $CHCl_3$ in Hexane	Brown viscous liquid	562.5
BE28	598-686	100% $CHCl_3$	Brown viscous liquid	260.0
BE29	687-776	10-50% MeOH in $CHCl_3$	Red brown viscous liquid	283.3
BE30	777-786	50-70% MeOH in $CHCl_3$	Gray brown viscous liquid	999.0
BE31	787-850	70-100% MeOH in $CHCl_3$	Black viscous liquid	1560.8

The procedure for separation of mixture BH1 from EtOAc crude of culture broth extract is shown in scheme 3.3.



Scheme 3.3 Isolation of compound BE1 from EtOAc crude of culture broth extract

3.19 Purification and properties of pure compounds from endophytic fungus isolate PcBr20

3.19.1 Purification and properties of mixture BH1

Mixture BH1 was purified by re-crystallization with $CHCl_3$ and hexane to obtain a colorless needle crystal (92.50 mg, 6.17% wt. by wt. of hexane crude extract from culture broth extract). Mixture BH1 has m.p. 115.5-116 ^{o}C and showed a sigle spot at the R_f value 0.25 on TLC plate using 15% MeOH in $CHCl_3$ as the mobile phase. TLC spots were visualized with UV lamp (254 nm) and with iodine vapour. Mixture BH1 is soluble in EtOAc, $CHCl_3$, MeOH, DMSO, H_2O, and slightly soluble in hexane.

Mixture BH1 is a colorless needle crystal (92.50 mg),$[\alpha]_D^{20}$ +4 (C 0.1, MeOH), UV λ_{max} (nm), MeOH (log ε): 227sh (4.49). (Figure 1 in Appendix B)

FT-IR spectrum (KBr), ν_{max}(cm^{-1}): 3455 (br), 3077 (m), 2960 (m), 2925 (m), 1707 (s), 1641 (m), 1587 (s), 1384 (s), 1279 (s), and 1034 (m). (Figure 2 in Appendix B)

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz.) δ (ppm): 0.97 (3H, t, J = 7.6), 1.41 (2H, dq, J = 7.6, 15.2), 1.69 (2H, dt, J = 7.2, 15.2, 2.46 (2H, q , J = 8.0), 2. 78 (2H, t, J = 7.6), 2.89 (2H, t, J = 7.2), 5.01 (2H, dd, J = 2.4, 13.2), 5.80 (1H, m), 7.80 (1H, d, J = 8.0), 8.18 (1H, d, J = 8.0), and 8.67 (1H, br s). (Figure 3 in Appendix B)

^{13}C-NMR spectrum ($CDCl_3$, 100 MHz.) δ (ppm): 13.7 (q), 22.2 (t), 32.3 (t), 32.7 (t), 32.9 (t), 34.6 (t), 116.4 (t), 124.3 (d), 136.2 (d), 138.6 (d), 138.7 (d), 142.1 (s), 143.2 (s), 144.6 (s), 144.8 (s), 147.6 (d), 147.8 (d), and 165.0 (s). (Figure 4 in Appendix B)

EI-MS spectrum (m/z) 70 eV: 339 (17), 313 (4), 269 (9), 180 (100), 162 (22), 135 (40), 119 (4), 92 (9), and 65 (3). (Figure 12 in Appendix B)

LC-MS spectrum (m/z): 358 (30), 180 (100), and 177.96 (50). (Figure 11 in Appendix B)

3.19.2 Purification and properties of the compound BE1

Compound BE1 was obtained from the elution of silica gel column chromatography with 50% $CHCl_3$ in hexane to obtain a white amorphous solid (5.2 g, 25.95% wt. by wt. of EtOAc crude extract from culture broth extract). Mixture BH1 has m.p. 190-191 ^{o}C and showed a sigle spot at the R_f value 0.25 on TLC plate using 15% MeOH in $CHCl_3$ as the mobile phase. TLC spots were visualized with UV lamp (254 nm) and with iodine vapour. Mixture BH1 is soluble in MeOH.

Compound BE1 is a white amorphous solid (5.2 g), $[\alpha]_D^{20}$ +9 (C 0.1, MeOH), UV λ_{max} (nm), MeOH (log ε): 223sh (3.59). (Figure 13 in Appendix B)

FT-IR spectrum, (KBr), ν_{max}(cm^{-1}): 2500-3600 (br), 2925 (m), 2855 (m), 1622 (s), 1567 (s), 1380 (s), 1256 (w), 1212 (w), 1116 (w), 1030 (w), 917 (w), and 804 (m). (Figure 14 in Appendix B)

LC-MS spectrum (m/z): 358 (20) and 179.98 (100). (Figure 11 in Appendix B)

3.20 Biological activity test

3.20.1 Antimicrobial activity test

3.20.1.1 Antimicrobial activity of the crude extracts and pool fractions

Evaluation of the antimicrobial activity of the fractions was determined by the agar well diffusion method (Weaver, Angel and Botlomley, 1994) in the same manner as described in section 3.7. Antimicrobial activity was performed against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. cerevisiae* TISTR 5169, and *C. albican* ATCC 10231.

3.20.1.2 Antimicrobial activity of pure compounds

Evaluation of the antimicrobial activity of pure compounds was 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, *S. cerevisiae* TISTR 5169, and *C. albican* ATCC 10231.

A. Preparation of pure compounds and antibiotic drug standards

Four mg of pure compounds and antibiotic drug standards were dissolved in 1 ml of 10% DMSO in sterile distilled water and kept in a refrigerator at 4 ^{o}C for bioassay. Antibacterial (Streptomycin and Penicillin G) and antifungal (Cyclohexamide and Ketokonazole) compound were used as positive controls.

B. Preparation of bacterial inoculum

A bacterial inoculum was prepared in the same manner as described in section 3.7.3. The final inoculum was diluted with NB to obtain a cell suspension containing approximately 10^6 CFU/ml.

C. Preparation of yeast inoculum

A yeast inoculum was performed in the same manner as described in section 3.7.4. The final inoculum was approximately 10^5 CFU/ml.

D. Assay procedure

Solutions of pure compounds and antibiotic drug standards were diluted with Mueller-Hinton Broth (MHB) and YMB for assays of antibacterial and antifungal (yeast form) activity respectively. Fifty µl of pure compound was dispensed into each well in sterile microtiter plates (96-well bottom wells). 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 ^{o}C and room temperature for bacterial and yeast, respectively.

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 Sunrise microplate reader (TECAN, AUSTRIA) before and after incubation. The lowest concentration of pure compound showing complete inhibition of growth was recorded as minimal inhibitory concentration (MIC).

For filamentous fungi results were determined by using the agar well diffusion method (Weaver, Angel, and Botlomley, 1994) in the same manner as described in section 3.7. Antimicrobial activity tests were performed against *Microsporum gypseam*, *Trichophyton mentagrophytes, Fusarium proliferatum,* and *Phytophthora parasitica*.

3.20.2 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 solubilization, 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 ^{o}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 ^{o}C for 4 to 24 h 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.

Samples were also tested for cytotoxic activity towards 5 cell lines, which contain of HEP-G2 (hepatoma), SW 620 (colon), Chago (lung), Kato-3 (gastric), and BT 474 (breast) following the experimental method of bioassay of cyototoxic activity.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Isolation of endophytic fungi

Sixty two endophytic fungal isolates were isolated from healthy young leaves, mature leaves, branches and bark of *Croton oblongifolius*. All endophytic fungal isolates were selected for further study, as shown in Table 4.1.

Table 4.1 Number and isolate of endophytic fungi

Plant section	Number of endophytic fungi (Isolates)	Endophytic fungi isolates
Young leaf	8	PcLy01-08
Mature leaf	14	PcLm01-14
Branch	23	PcBr01-23
Bark	17	PcBa01-17
Total (isolates)	62	62

4.2 Characterization and identification of endophytic fungi

Each fungal isolate was grown on MEA, for 2-4 weeks at room temperature. Colony morphology of the 62 fungal isolates is shown in Figures 4.1-4.4. A total of 62 isolates of endophytic fungi were identified. Twenty one fungal isolates were identified as belonging to typical genera of endophytes such as *Cladosporium*, *Colletotrichum*, *Fusarium*, *Pestalotia*, *Phomopsis*, and members of the Xylariaceae. Seven fungal isolates were identified as hyphomycetes. The remaining of 34 isolates of endophytic fungi did not produce conidia or sporulate on media and were therfore recorded as mycelia sterilia. The results and summary of identification of endophytic fungi is shown in Tables 4.2 and 4.3, respectively.

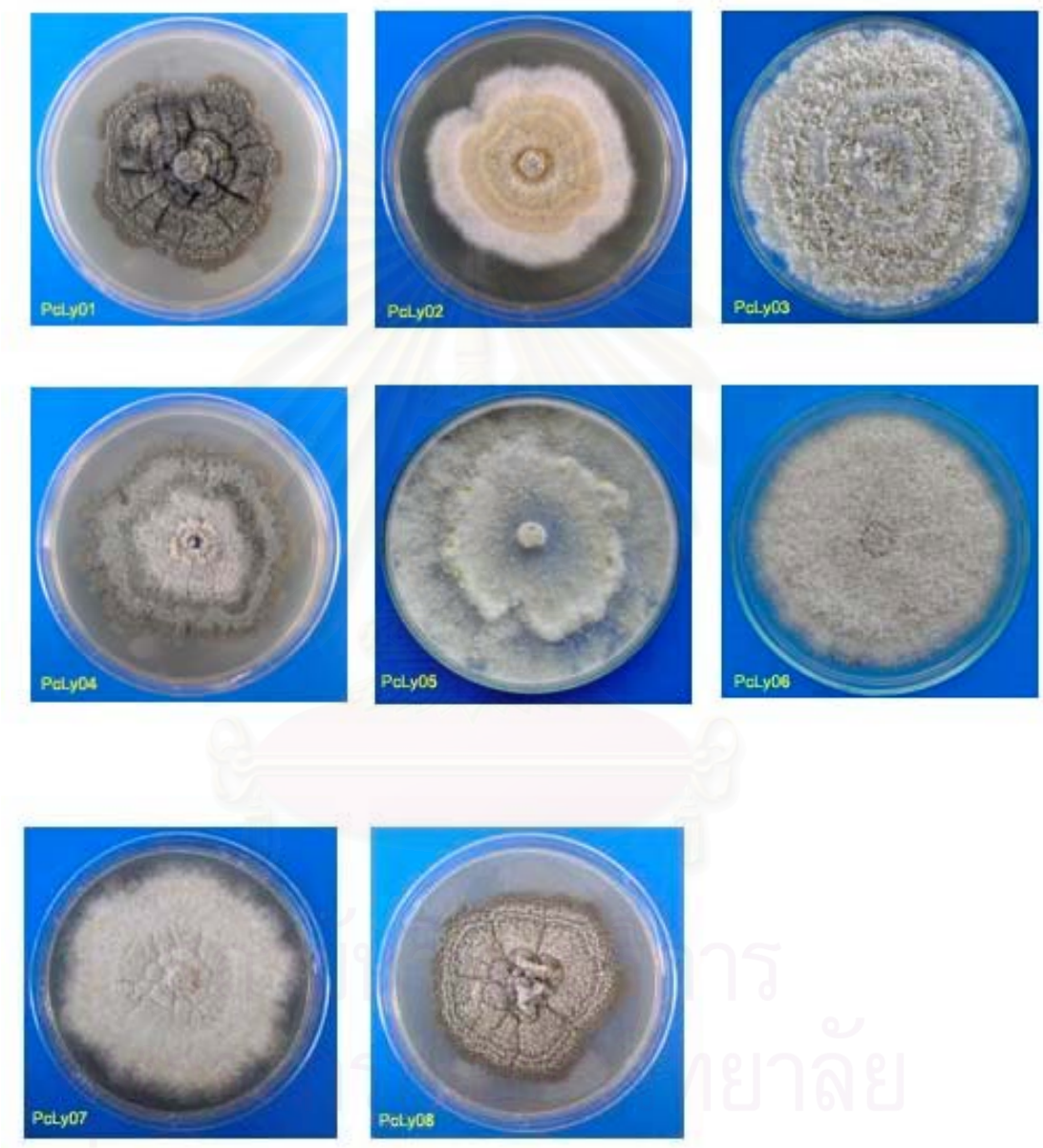


Figure 4.1 Colony characteristic of endophytic fungal isolates, PcLy01-08, on MEA after cultivation for 2-4 weeks at room temperature. Isolate number is shown in each picture.

Figure 4.2 Colony characteristic of endophytic fungal isolates, PcLm01-14, on MEA after cultivation for 2-4 weeks at room temperature. Isolate number is shown in each picture

.

Figure 4.2 (continued)

Figure 4.3 Colony characteristic of endophytic fungal isolates, PcBr01-23, on MEA after cultivation for 2-4 weeks at room temperature. Isolate number is shown in each picture.

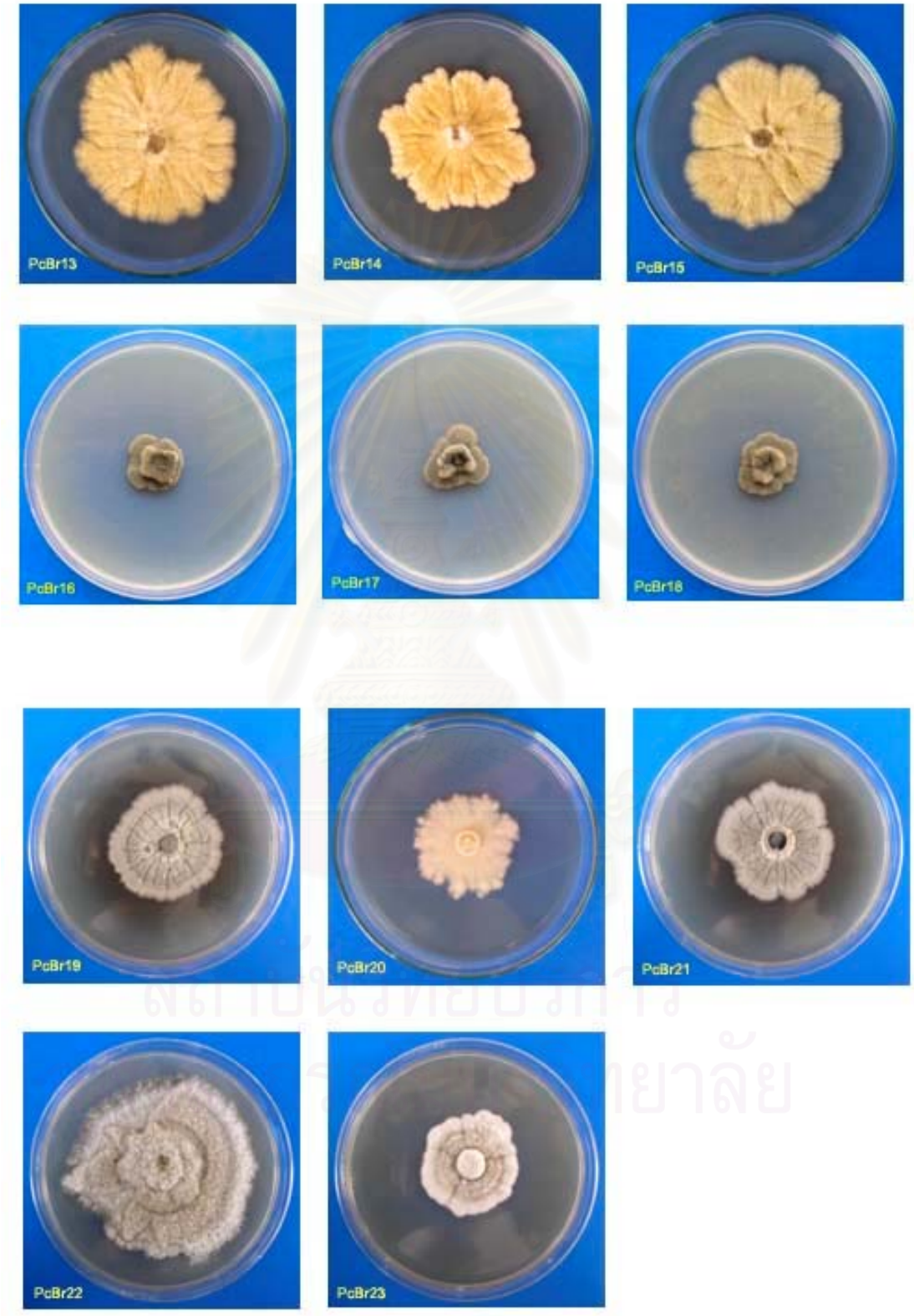


Figure 4.3 (continued)

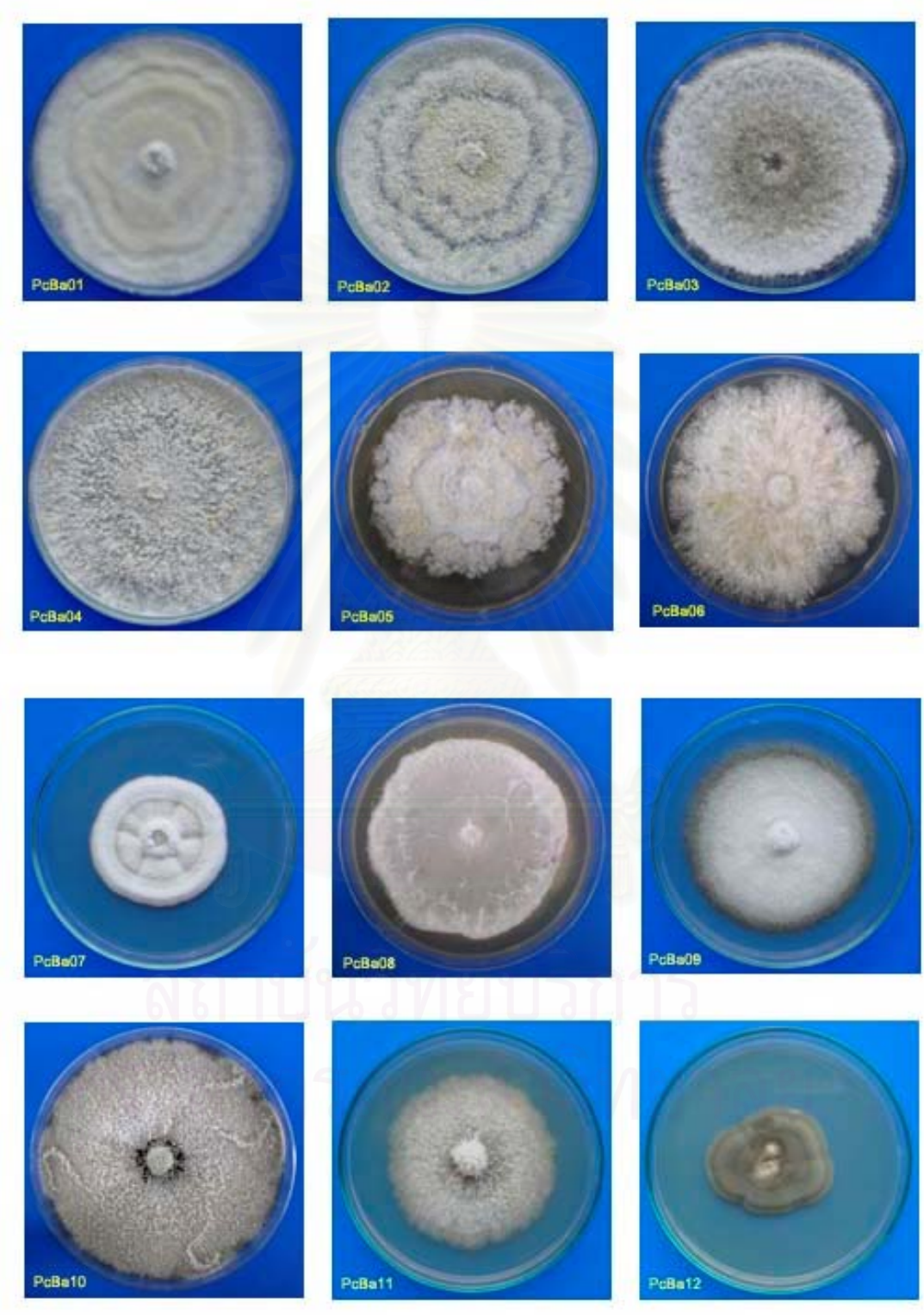


Figure 4.4 Colony characteristic of endophytic fungal isolates, PcBa01-17, on MEA after cultivation for 2-4 weeks at room temperature. Isolate number is shown in each picture

.

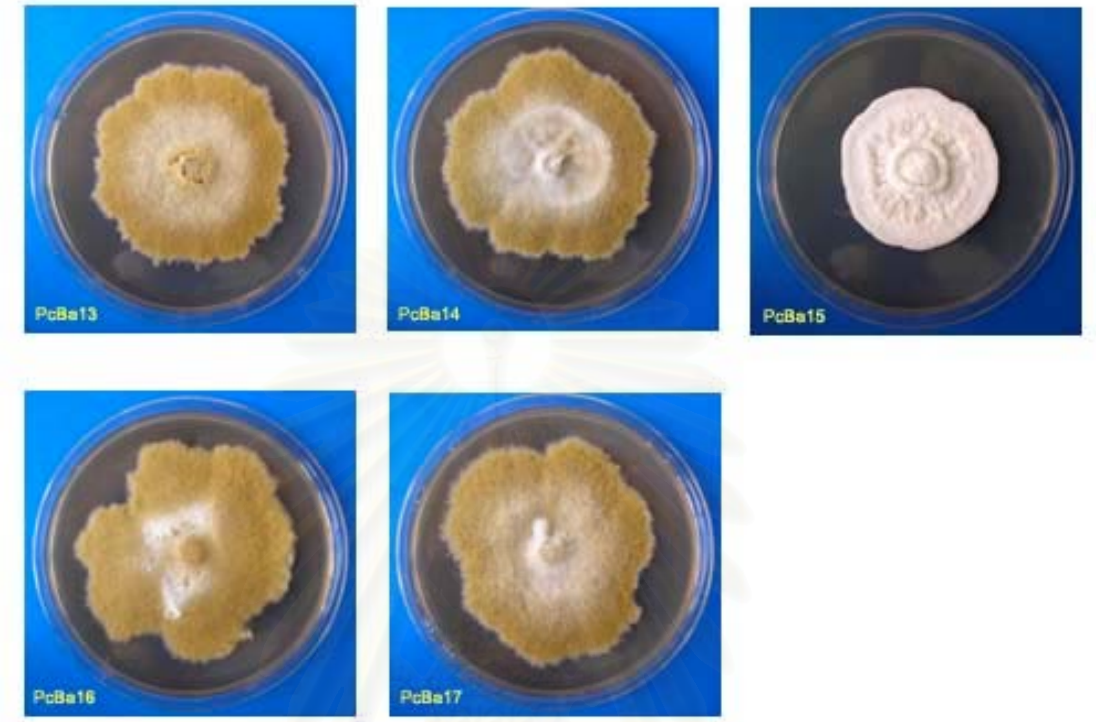


Figure 4.4 (continued)

Table 4.2 Identification of endophytic fungi

Plant section	Endophyte isolate	Species
Young leaf	PcLy01	Mycelia sterilia
	PcLy02	Mycelia sterilia
	PcLy03	Mycelia sterilia
	PcLy04	Mycelia sterilia
	PcLy05	Mycelia sterilia
	PcLy06	*Colletotrichum* sp.
	PcLy07	Mycelia sterilia
	PcLy08	Mycelia sterilia
Mature leaf	PcLm01	Mycelia sterilia
	PcLm02	*Colletotrichum* sp.
	PcLm03	Mycelia sterilia
	PcLm04	Mycelia sterilia
	PcLm05	Mycelia sterilia
	PcLm06	*Colletotrichum* sp.
	PcLm07	*Colletotrichum* sp.
	PcLm08	Mycelia sterilia
	PcLm09	Mycelia sterilia
	PcLm10	*Phomopsis* sp.
	PcLm11	*Colletotrichum* sp.
	PcLm12	*Phomopsis* sp.
	PcLm13	Mycelia sterilia
	PcLm14	Mycelia sterilia
Branch	PcBr01	*Fusarium* sp.
	PcBr02	*Fusarium* sp.
	PcBr03	Mycelia sterilia
	PcBr04	*Fusarium* sp.
	PcBr05	Xyraliaceae

Table 4.2 (continued)

Plant section	Endophyte isolate	Species
Branch	PcBr06	*Fusarium* sp.
	PcBr07	*Fusarium* sp.
	PcBr08	*Fusarium* sp.
	PcBr09	Hyphomycete
	PcBr10	*Fusarium* sp.
	PcBr11	Hyphomycete
	PcBr12	Mycelia sterilia
	PcBr13	*Fusarium* sp.
	PcBr14	*Fusarium* sp.
	PcBr15	*Fusarium* sp.
	PcBr16	Mycelia sterilia
	PcBr17	Mycelia sterilia
	PcBr18	Mycelia sterilia
	PcBr19	Mycelia sterilia
	PcBr20	*Fusarium* sp.
	PcBr21	Mycelia sterilia
	PcBr22	Mycelia sterilia
	PcBr23	Mycelia sterilia
Bark	PcBa01	Mycelia sterilia
	PcBa02	Mycelia sterilia
	PcBa03	*Pestalotia* sp.
	PcBa04	Mycelia sterilia
	PcBa05	Mycelia sterilia
	PcBa06	Mycelia sterilia
	PcBa07	Mycelia sterilia
	PcBa08	Hyphomycete
	PcBa09	Mycelia sterilia

Table 4.2 (continued)

Plant section	Endophyte isolate	Species
Bark	PcBa10	Mycelia sterilia
	PcBa11	Mycelia sterilia
	PcBa12	*Cladosporium* sp.
	PcBa13	Hyphomycete
	PcBa14	Hyphomycete
	PcBa15	Mycelia sterilia
	PcBa16	Hyphomycete
	PcBa17	Hyphomycete

Table 4.3 A summary of identification of endophytic fungi

Species	Plant section and isolation frequency				Total (isolates)
	Young leaf	Mature leaf	Branch	Bark	
Cladosporium sp.	0	0	0	1	1
Colletotrichum sp.	1	4	0	0	5
Fusarium sp.	0	0	11	0	11
Pestalotia sp.	0	0	0	1	1
Phomopsis sp.	0	2	0	0	2
Xylariaceae	0	0	1	0	1
Hyphomycetes	0	0	2	5	7
Mycelia sterilia	7	8	9	10	34
Total (isolates)	8	14	23	17	62

The fungal genera found in young and mature leaves, branches, and barks of *Croton oblongifolius* in this study were *Cladosporium*, *Colletotrichum*, *Fusarium*, *Pestalotia*, *Phomopsis*, members of the Xylariaceae and mycelia sterilia. All fungal genera isolate are well known as common endophytes on many different plants. For example, *Cladosporium* has been found in mangrove forest such as *Avicennia marian*, *Avicennia officinalis*, *Ceriops decandra* and *Lumnitzera racemosa* (Kumaresan and Suryanarayanan, 2001). *Colletotrichum* was found *on* wild banana (Photita et al., 2001). *Fusarium* has been found on *Ephedra* and *Rosmarinus* (Pelaez et al., 1998). *Phomopsis* has been found on *Eucalyptus globulus* (Bettucci et al., 1999). *Pestalotia* has been found on *Taxus baccata* (Strobel et al., 1996). *Xylaria* (anamorph) spp. has been found on palms (*Lucuala* sp.) (Frohlich, Hyde, and Petrini, 2000). Mycelia sterilia has been found on manuka (*Leptospermum scoparium*), estuarine mangrove forest (*Sesuvium portulacastrum*), and most Thai medicinal plants (Johnston, 1998; Suryanarayanan and Kumaresan, 2000; Panphut, 2001).

4.3 Taxonomy of endophytic fungi

Descriptions of the genus

CLADOSPORIUM (Ellis, 1971)

Cladosporium Link ex Fries; Link, 1815, *Magazin Ges, naturf. Freunde Berlin*, 7: 37-38; Fries, 1821, Syst. mycol., 1: XLVI. (Figure 4.5)

Colonies effuse or occasionally punctiform, often olivaceous but also sometimes grey, buff, brown or dark blackish brown, velvety, floccose or hairy. Mycelium immersed and often also superficial. Stroma sometimes present. Setae and hyphopodia absent, Conidophores macronematous or semimacronematous and sometimes also micronematous; macronematous conidiophores straight or flexuous, mostly unbranched or with branches restricted to the apical region forming a stipe and head, olivaceous brown or brown, smooth or verrucose. Ramo-conidia often present. Conidiogenous cells polyblastic, usually integrated, terminal and intercalary but sometimes discrete, sympodial, more or less cylindrical, cicatrized, scars usually promiinent. Conidia catenate as a rule but sometimes solitary especially in species with large conidia, often in branched chains, acropleurogenous, simple, cylindrical, doliiform, ellipsoidal, fusiform, ovoik, spherical or subspherical, often with a distinctly protuberant scar at each end or just at the base, pale to dark olivaceous brown or brown. Smooth, verruculose or echinulate, with 0-3 or occasionally more septa.

COLLETOTRICHUM (Sutton, 1980)

Collectotrichum Cda in Sturm, *Deutschlands Flora* 3: 41 (1831-1832). (Figure 4.6)

Mycelium immersed, branched, septate, hyaline, pale brown or dark brown. Conidiomata acervular, subcuticular, epidermal, subepidermal or peridermal, separate or confluent, composed of hyaline to dark brown, thin-or thick-walled textura angularis; dehiscence irregular. Sclerotia sometimes present in culture, dark brown to black, often confluent, occasionally setose. Setae in conidiomata or sclerotia, brown, smooth,

septate, tapered to the apices. Conidiophores hyaline to brown, septate, branched only at the base, smooth, formed from the upper cells of the conidiomata. Conidiogenous cells enteroblastic, phialidic, hyaline, smooth, determinate, cylindrical, intergrated or discrete, channel minute but occasionally collarette and periclinal thickening quite prominent. Conidia hyaline, aseptate (except prior to germination), straight or falcate, smooth, thin-walled, sometimes guttulate, muticate or with the apex prolonged into a simple cellular appendage. Appressoria brown, entire or with crenate to irregular margins, simple or repeatedly germinating to produce complex columns of several closely connected appressoria.

FUSARIUM (Subramanian, 1971)

Fusarium Link ex Fries. *Syst. Mycol.* 3: 469, 1932. (Figure 4.7)

Mycelium composed of hyaline, septate, branched hyphae. Conidial masses typically formed in sporodochia or in pionnotes 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 phialides, or else abstricted 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, occuring 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.

PESTALOTIA (Sutton, 1980)

Pestalotia de Not., Mem. *R. Accad. Sci.*, *Torino* 2, 3: 80 (1839). (Figure 4.8)

Mycelium immersed, branched, septate, brown. Conidiomata eustromatic, cupulate, separate or confluent, black or dark brown, at first immersed, then erumpent, composed of thick-walled, dark brown textura angularis, giving rise to thinner-walled, paler tissue in the conidiogenous region. Dehiscence irregular. Conidiophores hyaline. Branched irregularly, septate, smooth, formed from the upper cells of the pseudoparenchyma. Conidiogenous cells holoblastic, annellidic, indeterminate, integrated, cylindrical, hyaline, smooth, with 1-3 percurrent proliferations. Conidia fusiform, straight or slightly curved, 6-celled; basal cell hyaline, thin-walled, truncate, with an endogenous, cellular, simple or dichotomously branched appendage; apical cell conic, thin-walled, hyaline, with 3-9 apical, simple or dichotomously branched appendages; median cells separated from end cells by eusepta but from one another by distosepta; lumina reduced, medium brown.

PHOMOPSIS (Sutton, 1980)

Phomopsis (Sacc.) Sacc., *Annls mycol*. 3: 166 (1905). (Figure 4.9)

Mycelium immersed, branched, septate, hyaline to pale brown. Conidiomata eustromatic, immersed, brown to dark brown, septate or aggregated and confluent, globose, ampuliform or applanate, unilocular, multilocular or convoluted, thick-walled; walls of brown, thin- or thick-walled textura angularis, often somewhat darker in the upper region, lined by a layer of smaller-celled tissue. Ostiole single, or serveral in complex conidiomata, circular, often papillate. Conidiophores branched and septate at the base and above, occasionally short and only 1-2 septate, more frequently multiseptate and filiform, hyaline, formed from the inner cells of the locular walls.

Conidiogenous cells enteroblastic, phialidic, determinate, integrated, rarely discrete, hyaline, cylindrical, apertures apical on long or short lateral and main branches of the conidiophores, collarette, channel and periclinal thickening minute. Conidia of two basic type, but in some species with intermediates between the two: α-conidia hyaline, fusiform, straight, usually biguttulate (one guttule at each end) but sometimes with more guttules, aseptate; β-conidia hyaline, filiform, straight or more often hamate, eguttulate, aseptate.

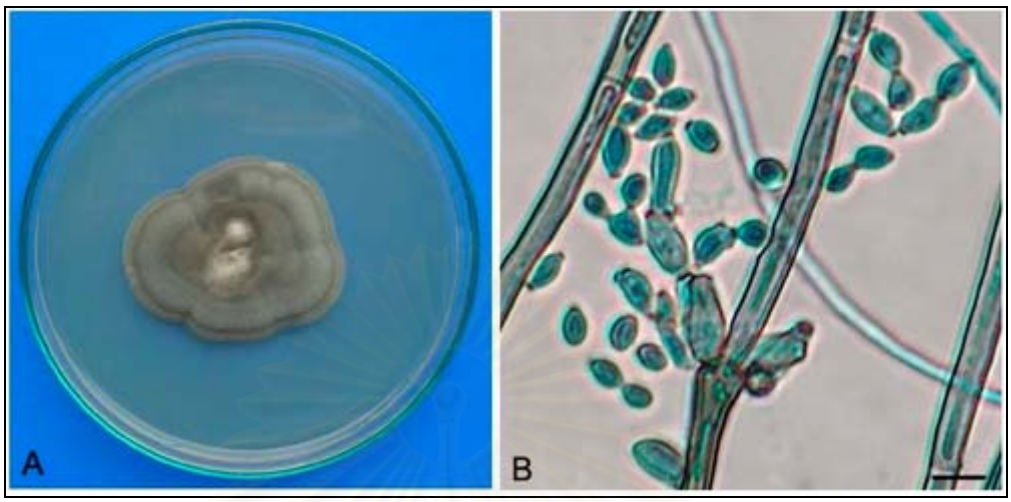


Figure 4.5 *Cladosporium* sp. A: Culture on MEA (14 days). B: Conidia. (Bar = 10 µm)

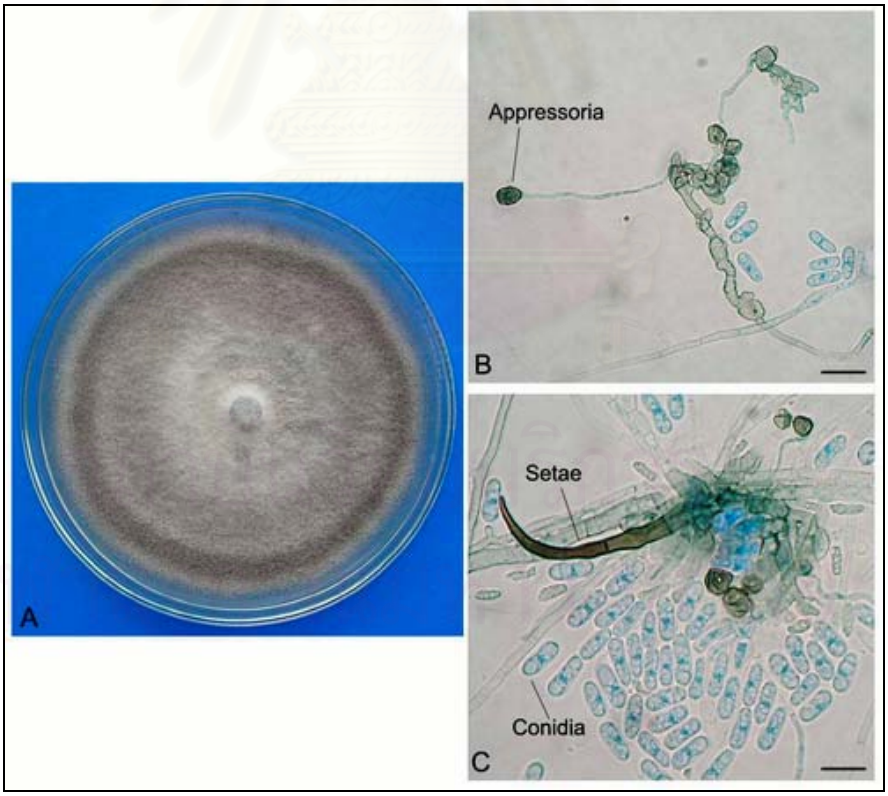


Figure 4.6 *Colletotrichum* sp. A: Culture on MEA (21 days). B: Appressoria and conidia. C: Setae, appressoria and conidia. (Bar = 5 µm)

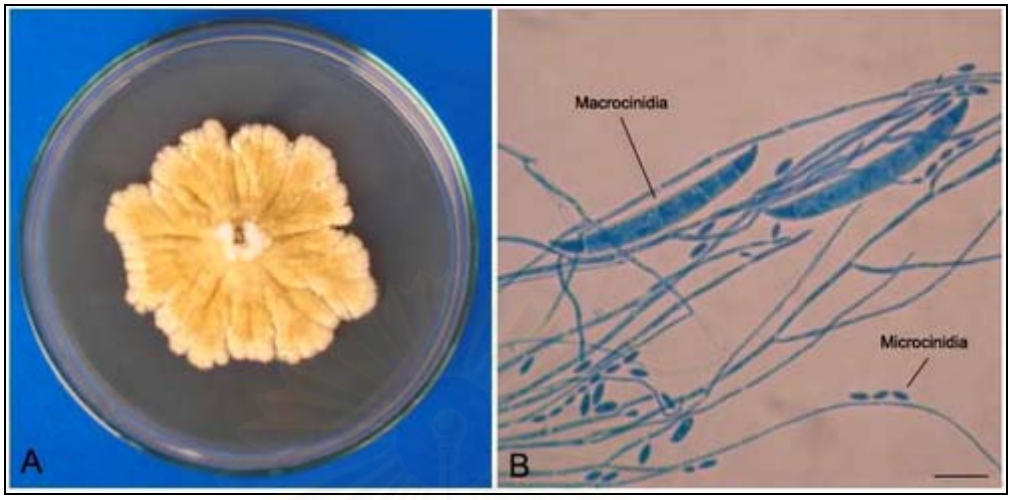


Figure 4.7 *Fusarium* sp. A: Culture on MEA (14 days). B: Microconidia and macroconidia. (Bar = 10 µm)

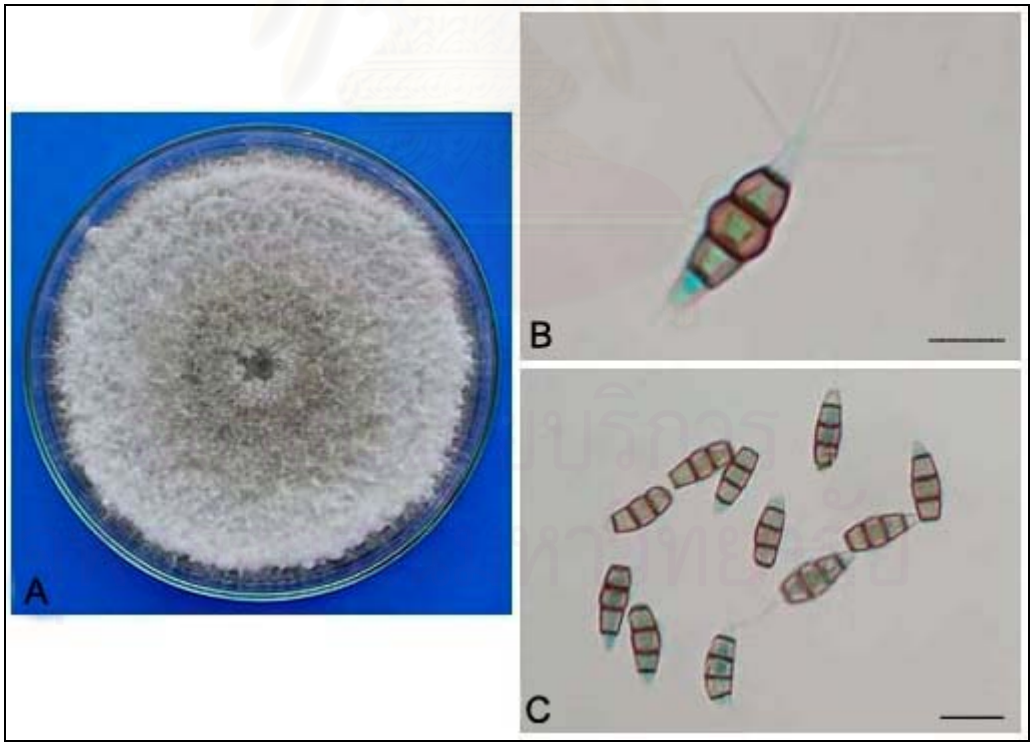


Figure 4.8 *Pestalotia* sp. A: Culture on MEA (14 days). B: Conidia. (Bar = 5 µm) C: Conidia. (Bar = 10 µm)

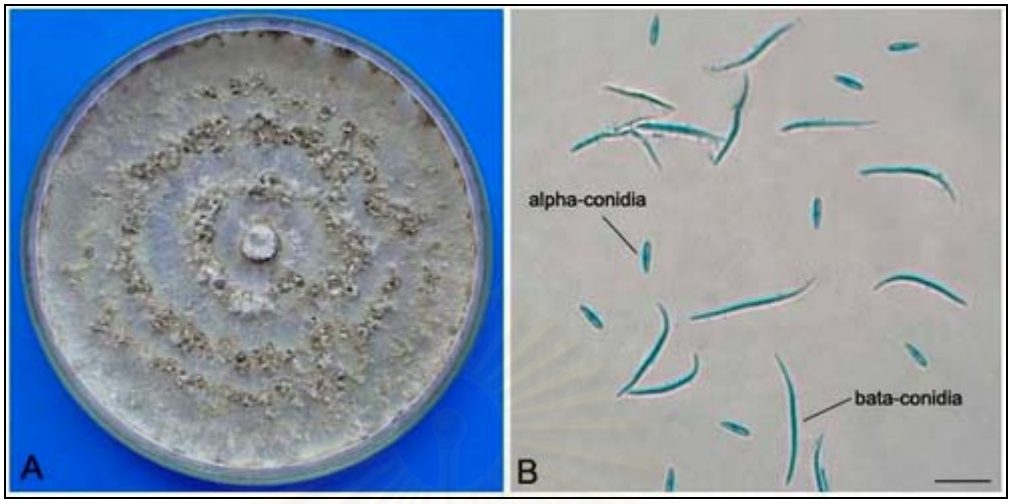


Figure 4.9 *Phomopsis* sp. A: Culture on MEA (14 days). B: Alpha and beta conidia. (Bar = 10 μm)

4.4 Crude extracts of endophytic fungi

Sixty two endophytic fungal isolates were grown in malt extract broth (MEB). Mycelium was separated from culture broth by filtration and extracted with MeOH. Culture broth was concentrated by evaporation, freeze dried and extracted with MeOH. From a total of 62 endophytic fungal cultures, a total of 124 samples were obtained as shown in Table 4.4. The culture broth extracts and mycelium extracts were further tested for antimicrobial activity and metabolite profiles.

Table 4.4 The culture broth extracts and mycelia extracts from endophytic fungi

Crude extracts	Number of samples
Culture broth	62
Mycelium	62
Total	124

4.5 Enumeration of test microorganisms

Viable counts of bacteria and yeast were performed for standardized inocula whose turbidity matched a 0.5 McFarland standard. Direct counting of conidia or spores of filamentous fungi in suspension was performed using a counting chamber. The CFU/ml and spores/ml values are shown in Table 4.5.

Table 4.5 Quantity of standardized inocula

Test microorganisms	Quantity
Bacillus subtilis ATCC 6633	3.4 X 10^7 CFU/ml
Staphylococcus aureus ATCC 25923	7 X 10^7 CFU/ml
Escherichia coli ATCC 25922	1.52 X 10^7 CFU/ml
Pseudomonas aeruginosa ATCC 27853	2.82 X 10^7 CFU/ml
Saccharomyces cerevisiae TISTR 5169	4.8 X 10^5 CFU/ml
Candida albicans ATCC 10231	5.67 X 10^5 CFU/ml
Microsporum gypseam (clinical isolate)	3.5 X10^6 spores/ml
Trichophyton mentagrophytes (clinical isolate)	4.2 X10^6 spores/ml

CFU: Colony forming unit

4.6 Determination of antimicrobial activity

Antimicrobial activities of culture broth extracts and mycelium extracts against test microorganisms were determined using the agar well diffusion method. The antimicrobial activities of the viroius isolates are shown in Figure 4.10, Tables 4.6- 4.13.

The results of antimicrobial activity assays of endophytic fungi isolated from each plant section which were active against at least one test microorganisms are summarized in Table 4.14-4.15 and in histograms in Figure 4.11-4.12.

The results showed that 36 isolates of culture broth or/and mycelium extracts displayed growth inhibition on at least one test microorganisms. For example 5 isolates

showed activity with culture broth and mycelium extracts, 26 isolates showed activity with broth and 5 isolates showed activity with mycelium.

Results of antimicrobial activity assays against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. cerevisiae* TISTR 5169*, C. albicans* ATCC 10231, *M. gypseam* and *T. mentagrophytes* are summarized in Table 4.14, and in histograms in Figure 4.12.

The results showed that 10 isolates of culture broth or/and mycelium extracts were active against *B. subtilis* ATCC 6633. Three isolates showed activity with culture broth and mycelium extracts, 4 isolates showed activity with broth and 3 isolates showed activity with mycelium.

Twenty seven isolates of culture broth or/and mycelium extracts were active against *S. aureus* ATCC 25923. Four isolates showed activity with culture broth and mycelium extracts, 18 isolates showed activity with broth and 5 isolates showed activity with mycelium.

Four isolates of culture broth or/and mycelium extracts were active against *E. coli* ATCC 25922. Three isolates showed activity with broth and 1 isolate showed activity with mycelium.

Two isolates of culture broth extracts were active against *P. aeruginosa* ATCC 27853.

Eight isolates of culture broth extracts were active against *S. cerevisiae* TISTR 5169. One isolate of culture broth and mycelium extracts were active against *C. albicans* ATCC 10231.

No fungal isolate inhibited the grownth of *M. gypseam*.

Two isolates of culture broth and mycelium extracts were active against *T. mentagrophytes*.

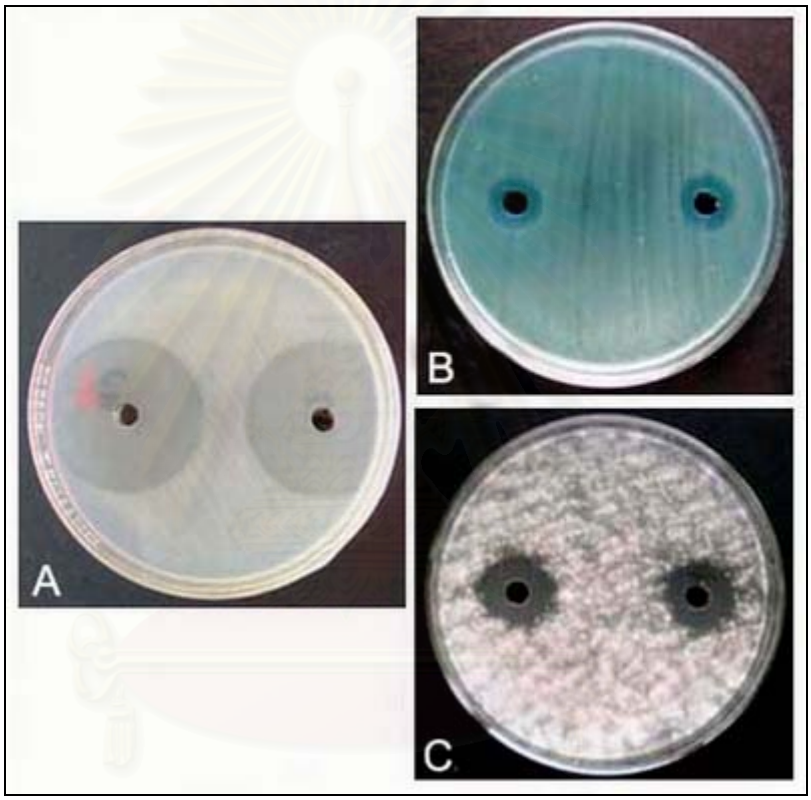


Figure 4.10 Agar well diffusion method for antimicrobial activity. A: Against *B. subtilis* ATCC 6633. B: Against *P. aeruginosa* ATCC 27853. C: Against *T. mentagrophyte.* Inhibition zone indicates growth inhibition.

Table 4.6 Antimicrobial activity of culture broth extracts of endophytic fungi isolated from young leaves

Isolates	Test microorganisms							
	Gram positive bacteria		Gram negative bacteria		Yeast		Filamentous fungi	
	B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*S. serevisiae* TISTR 5169	*C. albicans* ATCC 10231	*M. gypseam*	*T. mentagrophytes*
PcLy01	-	-	-	-	-	-	-	-
PcLy02	-	-	-	-	-	-	-	-
PcLy03	-	-	-	-	-	-	-	-
PcLy04	-	-	-	-	-	-	-	-
PcLy05	-	-	-	-	-	-	-	-
PcLy06	-	++	+	-	-	-	-	-
PcLy07	-	-	-	-	-	-	-	-
PcLy08	-	-	-	-	-	-	-	-

Activities were classified according to the diameter of the inhibition zones around the point of application of the sample ++, more than 15 mm; +, less than 15 mm; -, no inhibition.

Table 4.7 Antimicrobial activity of mycelium extracts of endophytic fungi isolated from young leaves

Isolates	Test microorganisms							
	Gram positive bacteria		Gram negative bacteria		Yeast		Filamentous fungi	
	B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*S. serevisiae* TISTR 5169	*C. albicans* ATCC 10231	*M. gypseam*	*T. mentagrophytes*
PcLy01	-	-	-	-	-	-	-	-
PcLy02	-	-	-	-	-	-	-	-
PcLy03	-	-	-	-	-	-	-	-
PcLy04	-	+	-	-	-	-	-	-
PcLy05	-	-	-	-	-	-	-	-
PcLy06	-	-	-	-	-	-	-	-
PcLy07	-	-	-	-	-	-	-	-
PcLy08	-	-	-	-	-	-	-	-

Activities were classified according to the diameter of the inhibition zones around the point of application of the sample ++, more than 15 mm; +, less than 15 mm; -, no inhibition.

Table 4.8 Antimicrobial activity of culture broth extracts of endophytic fungi isolated from mature leaves

Isolates	Test microorganisms							
	Gram positive bacteria		Gram negative bacteria		Yeast		Filamentous fungi	
	B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*S. serevisiae* TISTR 5169	*C. albicans* ATCC 10231	*M. gypseam*	*T. mentagrophytes*
PcLm01	-	-	-	-	-	-	-	-
PcLm02	-	+	-	-	-	-	-	-
PcLm03	-	-	-	-	-	-	-	-
PcLm04	+	-	-	-	-	-	-	-
PcLm05	-	-	-	-	-	-	-	-
PcLm06	-	+	-	-	-	-	-	-
PcLm07	+	-	-	-	-	-	-	-
PcLm08	-	-	+	-	-	-	-	-
PcLm09	-	-	-	-	-	-	-	-
PcLm10	-	-	-	-	-	-	-	-
PcLm11	-	-	-	-	-	-	-	-
PcLm12	-	-	-	-	-	-	-	-
PcLm13	-	-	-	-	-	-	-	-
PcLm14	+	+	-	-	-	-	-	-

Activities were classified according to the diameter of the inhibition zones around the point of application of the sample ++, more than 15 mm; +, less than 15 mm; -, no inhibition.

Table 4.9 Antimicrobial activity of mycelium extracts of endophytic fungi isolated from mature leaves

Isolates	Test microorganisms							
	Gram positive bacteria		Gram negative bacteria		Yeast		Filamentous fungi	
	B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*S. serevisiae* TISTR 5169	*C. albicans* ATCC 10231	*M. gypseam*	*T. mentagrophytes*
PcLm01	-	-	-	-	-	-	-	-
PcLm02	-	-	-	-	-	-	-	-
PcLm03	-	-	-	-	-	-	-	-
PcLm04	-	-	-	-	-	-	-	-
PcLm05	-	-	-	-	-	-	-	-
PcLm06	-	-	-	-	-	-	-	-
PcLm07	-	-	-	-	-	-	-	-
PcLm08	-	+	-	-	-	-	-	-
PcLm09	-	+	-	-	-	-	-	-
PcLm10	-	-	+	-	-	-	-	-
PcLm11	-	-	-	-	-	-	-	-
PcLm12	+	+	-	-	-	-	-	-
PcLm13	-	-	-	-	-	-	-	-
PcLm14	+	+	-	-	-	-	-	-

Activities were classified according to the diameter of the inhibition zones around the point of application of the sample ++, more than 15 mm; +, less than 15 mm; -, no inhibition.

Table 4.10 Antimicrobial activity of culture broth extracts of endophytic fungi isolated from branches

Isolates	Test microorganisms							
	Gram positive bacteria		Gram negative bacteria		Yeast		Filamentous fungi	
	B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*S. serevisiae* TISTR 5169	*C. albicans* ATCC 10231	*M. gypseam*	*T. mentagrophytes*
PcBr01	-	++	-	-	-	-	-	-
PcBr02	-	++	-	-	-	-	-	-
PcBr03	-	-	-	+	-	-	-	-
PcBr04	-	+	-	-	-	-	-	-
PcBr05	-	-	-	-	+	-	-	-
PcBr06	-	++	-	-	+	-	-	-
PcBr07	-	+	-	-	-	-	-	-
PcBr08	-	++	-	-	-	-	-	-
PcBr09	-	++	-	-	-	-	-	-
PcBr10	-	++	-	-	+	-	-	-
PcBr11	-	+	-	-	-	-		
PcBr12	-	-	-	-	-	-	-	-
PcBr13	-	++	-	-	+	-	-	-
PcBr14	-	-	-	-	+	-	-	-
PcBr15	-	+	-	-	-	-	-	-
PcBr16	-	-	-	-	-	-	-	-
PcBr17	-	-	-	-	-	-	-	-
PcBr18	-	-	-	-	-	-	-	-
PcBr19	-	-	-	-	-	-	-	-
PcBr20	++	++	++	-	++	++	-	++
PcBr21	-	-	-	-	-	-	-	-
PcBr22	-	-	-	-	-	-	-	-
PcBr23	-	-	-	-	-	-	-	-

Activities were classified according to the diameter of the inhibition zones around the point of application of the sample ++, more than 15 mm; +, less than 15 mm; -, no inhibition.

Table 4.11 Antimicrobial activity of mycelium extracts of endophytic fungi isolated from branches

Isolates	Test microorganisms							
	Gram positive bacteria		Gram negative bacteria		Yeast		Filamentous fungi	
	B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*S. serevisiae* TISTR 5169	*C. albicans* ATCC 10231	*M. gypseam*	*T. mentagrophytes*
PcBr01	-	-	-	-	-	-	-	-
PcBr02	-	-	-	-	-	-	-	-
PcBr03	-	-	-	-	-	-	-	-
PcBr04	-	-	-	-	-	-	-	-
PcBr05	-	-	-	-	-	-	-	-
PcBr06	-	-	-	-	-	-	-	-
PcBr07	-	-	-	-	-	-	-	-
PcBr08	-	-	-	-	-	-	-	-
PcBr09	-	-	-	-	-	-	-	-
PcBr10	-	-	-	-	-	-	-	-
PcBr11	-	-	-	-	-	-	-	-
PcBr12	+	+	-	-	-	-	-	-
PcBr13	-	-	-	-	-	-	-	-
PcBr14	-	-	-	-	-	-	-	-
PcBr15	-	-	-	-	-	-	-	-
PcBr16	-	-	-	-	-	-	-	-
PcBr17	-	-	-	-	-	-	-	-
PcBr18	-	-	-	-	-	-	-	-
PcBr19	-	-	-	-	-	-	-	-
PcBr20	++	++	++	-	-	+	-	+
PcBr21	-	-	-	-	-	-	-	-
PcBr22	-	-	-	-	-	-	-	-
PcBr23	-	-	-	-	-	-	-	-

Activities were classified according to the diameter of the inhibition zones around the point of application of the sample ++, more than 15 mm; +, less than 15 mm; -, no inhibition.

Table 4.12 Antimicrobial activity of culture broth extracts of endophytic fungi isolated from bark

Isolates	Test microorganisms							
	Gram positive bacteria		Gram negative bacteria		Yeast		Filamentous fungi	
	B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*S. serevisiae* TISTR 5169	*C. albicans* ATCC 10231	*M. gypseam*	*T. mentagrophytes*
PcBa01	++	-	-	++	-	-	-	-
PcBa02	-	-	-	-	-	-	-	-
PcBa03	-	-	-	-	-	-	-	-
PcBa04	-	-	-	-	-	-	-	-
PcBa05	-	-	-	-	-	-	-	-
PcBa06	-	-	-	-	-	-	-	-
PcBa07	-	++	-	-	-	-	-	-
PcBa08	+	++	-	-	-	-	-	-
PcBa09	-	+	-	-	-	-	-	+
PcBa10	-	++	-	-	-	-	-	-
PcBa11	-	++	-	-	-	-	-	-
PcBa12	+	-	-	-	-	-	-	-
PcBa13	-	-	-	-	-	-	-	-
PcBa14	-	++	-	-	+	-	-	-
PcBa15	-	-	-	-	-	-	-	-
PcBa16	-	-	-	-	-	-	-	-
PcBa17	-	-	-	-	+	-	-	-

Activities were classified according to the diameter of the inhibition zones around the point of application of the sample ++, more than 15 mm; +, less than 15 mm; -, no inhibition.

Table 4.13 Antimicrobial activity of mycelium extracts of endophytic fungi isolated from bark

Isolates	Test microorganisms							
	Gram positive bacteria		Gram negative bacteria		Yeast		Filamentous fungi	
	B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*S. serevisiae* TISTR 5169	*C. albicans* ATCC 10231	*M. gypseam*	*T. mentagrophytes*
PcBa01	-	-	-	-	-	-	-	-
PcBa02	-	-	-	-	-	-	-	-
PcBa03	-	-	-	-	-	-	-	-
PcBa04	-	-	-	-	-	-	-	-
PcBa05	-	-	-	-	-	-	-	-
PcBa06	-	-	-	-	-	-	-	-
PcBa07	-	-	-	-	-	-	-	-
PcBa08	+	+	-	-	-	-	-	-
PcBa09	+	+	-	-	-	-	-	+
PcBa10	-	-	-	-	-	-	-	-
PcBa11	-	-	-	-	-	-	-	-
PcBa12	-	-	-	-	-	-	-	-
PcBa13	-	-	-	-	-	-	-	-
PcBa14	-	-	-	-	-	-	-	-
PcBa15	-	-	-	-	-	-	-	-
PcBa16	-	-	-	-	-	-	-	-
PcBa17	-	-	-	-	-	-	-	-

Activities were classified according to the diameter of the inhibition zones around the point of application of the sample ++, more than 15 mm; +, less than 15 mm; -, no inhibition.

Table 4.14 A summary of the agar well diffusion method assay results for the antimicrobial activity of endophytic fungi isolated from each plant section

Crude extracts	Active isolate frequency of endophytic fungi from each plant section				Total (isolates)
	Young leaf	Mature leaf	Branch	Bark	
Culture broth and mycelium	0	2	1	2	5
Culture broth	1	4	14	7	26
Mycelium	1	3	1	0	5
Total	2	9	16	9	36

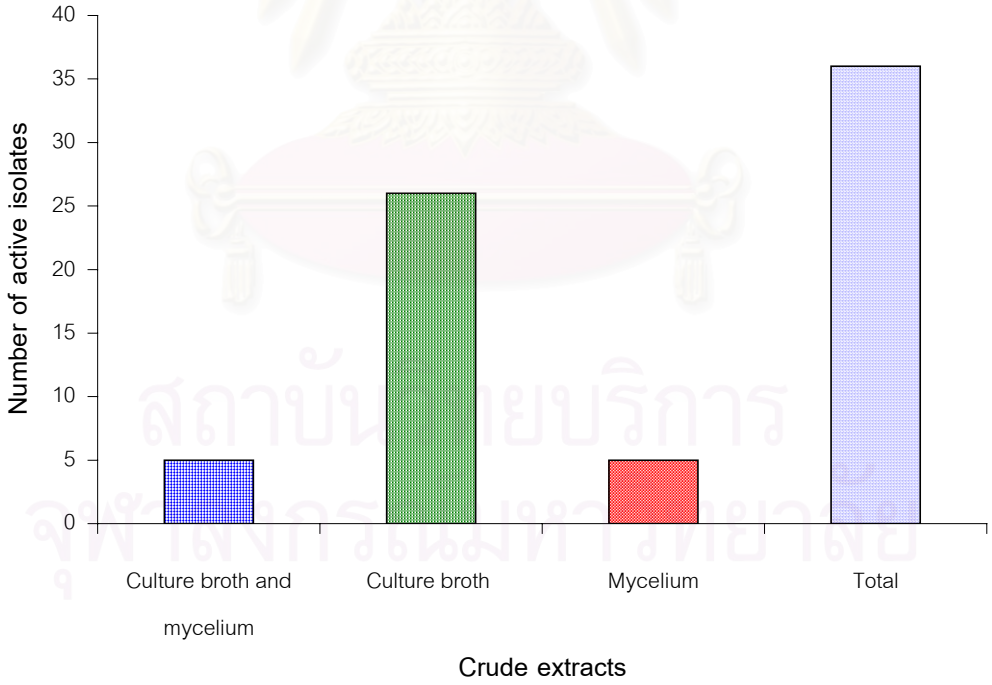


Figure 4.11 A summary of the agar well diffusion method assay results for the antimicrobial activity of endophytic fungi

Table 4.15 A summary of the agar well diffusion method assay results for the antimicrobial activity of endophytic fungi

Crude extracts	Test microorganisms and number of active isolate frequency							
	Gram positive bacteria		Gram negative bacteria		Yeast		Filamentous fungi	
	B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*S. serevisiae* TISTR 5169	*C. albicans* ATCC 10231	*M. gypseam*	*T. mentagrophytes*
Culture broth and mycelium	3	4	0	0	0	1	0	2
Culture broth	4	18	3	2	8	0	0	0
Mycelium	3	5	1	0	0	0	0	0
Total	10	27	4	2	8	1	0	2

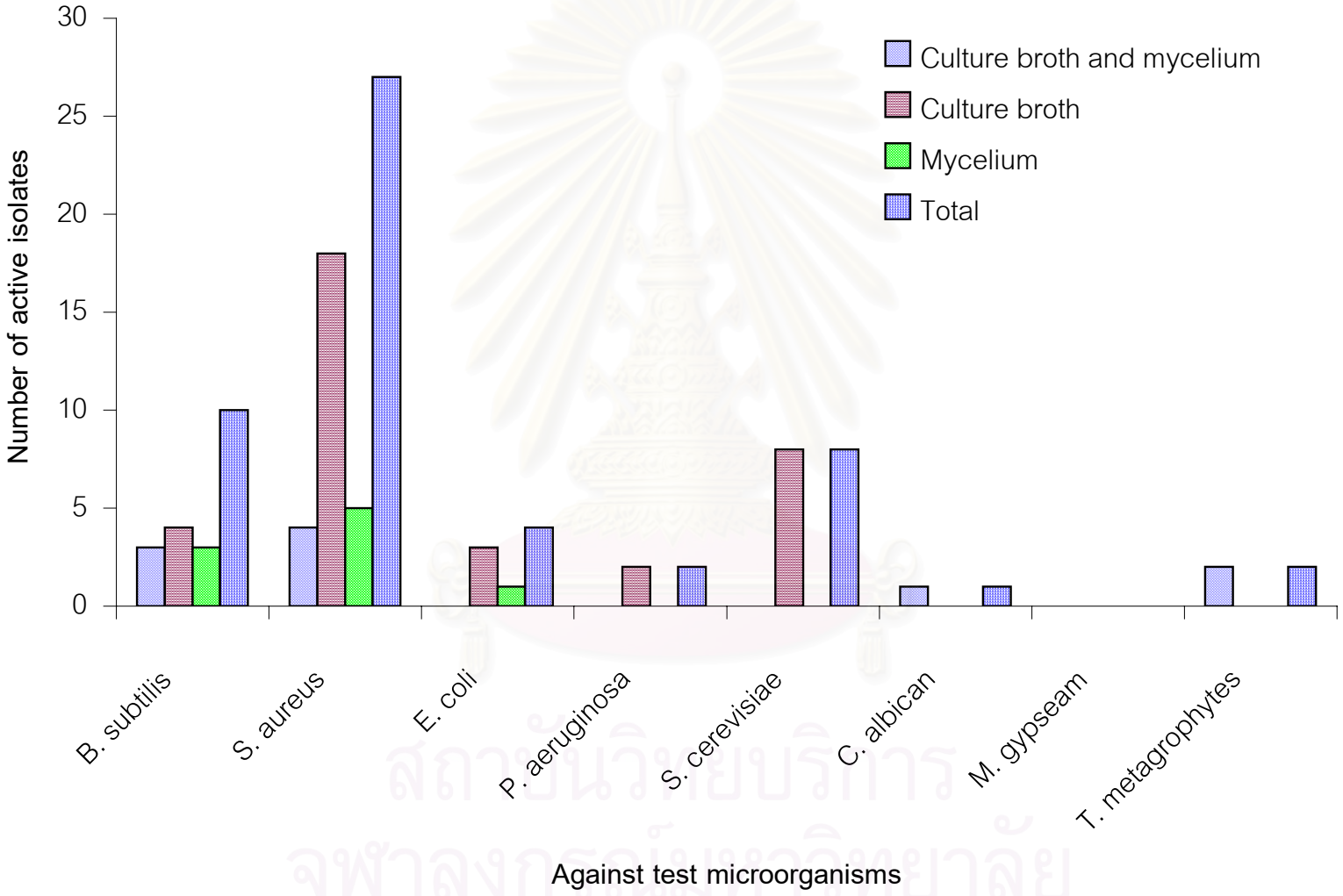


Figure 4.12 A summary of the agar well diffusion method assay results for the antimicrobial activity of endophytic fungi

All endophytic fungal isolates from *C. oblongifolius* were assessed for antimicrobial activities. Fifty eight percent of fungal isolate could produce a bioactive compound that was active against at least of the test microorganisms. Most biologically active compounds of endophytic fungi were produced and secreted into the culture broth. In contrast, some bioactive compounds were produced and accumulated within mycelium. Additionally, some bioactive compounds were produced and accumulated within mycelium and also secreted into the culture broth. Panphut (2001) reported most active metabolites of endophytic fungi from Thai medicinal plants were found in culture broth extracts rather than in mycelium extracts.

Recently, several researchers worked on antimicrobial activities of endophytic fungi from many different plants. For example, Schulz et al. (2002) reported that 80% of the endophytic fungi from plants inhibited growth of at least one of the test organisms showing antibacterial, fungicidal, algicidal or herbicidal activities. Furthermore, Chareprasert (2001) reported that 51% of endophytic fungi from teak (*Tectona grandis* L.) and rain tree (*Samanea saman* Merr.) could produce substances which inhibited at least one of the test organisms. Additionally, Rodrigues and Petrini (1997) reported antibacterial or antifungal activity for more than 30% of endophytic isolates from ericaceous plants. These observations suggest that endophytic fungi may have pharmaceutical potential and could be a promising source for antimicrobial compounds.

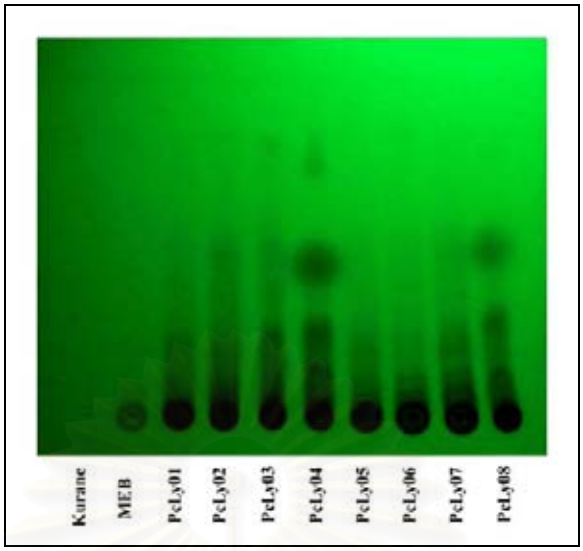
4.7 Determination of metabolite profiles

Metabolite profiles of culture broth extracts and mycelium extracts were examined by TLC technique and detected under UV light at 254 and 365 nm and with iodine vapor. Kurane is a chemical compound that is obtained from the bark of *Croton oblongifolius*. It was used as positive control. MEB extract was used as a negative control. The result of metabolite profiles is shown in Figures 4.13-4.16.

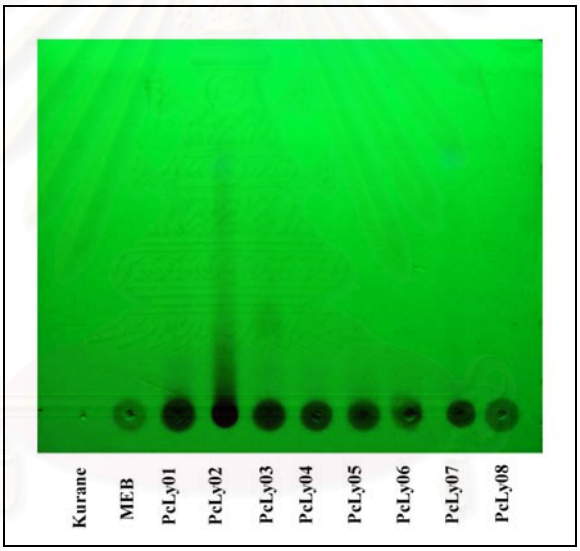
The results showed that no compound from the metabolite profiles of culture broth and mycelial extracts matched with Kurane. Kurane is detected with iodine vapor only. The R_f value of Kurane was 0.7 in 15% MeOH in $CHCl_3$ system and 0.55 in 5% MeOH in $CHCl_3$ system.

For metabolite profiles of culture broth extracts was detected under UV light at wavelength 254 and 365 nm and with iodine vapor, the result showed variety of metabolite profiles. Most spots in the metabolite profiles from culture broth extracts were detected under UV light at 254 nm while some compounds showed spots when detected under UV light at 365 nm and with iodine vapor.

For metabolite profiles of mycelia extracts was detected under UV light at 254 and 365 nm and with iodine vapor, the results showed similar patterns of metabolite profiles. Most spots in the metabolite profiles were detected under UV light at 365 nm and with iodine vapor, while some compounds showed spots when detected under UV light at 254 nm. The same pattern of spots showed R_f value was 0.56 and 0.71 in 5% MeOH in $CHCl_3$ system when detected under UV light at 365 nm and R_f value was 0.4 in 5% MeOH in $CHCl_3$ system when detected with iodine vapor.

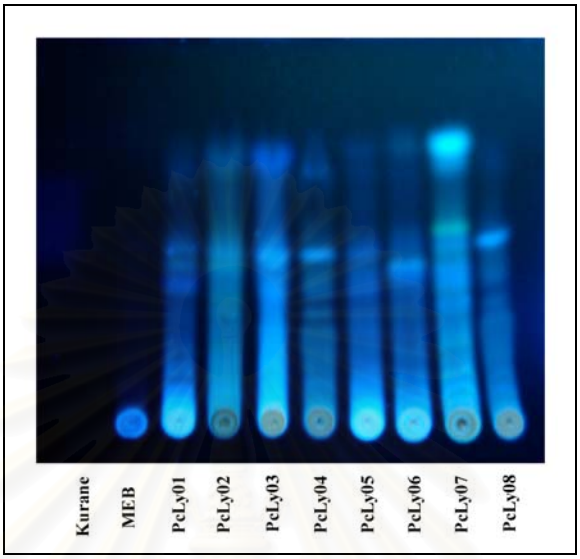


A1

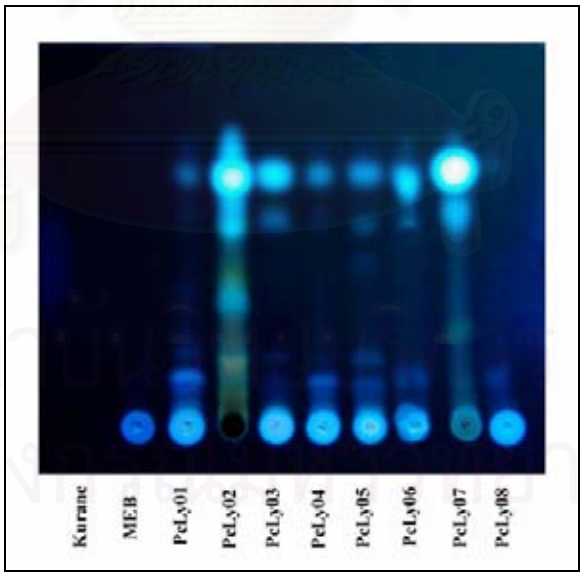


B1

Figure 4.13 The metabolite profiles of culture broth extracts (A1, A2 and A3) and mycelia extracts (B1, B2 and B3) of fungal isolate PcLy01-08 from young leaf on TLC sheets. A1, A2 and A3; the system solvent was used as 15% MeOH in $CHCl_3$. B1, B2 and B3; the system solvent was used as 5% MeOH in $CHCl_3$. A1 and B1: Detected under UV light at wavelength 254 nm. A2 and B2: Detected under UV light at wavelength 365 nm. A3 and B3: Detected with iodine vapor

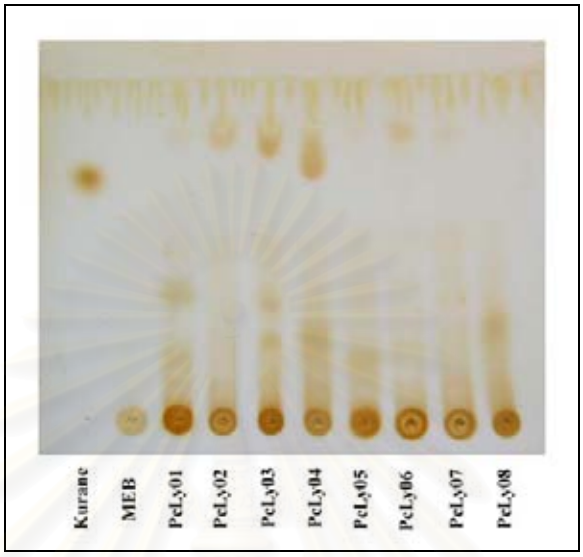


A2



B2

Figure 4.13 (continued)



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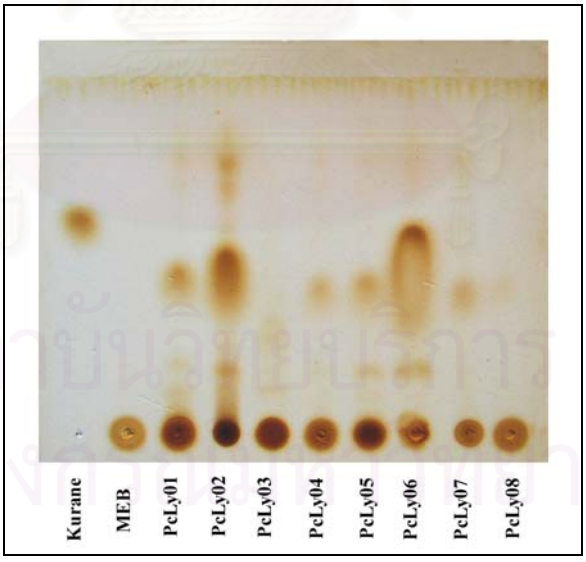
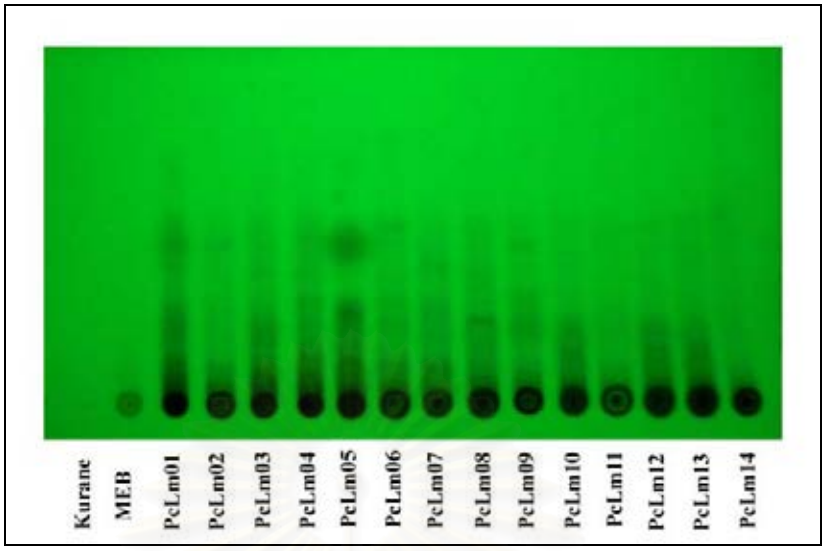
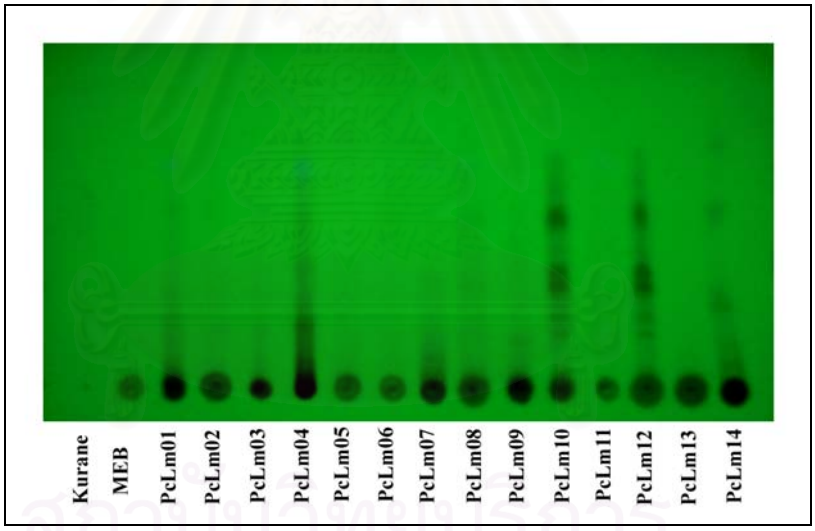


B3

Figure 4.13 (continued)

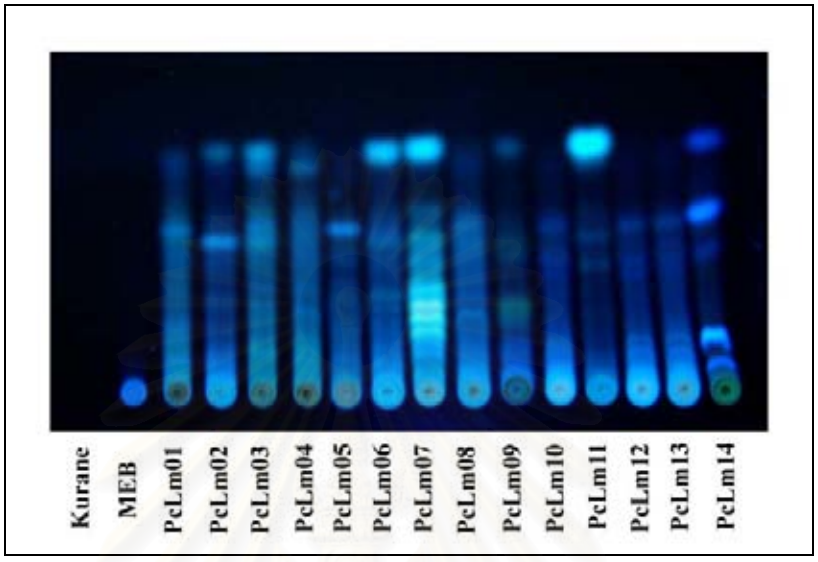


A1

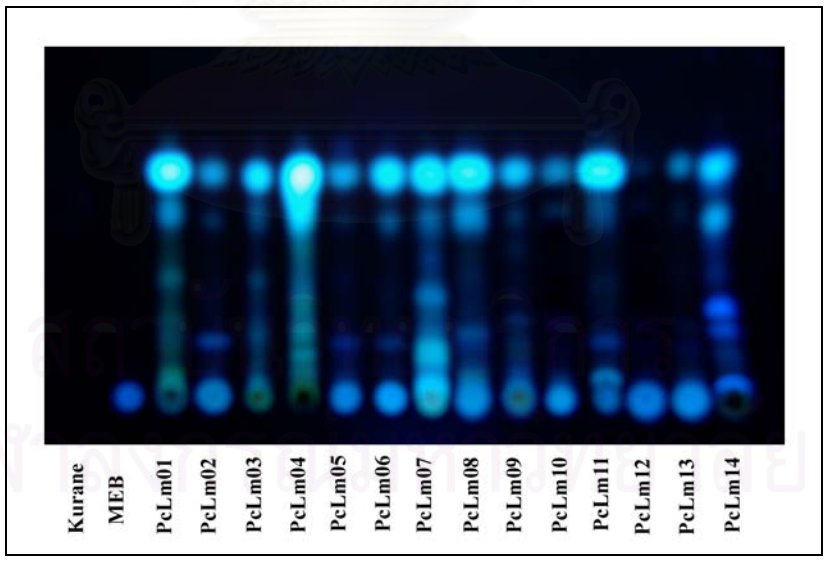


B1

Figure 4.14 The metabolite profiles of culture broth extracts (A1, A2 and A3) and mycelia extracts (B1, B2 and B3) of fungal isolate PcLm01-14 from mature leaf on TLC sheets. A1, A2 and A3; the system solvent was used as 15% MeOH in $CHCl_3$. B1, B2 and B3; the system solvent was used as 5%MeOH in $CHCl_3$. A1 and B1: Detected under UV light at wavelength 254 nm. A2 and B2: Detected under UV light at wavelength 365 nm. A3 and B3: Detected with iodine vapor.

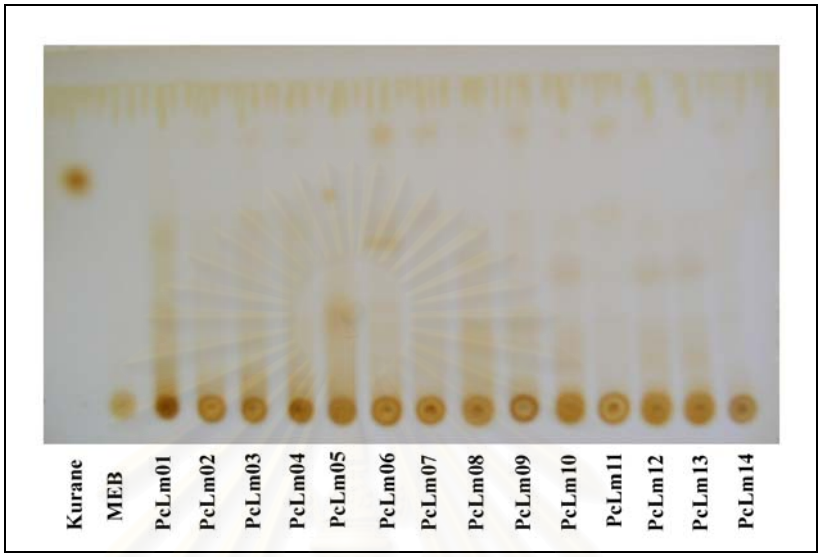


A2

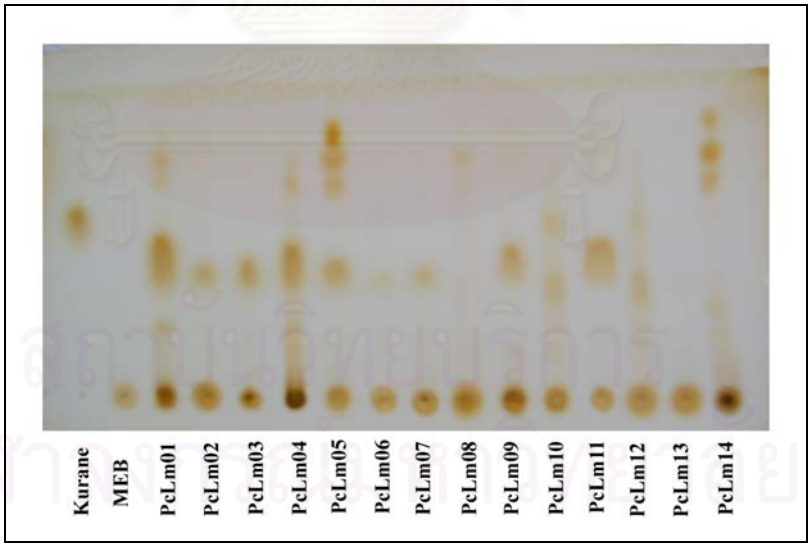


B2

Figure 4.14 (continued)

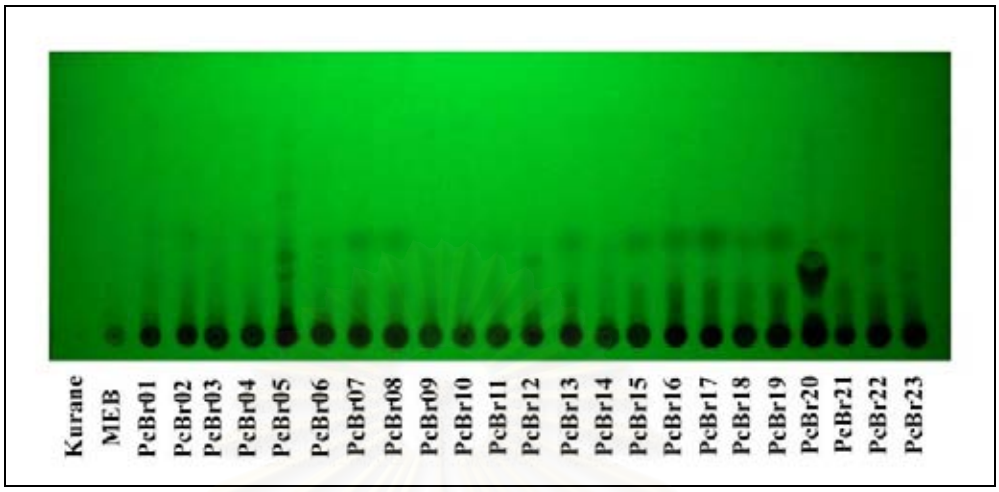


A3

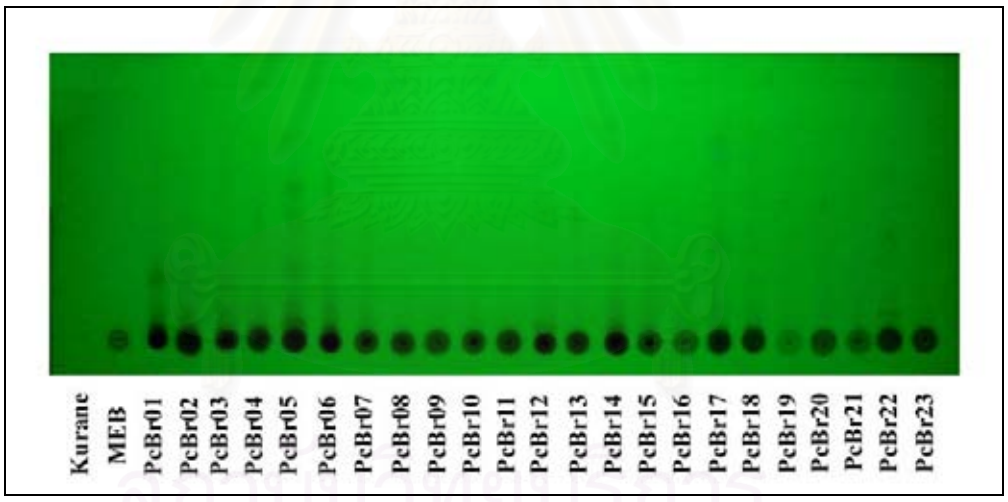


B3

Figure 4.14 (continued)

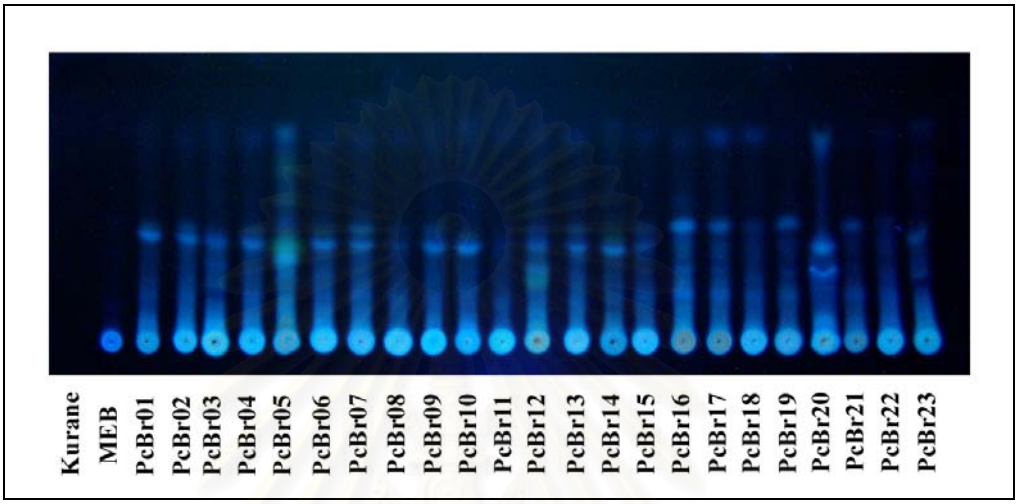


A1



B1

Figure 4.15 The metabolite profiles of culture broth extracts (A1, A2 and A3) and mycelia extracts (B1, B2 and B3) of fungal isolate PcBr01-23 from branch on TLC sheets. A1, A2 and A3; the system solvent was used as 15% MeOH in $CHCl_3$. B1, B2 and B3; the system solvent was used as 5% MeOH in $CHCl_3$. A1 and B1: Detected under UV light at wavelength 254 nm. A2 and B2: Detected under UV light at wavelength 365 nm. A3 and B3: Detected with iodine vapor.



A2

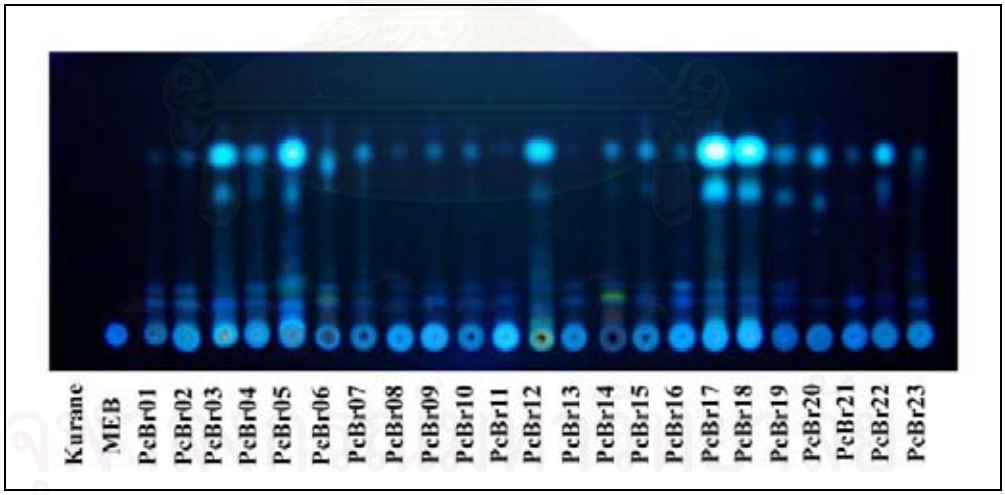


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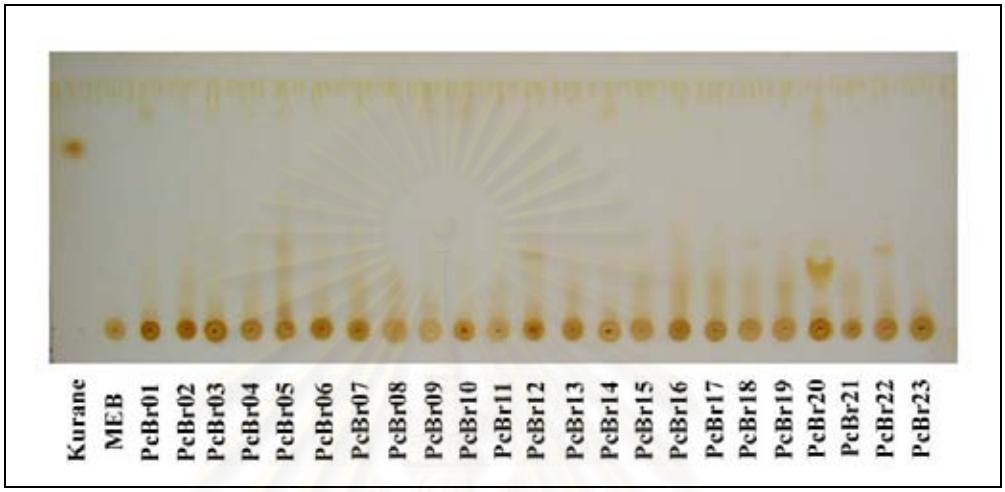
Figure 4.15 (continued)



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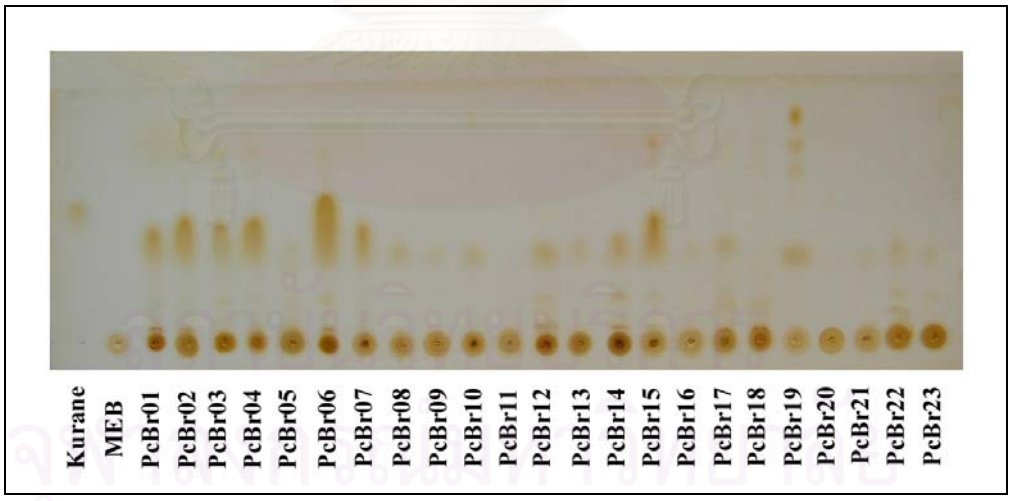
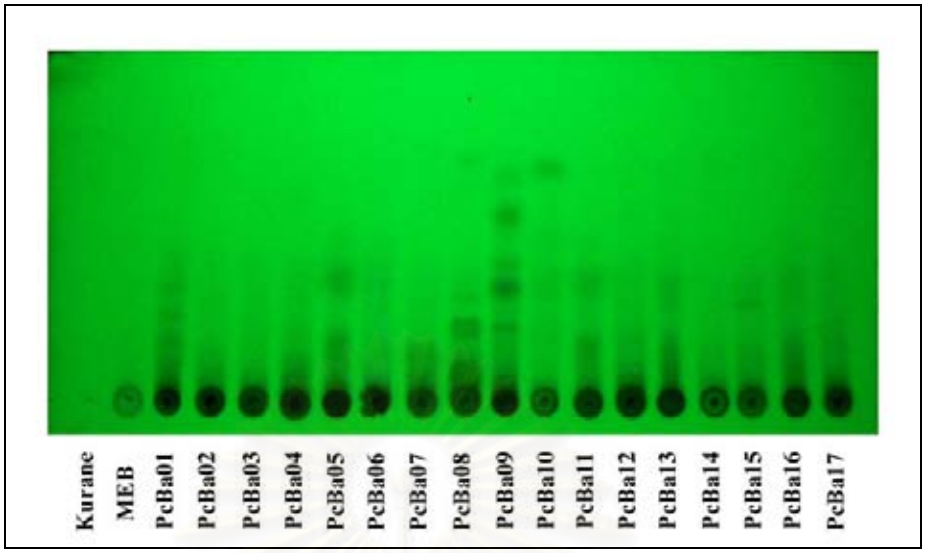
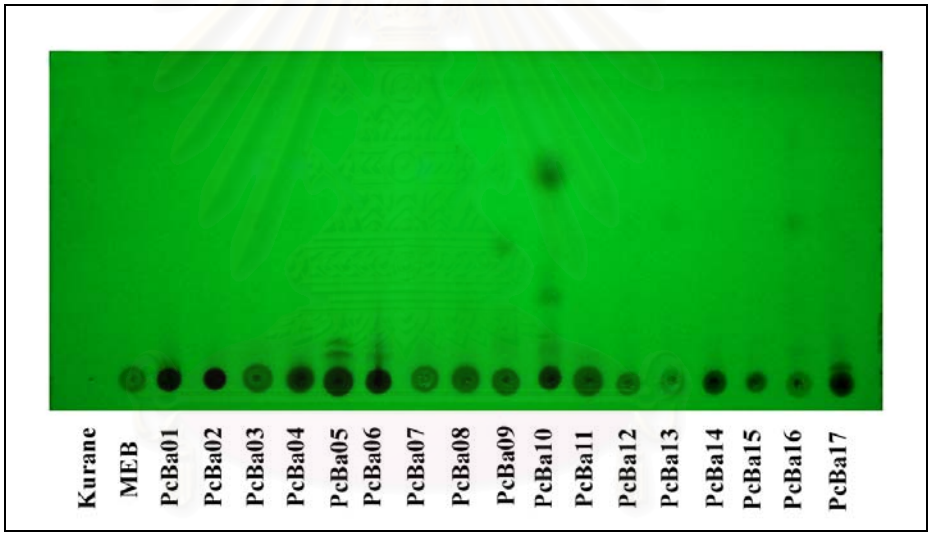


B3

Figure 4.15 (continued)

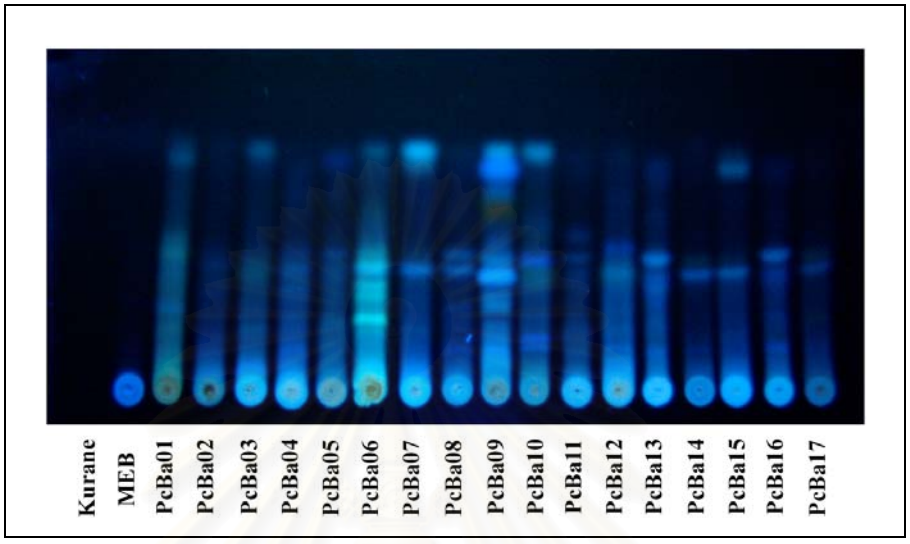


A1

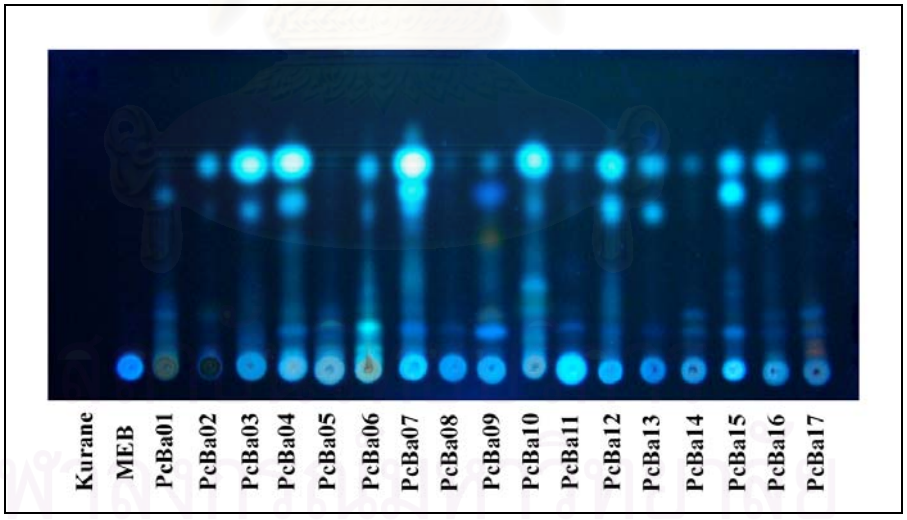


B1

Figure 4.16 The metabolite profiles of culture broth extracts (A1, A2 and A3) and mycelia extracts (B1, B2 and B3) of fungal isolate PcBa01-17 from bark on TLC sheets. A1, A2 and A3; the system solvent was used as 15% MeOH in $CHCl_3$. B1, B2 and B3; the system solvent was used as 5% MeOH in $CHCl_3$. A1 and B1: Detected under UV light at wavelength 254 nm. A2 and B2: Detected under UV light at wavelength 365 nm. A3 and B3: Detected with iodine vapor.

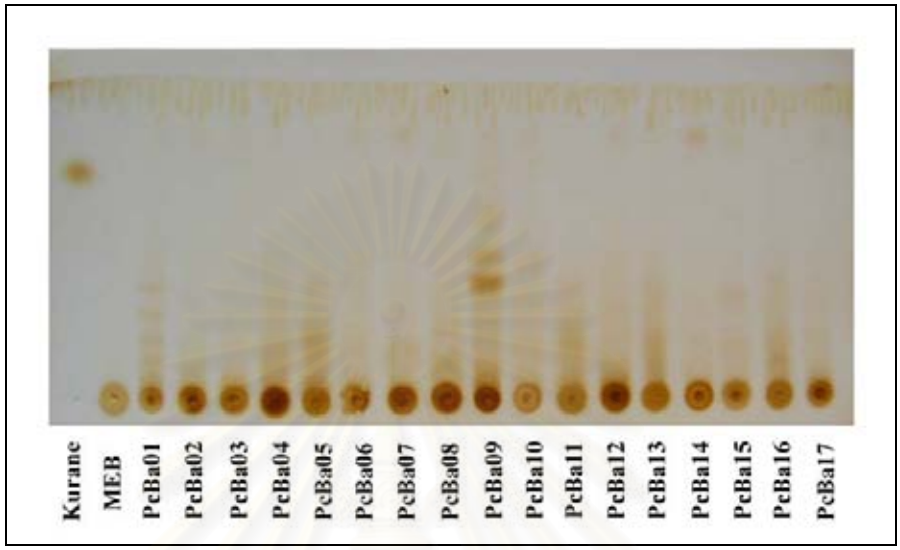


A2

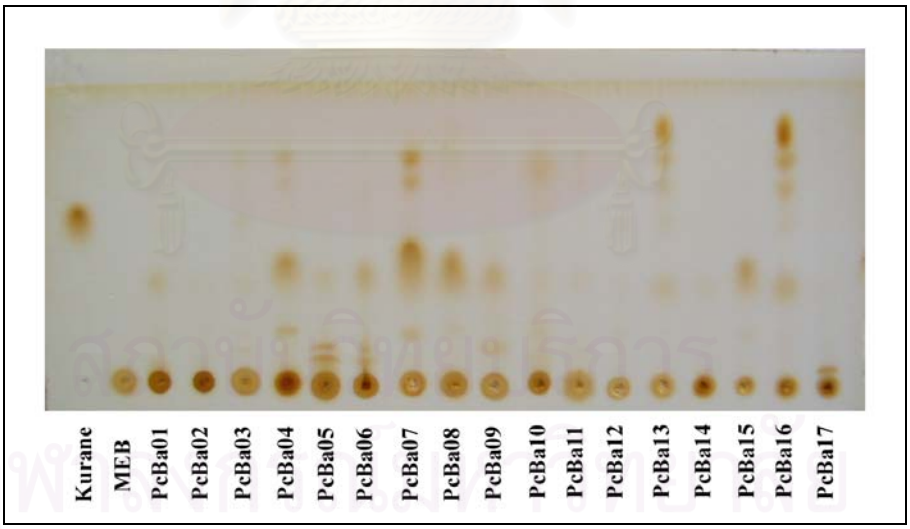


B2

Figure 4.16 (continued)



A3



B3

Figure 4.16 (continued)

Metabolite profiles of culture broth extracts and mycelia extracts were examined by TLC technique and detected under UV light at wavelength of 254 and 365 nm and also with iodine vapor.

In this research, 100% of endophytic fungi could produce at least one compound in culture broth, but only 50% of fungal endophyte produced compound with antimicrobial activities. Meanwhile, 50% of fungal endophyte compounds showed no antimicrobial activities. These data indicate that the remaining 50% of fungal endophytes may show some one activity such as anticancer drugs, antioxidant, antiviral, insecticidal, antialgal, herbicidal, or antifeedant.

Spots in the metabolite profiles of mycelia extracts were showed similar patterns. The similar patterns may be result of primary metabolite that are a major component of the fungal cell membrane such as ergosterol (Lu et al., 2000; Watcharadit, 2002).

4.8 Identification of fungal endophyte PcBr20

Fungal isolate PcBr20 was chosen for further study for bioactive compounds. This was because the culture broth extracts were active against a large number of test microorganisms such as *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *S. cerevisiae* TISTR 5169, *C. albicans* ATCC 10231, and *T. mentagrophytes* and the mycelia extracts against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *C. albican* ATCC 10231, and *T. mentagrophytes*.

4.8.1 Morphology identification

Fungal isolate PcBr20 was identified as *Fusarium* sp. Descriptions of the genus Fusarium is described in section 4.5. The fungus was grown on V8 agar, for 10 days at room temperature. Colony morphology and slide culture of isolate PcBr20 is shown in Figures 4.17.

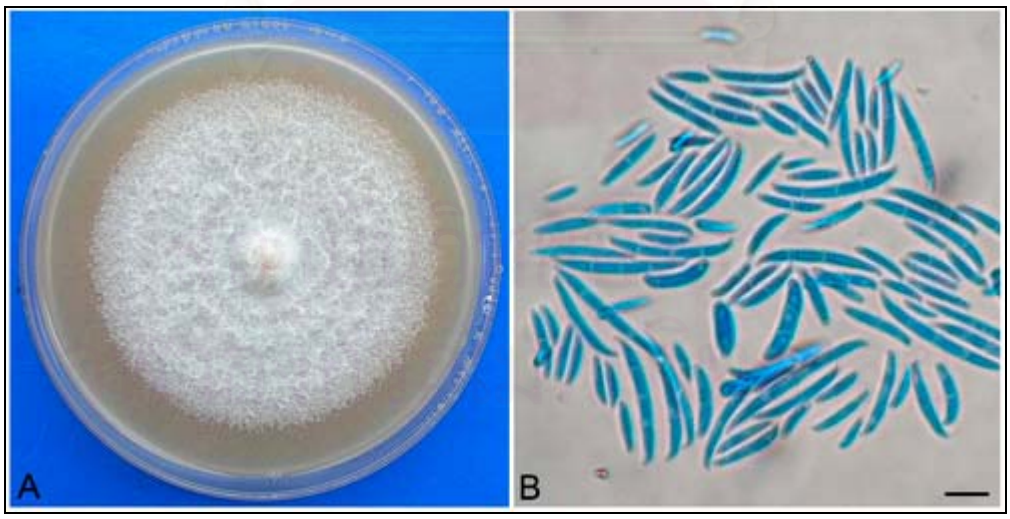
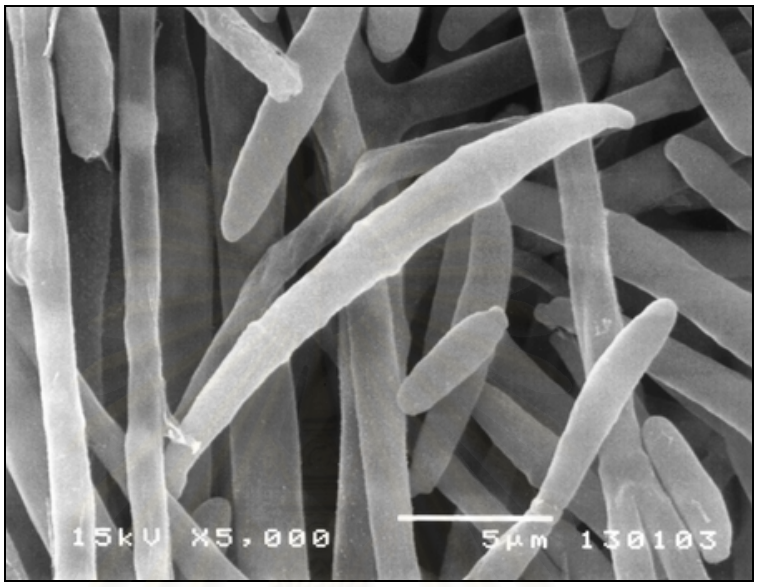
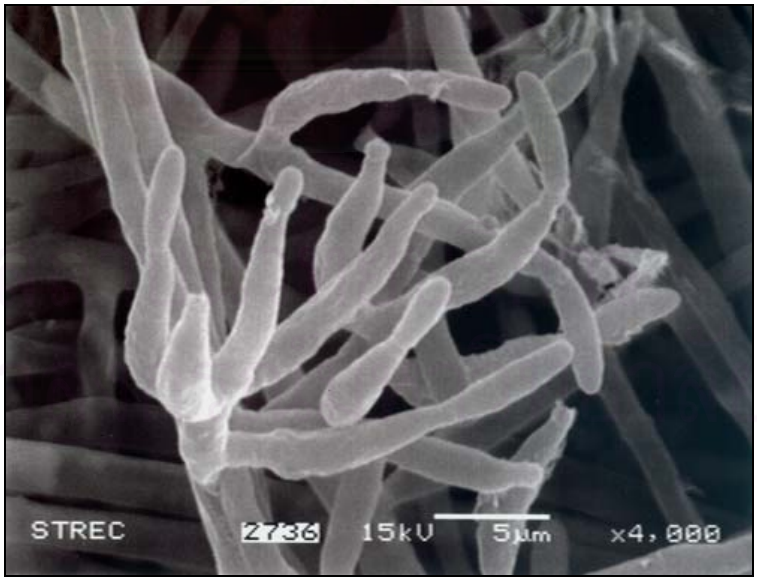


Figure 4.17 Endophytic fungal isolate PcBr20. A: Culture on V-8 agar (10 days). B: Macroconidia. (Bar = 5 µm)



A



B

Figure 4.18 Scanning electron microscope of endophytic fungal isolate, PcBr20. A: conidia. B: conindia and conidiophore.

4.8.2 Molecular identification

Endophytic fungus isolate PcBr20 was sent for identification by molecular methods at the Asian Natural Environmental Science Center, the University of Tokyo, Japan.

Sequencing of the nucleotide sequences of partial 18S, ITS region of the isolate, PcBr20 resulted in a 583 bp fragment, as shown in Figure 4.19.

```
   1
5'CTTGGTCATT     TAGAGGAAGT     AAAAGTCGTA     ACAAGGTCTC
  CGTTGGTGAA     CCAGCGGAGG     GATCATTACC     GAGTTTACAA
  CTCCCAAACC     CCTGTGAACA     TACCAATTGT     TGCCTCGGCG
  GATCAGCCCG     CTCCCGGTAA     AACGGGACGG     CCCGCCAGAG
  GACCCCTAAA     CTCTGTTTCT     ATATGTAACT     TCTGAGTAAA
  ACCATAAATA     AATCAAAACT     TTCAACAACG     GATCTCTTGG
  TTCTGGCATC     GATGAAGAAC     GCAGCAAAAT     GCGATAAGTA
  ATGTGAATTG     CAGAATTCAG     TGAATCATCG     AATCTTTGAA
  CGCACATTGC     GCCCGCCAGT     ATTCTGGCGG     ATTGCCTGTT
  CGAGCGTCAT     TTCAACCCTC     AAGCCCAGCT     TGGTGTTGGG
  ACTCGCGAGT     CAAATCGCGT     TCCCCAAATT     GATTGGCGGT
  CACGTCGARC     TTCCATAGCG     TAGTAGTAAA     ACCCTCGTTA
  CTGGTAATCG     TCGCGGCCAC     GCCGTTAAAC     CCCAACTTCT
  GAATGTTGAC     CTCGGATCAG     GTAGGAATAC     CCGCTGAACT
  TAAGCATATC     AATAAGCGGA     GGA 3'
                                   583
```

Figure 4.19 Nucleotide sequences of partial 18S region, complete ITS region of the isolate PcBr20

A blast search was performed to find a similar sequence to ITS region of fungal isolate PcBr20 in the Genbank DNA database. The ITS region of this isolate was similar to 99.291% identity of *Gibberella sacchari*, as shown in Figure 4.20. The reference was reported by Buzina et al., 2003.

```
>>AF455450|AF455450.1 Gibberella sacchari isolate wb395   (564 nt)
 initn: 2782 init1: 1650 opt: 2778 Z-score: 854.3 expect() 3.2e-40
 99.291% identity in 564 nt overlap (21-583:1-564)

               10        20        30        40        50        60
       CTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGG
                          :::::::::::::::::::::::::::::::::::::::
AF4554                    AAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGG
                                  10        20        30        40

               70        80        90       100       110       120
       GATCATTACCGAGTTTACAACTCCCAAACCCCTGTGAACATACCAATTGTTGCCTCGGCG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AF4554 GATCATTACCGAGTTTACAACTCCCAAACCCCTGTGAACATACCAATTGTTGCCTCGGCG
               50        60        70        80        90       100

              130       140       150       160       170       180
       GATCAGCCCGCTCCCGGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTCTGTTTCT
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AF4554 GATCAGCCCGCTCCCGGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTCTGTTTCT
              110       120       130       140       150       160

              190       200       210       220       230       240
       ATATGTAACTTCTGAGTAAAACCATAAATAAATCAAAACTTTCAACAACGGATCTCTTGG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AF4554 ATATGTAACTTCTGAGTAAAACCATAAATAAATCAAAACTTTCAACAACGGATCTCTTGG
              170       180       190       200       210       220

              250       260       270       280       290       300
       TTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AF4554 TTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAG
              230       240       250       260       270       280

              310       320       330       340        350      360
       TGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGC-GGATTGCCTGT
       :::::::::::::::::::::::::::::::::::::::::::::::: ::  :::::::
AF4554 TGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGT
              290       300       310       320       330       340

              370       380       390       400       410       420
       TCGAGCGTCATTTCAACCCTCAAGCCCAGCTTGGTGTTGGGACTCGCGAGTCAAATCGCG
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AF4554 TCGAGCGTCATTTCAACCCTCAAGCCCAGCTTGGTGTTGGGACTCGCGAGTCAAATCGCG
              350       360       370       380       390       400

              430       440       450       460       470       480
       TTCCCCAAATTGATTGGCGGTCACGTCGARCTTCCATAGCGTAGTAGTAAAACCCTCGTT
       ::::::::::::::::::::::::::::::.:::::::::::::::::::::::::::::
AF4554 TTCCCCAAATTGATTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAGTAAAACCCTCGTT
              410       420       430       440       450       460
```

Figure 4.20 Alignment data of ITS region of isolate PcBr20 and 1 reference taxa.

```
               490       500       510       520       530       540
       ACTGGTAATCGTCGCGGCCACGCCGTTAAACCCCAACTTCTGAATGTTGACCTCGGATCA
       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AF4554 ACTGGTAATCGTCGCGGCCACGCCGTTAAACCCCAACTTCTGAATGTTGACCTCGGATCA
               470       480       490       500       510       520

               550       560       570       580
       GGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA
       :::::::::::::::::::::::::::::::::::::::::::
AF4554 GGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA
               530       540       550       560
```

Figure 4.20 (continued)

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 PcBr20 was identified as belonging to the genera *Fusarium*.

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). The nucleotide sequence of the ITS region of fungal isolate PcBr20 was similar to 99.291% identity of *Gibberella sacchari* reported by Buzina et al., 2003.

The genus *Fusarium* is well known and is the anamorph in imperfect fungi or deuteromycetes and the genus *Gibberella* is a well known telemorph in ascomycetes. In this research, only the anamorph was studied as the sexual stage of fungal isolate PcBr20 did not appear on microbiological media.

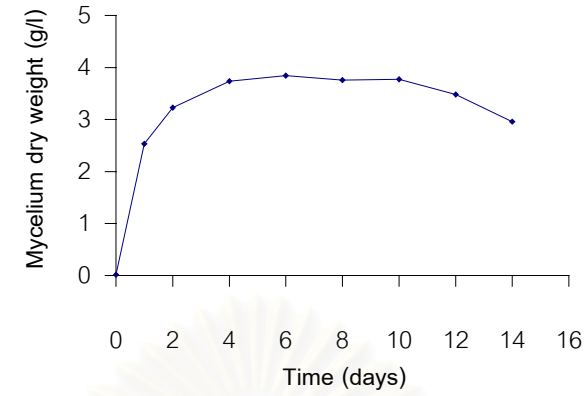
4.9 Determination of growth profile, pH, and antimicrobial activity of culture filtrate from fungal isolate PcBr20

Growth profile of fungal endophyte isolate PcBr20 was observed from mycelia dry weight. The culture filtrate of fungal isolate PcBr20 was measured pH and determined using the agar well diffusion method for antimicrobial acitivity. Figure 4.21 show the growth profile of fungal isolate PcBr20 (A), pH (B) and antimicrobial acitivity (C) of culture broth from fungal isolate PcBr20 at day 1 to day 14. The raw data of mycelium dry weight, pH, and inhibition zone are shown in table 1, Appendix C.

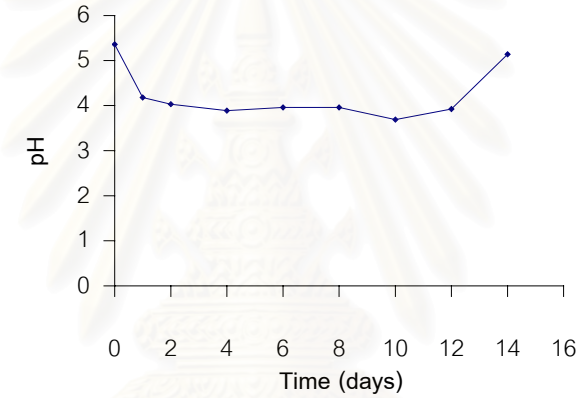
Cultivation of fungal isolate PcBr20 was done in MEB medium for 14 days. Fungal isolate PcBr20 grow in the log phase. Then the mycelium increased up to highest amount wihtin 4 days. The growth of mycelium was in the form of stationary phase and continued to death phase in 14 days.

The initial pH of MEB medium is 5.36. The pH decreased in first day (pH 4.18) until day 12 (pH 3.92). Then pH increased up to 5.14 amounts within day 14.

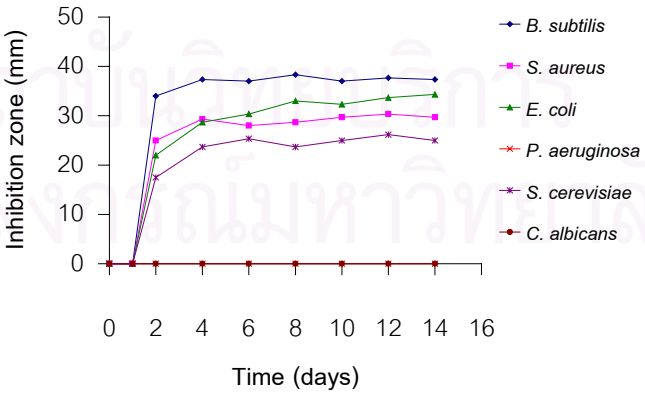
The culture filtrate of fungal isolate PcBr20 show antimicrobial acitivity with *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, and *S. cerivisiae* TISTR 5169 at day 2 and incresed up to the highest amount within day 4, which stationary and continued until day 14.



A



B



C

Figure 4.21 Growth profile (A), pH (B) and antimicrobial activity (C) of fungal isolate PcBr20

4.10 Cultivation and extraction

Fungal isolate PcBr20 was cultivated in malt extract broth totalling 17 l to yield 1.72 g of crude EtOAc, and 3.2 g of crude MeOH from mycelia extracts and 1.5 g of crude hexane, and 20.04 g of crude EtOAc from cultivation broth.

4.11 Isolation and purification of bioactive compounds in crude hexane and EtOAc from broth culture

Two compounds were isolated from the culture broth of the endophytic fungus isolate PcBr20. Mixture BH1 (92.5 mg, 6.17% yield of the hexane extract) were obtained from crystalization as a colorless needle crystal and compound BE1 (5.2 g, 25.95% yield of the EtOAc extract) were obtained from fracton BE08-11 as an amorphous white solid. Chemical structrues of these compounds were determined by analyes of spectroscopic data, including IR, UV, NMR and Mass spectra, as well as by comparison their spectral data with those of published values.

4.12 Structure elucidation of the pure compounds from endophytic fungus isolate PcBr20

4.12.1 Structure elucidation of mixture BH1

Mixture BH1 was a colorless needle crystal, m.p. 115.5-116 ^{o}C. The structure of mixture BH1 was elucidated by using spectroscopic techniques.

The IR spectra of mixture BH1 is shown in appendix B, Figure 1 and the absorption peaks were assigned as table 4.16. Its IR spectrum indicated important absorption bands at 3300-3600 cm^{-1} (O-H stretching vibration of carboxylic acid), 2960, 2925, and 2863 cm^{-1} (C-H stretching vibration), 1707 cm^{-1} (C=O vibration of carbonyl group), and 1384, 1310, and 1228 (C-N stretching vibration of amine).

Table 4.16 The IR absorption band assingment of mixture BH1

Wave number (cm^{-1})	Intensity	Vibration
3300-3600	Broad	O-H stretching vibration of acid
2960, 2925	Medium	C-H stretching vibration of $-CH_3$
2863	Medium	C-H stretching vibration of $-CH_2$
1707	Strong	C=O stretching vibration of acid
1641, 1462	weak	C=C stretching vibration of aromatic ring
1587	Medium	N-H bending vibration of amine
1384	Strong	C-N stretching vibration of amine
1310, 1228	Medium	C-N stretching vibration of amine
1034	Medium	C-O stretching vibration

The ^{1}H-NMR spectrum (Figure 3 in Appendix B) of mixture BH1 showed a methyl proton at 0.97 ppm, three aliphatic methylene protons at 1.41, 1.69, and 2.89 ppm, two benzylic protons at 2.46 and 2.78 ppm, two vinylic proton at 5.01 and 5.80 ppm and three aromatic protons at 7.80, 8.18, and 8.67 ppm.

The ^{13}C-NMR spectrum (Figure 4 in Appendix B) of mixture BH1 showed 18 singnals, which the carbonyl group of carboxylic acid corresponded to the signal at 165.0 ppm.

DEPT experiments (Figure 5 in Appendix B) of mixture BH1 showed six methylene carbons at 22.2, 32.3, 32.7, 32.8, 34.6, and 116.4 ppm, one methyl carbons at 13.7 ppm, which indicated that the carbon signals at 142.1, 143.2, 144.6, and 144.8 ppm were quaternary, and six methine carbons at 124.3, 136.2, 138.6, 138.7, 147.6, 147.8 and ppm.

. The EI mass spectrum (Figure 11 in Appendix B) showed the MH^{+} ion at m/z 178 and 180 indicated a compound with an add number of nitrogen atoms. If it is assumed that this mixture contains carbons, protons, oxygen, and nitrogens, then the molecular formula of $C_{10}H_{13}O_2N$ and $C_{10}H_{11}O_2N$. The molecular formula, $C_{10}H_{13}O_2N$ of this mixture BH1 indicated five degree of unsaturation; therefore, mixture BH1 must consist of one

aromatic ring and one carbonyl and the molecular formula, $C_{10}H_{11}O_2N$ of this mixture BH1 indicated six degree of unsaturation; therfore, mixture BH1 must consist of one aromatic ring and one carbonyl in addition to one double bone.

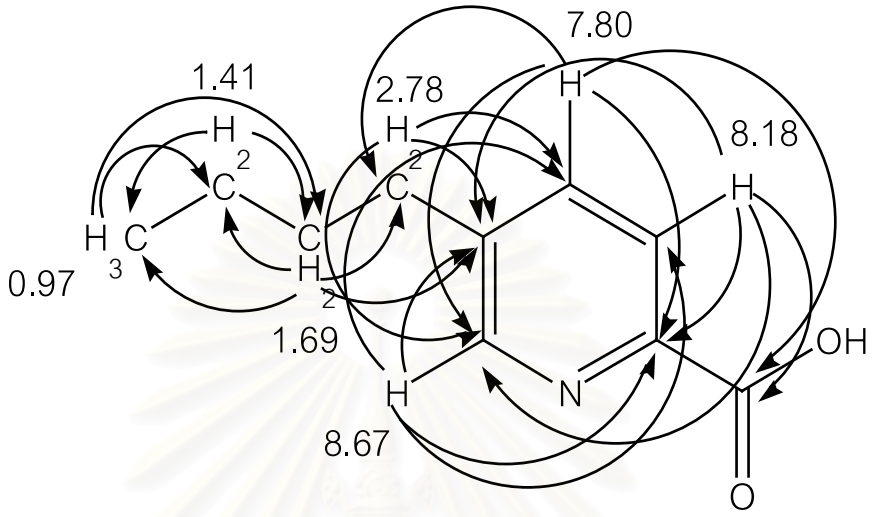
The information from 2D-NMR techniques; HSQC correlation (Table 4.17 and 4.18, Figure 6 in Appendix B), HMBC correlation (Table 4.18, Figure 4.22, and Figure 7 in Appendix B), and COSY correlation (Table 4.18, Figure 4.23, and Figure 8 in Appendix B) were used to assist the interpretation of the mixture BH1 structure.

Table 4.17 HSQC spectral data of mixture BH1

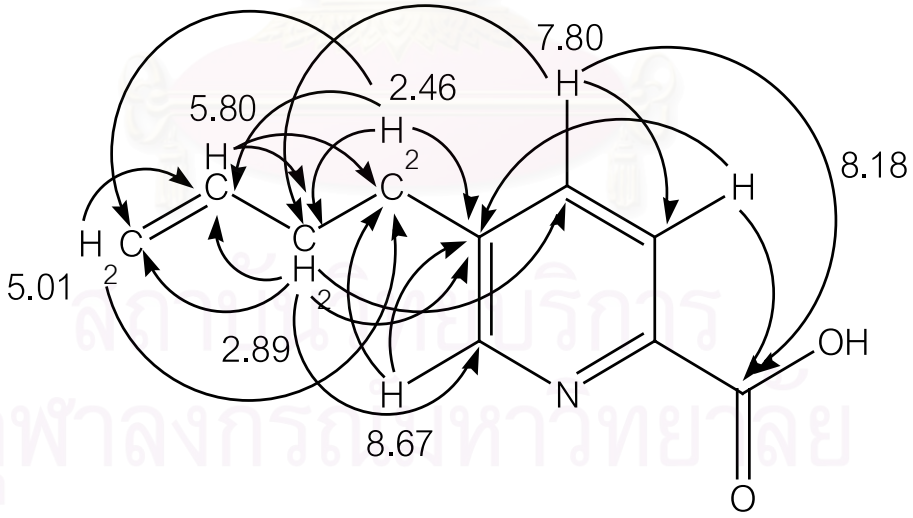
^{13}C-NMR (ppm)	^{1}H-NMR (ppm), coupling constant (Hz)
144.8 (s), 144.6 (s)	-
124.3 (d)	8.18 (1H, d, J = 8.0)
138.7 (d), 138.6 (d)	7.80 (1H, d br, J = 8.0)
143.2 (s), 142.1 (s)	-
147.8 (d), 147.6 (d)	8.67 (1H, s br)
32.7 (t), 34.6 (t)	2.78 (2H, t, J = 7.6), 2.46 (2H, q, J = 8.0)
32.8 (t), 32.3 (t)	1.69 (2H, dt, J = 7.2,15.2), 2.89 (2H, t, J = 7.2)
22.2 (t), 136.2 (d)	1.41 (2H, dq, J = 7.6, 15.2), 5.80 (1H, m)
13.7 (q), 116.4 (t)	0.97 (3H, t, J = 7.6), 5.01 (2H, dd, J = 2.4, 13.2)
165.0 (s)	-

Table 4.18 HSQC, HMBC, and COSY spectral data of mixture BH1

Position	δ_C	δ_H	HMBC (H to C)	COSY
1	-	-	-	-
2	144.8 (s)	-	-	-
3	124.3 (d)	8.18 (1H, d, J = 8.0)	C-2, C-5, C-6,C-11	H-4 (7.80)
4	138.7 (d)	7.80 (1H, d br, J = 8.0)	C-2, C-6, C-7, C-11	H-3 (8.18), H-6 (8.67)
5	143.2 (s)	-	-	-
6	147.8 (d)	8.67 (1H, s br)	C-2, C-3, C-4, C-5	H-4 (7.80)
7	32.7 (t)	2.78 (2H, t, J = 7.6)	C-4, C-5, C-6, C-8, C-10	H-8 (1.69)
8	32.8 (t)	1.69 (2H, dt, J = 7.2,15.2)	C-5, C-7, C-9, C-10	H-7(2.78), H-9 (1.41)
9	22.2 (t)	1.41 (2H, dq, J = 7.6, 15.2)	C-8, C-10	H-10 (0.94), H-8 (1.69)
10	13.7 (q)	0.97 (3H, t, J = 7.6)	C-8, C-9	H-9 (1.41)
11	165.0 (s)	-	-	-
1’	-	-	-	-
2’	144.6 (s)	-	-	-
3’	124.3 (d)	8.18 (1H, d, J = 8.0)	C-5’, C-11’	H-4’ (9.80)
4’	138.6 (d)	7.80 (1H, d br, J = 8.0)	C-3’, C-8’, C-11’	H-3’ (8.18), H-6’ 8.67)
5’	142.1 (s)	-	-	-
6’	147.6 (d)	8.67 (1H, s br)	C-5’, C-7’	H-4’ (7.80)
7’	34.6 (t)	2.46 (2H, q, J = 8.0)	C-5’, C-8’, C-9’, C-10’	H-8’ (2.89)
8’	32.3 (t)	2.89 (2H, t, J = 7.2)	C-4’, C-5’, C-6’, C-7’, C-9’	H-7’ (2.46)
9’	136.2 (d)	5.80 (1H, m)	C-7’, C-8’	H-10’ (5.01)
10’	116.4 (t)	5.01 (2H, dd, J = 2.4, 13.2)	C-7’, C-9’	H-9’ (5.08)
11’	165.0 (s)	-	-	-

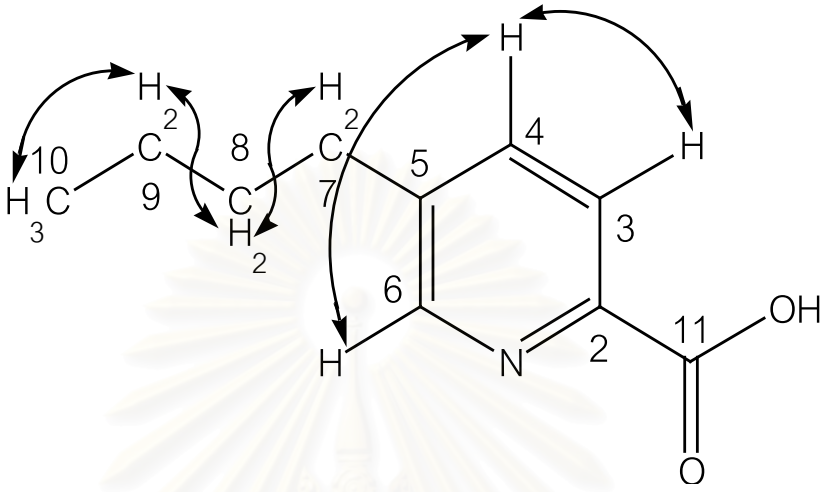


Fusaric acid

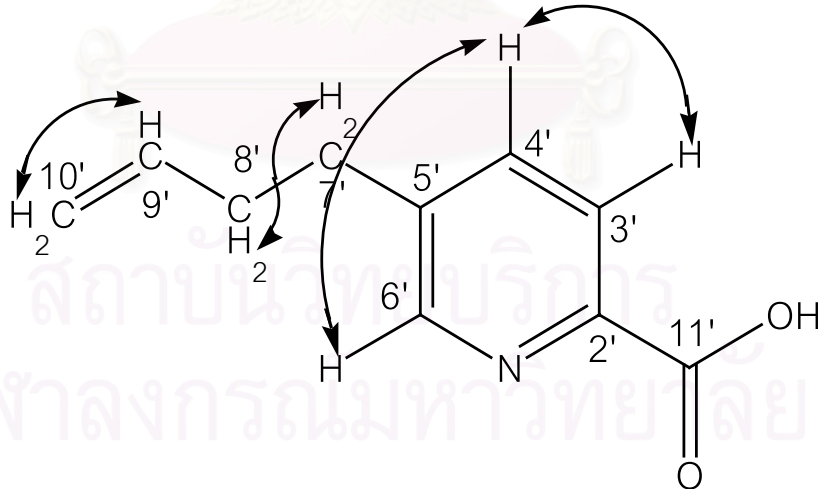


Dehydrofusaric acid

Figure 4.22 HMBC correlation of mixure BH1



Fusaric acid



Dehydrofusaric acid

Figure 4.23 COSY correlation of mixure BH1

Mixture BH1 showed spectral data indentical to that of fusaric acid and dehydrofusaric acid, which was reported in the literature (Abraham and Hanssen, 1992). The ^{1}H-NMR and ^{13}C-NMR signal of mixture BH1 and fusaric acid and dehydrofusaric acid are presented in Table 4.19 and 4.20 as follows.

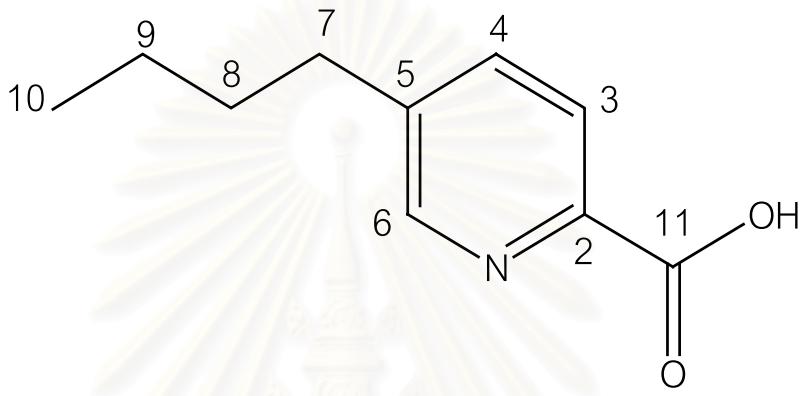
Table 4.19 ^{1}H-NMR spectral data of mixture BH1, fusaric acid and dehydrofusaric acid in $CDCl_3$

Position	^{1}H-NMR chemical shifts (ppm)		
	Mixture BH1	Fusaric acid	Dehydrofusaric acid
1,1'	-	-	-
2,2'	-	-	-
3,3'	8.18 (d, J = 8.0)	8.19 (d, J = 5)	8.19 (d, J = 5)
4,4'	7.80 (d br, J = 8.0)	7.80 (d br, J = 5)	7.80 (d br, J = 5)
5,5'	-	-	-
6,6'	8.67 (s br)	8.65 (s br)	8.65 (s br)
7,7'	2.78 (t, J = 7.6), 2.46 (q, J = 8.0)	2.74 (t, J = 8)	2.85 (t, J = 8)
8,8'	1.69 (dt, J = 7.2,15.2), 2.89 (t, J = 7.2)	1.66 (tt, J = 8)	2.44 (dt, J = 8)
9,9'	1.41 (dq, J = 7.6, 15.2), 5.80 (m)	1.37 (tq, J = 9)	5.82 (m, J = 7)
10,10'	0.97 (t, J = 7.6), 5.01 (dd, J = 2.4, 13.2)	0.94 (t, J = 7)	5.02 (m)
11,11'	-	-	-

Table 4.20 ^{13}C-NMR spectrum of mixture BH1, fusaric acid and dehydrofusaric acid in $CDCl_3$

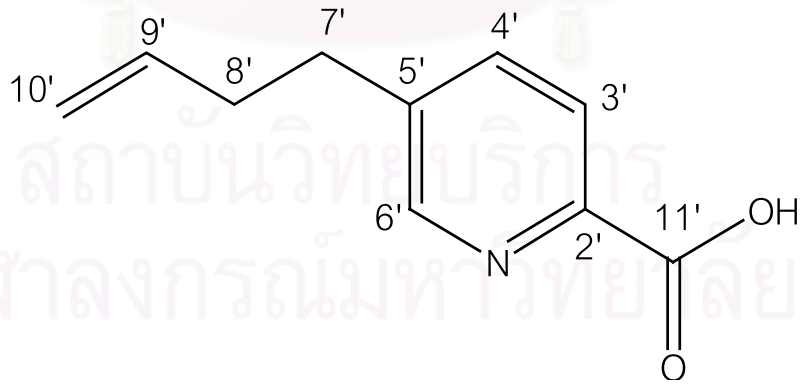
Position	^{13}C-NMR chemical shifts (ppm)		
	Mixture BH1	Fusaric acid	Dehydrofusaric acid
1,1'	-	-	-
2,2'	144.8 (s), 144.6 (s)	144.8 (s)	145.0 (s)
3,3'	124.3 (d)	124.7 (d)	124.7 (d)
4,4'	138.7 (d), 138.6 (d)	138.1 (d)	138.2 (d)
5,5'	143.2 (s), 142.1 (s)	142.0 (s)	141.9 (s)
6,6'	147.8 (d), 147.6 (d)	148.1 (d)	148.3 (d)
7,7'	32.7 (t), 34.6 (t)	34.4 (t)	32.3 (t)
8,8'	32.8 (t), 32.3 (t)	32.7 (t)	32.8 (t)
9,9'	22.2 (t), 136.2 (d)	22.1 (t)	136.3 (d)
10,10'	13.7 (q), 116.4 (t)	13.6 (q)	116.2 (t)
11,11'	165.0 (s)	165.3 (s)	165.3 (s)

From all of the data, it could be concluded that mixture BH1 was a mixture of fusaric acid and dehydrofusaric acid. The structure of this mixture was presented in Figure 4.24.



Fusaric acid

(5-butylpicolinic acid)



Dehydrofusaric acid

Figure 4.24 Sturcture of mixure BH1

4.12.2 Strucuter elucidation of compound BE1

Compound BE1 was a white amorphous solid, m.p. 190-191 ^{o}C. The structure of compound BE1 is still investigation.

IR spectrum of Compound BE1 is shown in appendix B, Figure 13 and summarized in Table 4.21.

Table 4.21 The IR absorption band assignment of mixture BH1

Wave number (cm^{-1})	Intensity	Vibration
2500-3500	Broad	O-H streching vibration of acid
2925	Medium	C-H streching vibration of -CH_3
2855	Medium	C-H streching vibration of -CH_2
1622 , 1567	Strong	C=C streching vibration of olefin
1380	Strong	C-N streching vibration of amine
1256, 1212, 1030	Weak	C-O streching vibration
804, 711	Medium	C-H out of plane bending vibration

4.13 Biological activity

4.13.1 Antimicrobial activity

4.13.1.1 Antimicrobial activity of the crude extracts from endophytic fungus isolate PcBr20

The animicrobial activity of the crude extacts from endophytic fungus isolate PcBr20 was evaluated by the agar well diffusion method. The fractions were examined at a concentration of 10 mg/ml (1 mg/well; 7mm diameter). The antimicrobial activity was calculated from the inhibition zones (mm) of test microorganisms, including the bacterial strains *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and fungi, yeast form strains *C. albican* ATCC 10231. Antimicrobial activity of the fractions from crude extracts is shown in Table 4.22.

Table 4.22 Antimicrobial activity of the crude extacts from endophytic fungus isolate PcBr20

Test microorganisms	Crude extracts			
	Mycelia		Culture broth	
	Crude EtOAc	Crude MeOH	Crude Hexane	Crude EtoAc
B. subtilis ATCC 6633	++	-	++	++
S. aureus ATCC 25923	++	-	++	++
E. coli ATCC 25922	++	-	++	++
P. aeruginosa ATCC 27853	-	-	+	+
S. serevisiae TISTR 5169	ND	ND	ND	ND
C. albicans ATCC 10231	+	-	+	+
M. gypseam	ND	ND	ND	ND
T. mentagrophytes	ND	ND	ND	ND

Activities were classified according to the diameter of the inhibition zones around the point of application of the sample ++, more than 15 mm; +, less than 15 mm; -, no inhibition; ND, not dertermined.

4.13.1.2 Antimicrobial activity of the fractions from crude extracts

The animicrobial activity of the fractions from crude extracts was evaluated by the agar well diffusion method. The fractions were examined at a concentration of 1000 μg/ml (0.1 mg/well; 7mm diameter). The antimicrobial activity was calculated from the inhibition zones (mm) of test microorganisms, including the bacterial strains *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and fungi, yeast form strains *C. albican* ATCC 10231. Antimicrobial activity of the fractions from crude extracts is shown in Table 4.23-4.25.

Table 4.23 Antimicrobial activity of the fraction of EtOAc crude from mycelia extracts

Fraction code	Test microorganisms							
	Gram positive bacteria		Gram negative bacteria		Yeast		Filamentous fungi	
	B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*S. serevisiae* TISTR 5169	*C. albicans* ATCC 10231	*M. gypseam*	*T. mentagrophytes*
ME01	-	-	-	-	ND	-	ND	ND
ME02	-	-	-	-	ND	-	ND	ND
ME03	-	-	-	-	ND	-	ND	ND
ME04	+	-	-	-	ND	-	ND	ND
ME05	-	-	-	-	ND	-	ND	ND
ME06	+	-	-	-	ND	-	ND	ND
ME07	-	-	-	-	ND	-	ND	ND
ME08	-	-	-	-	ND	-	ND	ND
ME09	-	-	-	-	ND	-	ND	ND
ME10	-	-	-	-	ND	-	ND	ND
ME11	-	-	-	-	ND	-	ND	ND
ME12	-	-	-	-	ND	-	ND	ND
ME13	-	-	-	-	ND	-	ND	ND
ME14	-	-	-	-	ND	-	ND	ND
ME15	-	-	-	-	ND	-	ND	ND
ME16	-	-	-	-	ND	-	ND	ND
ME17	-	-	-	-	ND	-	ND	ND

Activities were classified according to the diameter of the inhibition zones around the point of application of the sample ++, more than 15 mm; +, less than 15 mm; -, no inhibition; ND, not dertermined.

Table 4.23 (continued)

Fraction code	Test microorganisms							
	Gram positive bacteria		Gram negative bacteria		Yeast		Filamentous fungi	
	B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*S. serevisiae* TISTR 5169	*C. albicans* ATCC 10231	*M. gypseam*	*T. mentagrophytes*
ME18	-	-	-	-	ND	-	ND	ND
ME19	-	-	-	-	ND	-	ND	ND
ME20	(+)	(+)	-	-	ND	-	ND	ND
ME21	++	++	+	-	ND	(+)	ND	ND
ME22	+	++	-	-	ND	-	ND	ND
ME23	++	++	++	-	ND	(+)	ND	ND
ME24	++	++	++	-	ND	(+)	ND	ND
ME25	++	++	+	-	ND	-	ND	ND

Activities were classified according to the diameter of the inhibition zones around the point of application of the sample ++, more than 15 mm; +, less than 15 mm; (+), hazy inhibition zone; -, no inhibition; ND, not dertermined.

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Table 4.24 Antimicrobial activity of the fraction of EtOAc crude from mycelia extracts (recolumn fraction code ME25)

Fraction code	Test microorganisms							
	Gram positive bacteria		Gram negative bacteria		Yeast		Filamentous fungi	
	B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*S. serevisiae* TISTR 5169	*C. albicans* ATCC 10231	*M. gypseam*	*T. mentagrophytes*
ME2501	-	-	-	-	ND	-	ND	ND
ME2502	-	-	-	-	ND	-	ND	ND
ME2503	-	-	-	-	ND	-	ND	ND
ME2504	++	++	+	-	ND	-	ND	ND
ME2505	++	+	-	-	ND	-	ND	ND
ME2506	-	-	-	-	ND	-	ND	ND
ME2507	-	-	-	-	ND	-	ND	ND
ME2508	++	-	-	-	ND	-	ND	ND
ME2509	-	-	-	-	ND	-	ND	ND

Activities were classified according to the diameter of the inhibition zones around the point of application of the sample ++, more than 15 mm; +, less than 15 mm; (+), hazy inhibition zone; -, no inhibition; ND, not dertermined.

Table 4.25 Antimicrobial activity of the fraction of EtOAc crude from culture broth extracts

Fraction code	Test microorganisms							
	Gram positive bacteria		Gram negative bacteria		Yeast		Filamentous fungi	
	B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*S. serevisiae* TISTR 5169	*C. albicans* ATCC 10231	*M. gypseam*	*T. mentagrophytes*
BE01	-	-	-	-	ND	-	ND	ND
BE02	-	-	-	-	ND	-	ND	ND
BE03	-	-	-	-	ND	-	ND	ND
BE04	-	-	-	-	ND	-	ND	ND
BE05	-	-	-	-	ND	-	ND	ND

Activities were classified according to the diameter of the inhibition zones around the point of application of the sample ++, more than 15 mm; +, less than 15 mm; (+), hazy inhibition zone; -, no inhibition; ND, not dertermined.

Table 4.25 (continued)

Fraction code	Test microorganisms							
	Gram positive bacteria		Gram negative bacteria		Yeast		Filamentous fungi	
	B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*S. serevisiae* TISTR 5169	*C. albicans* ATCC 10231	*M. gypseam*	*T. mentagrophytes*
BE06	-	-	-	-	ND	-	ND	ND
BE07	-	-	-	-	ND	-	ND	ND
BE08	++	++	++	+	ND	++	ND	ND
BE09	++	++	++	+	ND	++	ND	ND
BE10	++	++	++	+	ND	++	ND	ND
BE11	++	++	++	+	ND	++	ND	ND
BE12	++	++	++	-	ND	-	ND	ND
BE13	++	++	++	-	ND	-	ND	ND
BE14	++	++	++	-	ND	-	ND	ND
BE15	++	++	+	-	ND	-	ND	ND
BE16	++	++	++	-	ND	-	ND	ND
BE17	++	++	++	-	ND	-	ND	ND
BE18	++	++	++	-	ND	-	ND	ND
BE19	++	++	++	-	ND	-	ND	ND
BE20	++	++	++	-	ND	-	ND	ND
BE21	++	++	+	-	ND	-	ND	ND
BE22	++	++	-	-	ND	-	ND	ND
BE23	++	++	-	-	ND	-	ND	ND
BE24	++	-	-	-	ND	-	ND	ND
BE25	++	+	-	-	ND	-	ND	ND
BE26	++	++	-	-	ND	-	ND	ND
BE27	++	++	-	-	ND	-	ND	ND
BE28	+	+	-	-	ND	-	ND	ND
BE29	-	-	-	-	ND	-	ND	ND
BE30	-	-	-	-	ND	-	ND	ND
BE31	-	-	-	-	ND	-	ND	ND

Activities were classified according to the diameter of the inhibition zones around the point of application of the sample ++, more than 15 mm; +, less than 15 mm; (+), hazy inhibition zone; -, no inhibition; ND, not dertermined.

4.13.1.3 Antimicrobial activity of pure compounds

The animicrobial activity of pure compounds was evaluated by the antimicrobial susceptibility test, broth microdilution method. The pure compound was examined at a concentration of ($0.24x10^{-3}$)–1,000 µg/ml (two-fold dilution). Antimicrobial activity tests were performed against *B. subtilis* ATCC 6633*, S. aureus* ATCC 25923*, E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. cerevisiae* TISTR 5169, and *C. albicans* ATCC 10231. The lowest concentration of pure compound showing complete inhibition of growth is recorded as the minimal inhibitory concentration (MIC). Antimicrobial activity of pure compounds is shown in Figure 4.25 and Table 4.26.

For filamentous fungi results were determined by using the agar well diffusion method. The pure compound was examined at a concentration of 1000 µg/ml. Antimicrobial activity was tested against *M. gypseam*, *T. mentagrophytes*, *F. proliferatum*, and *P. parasitica*. Antimicrobial activity of isolated compounds is shown in Table 4.27.

Table 4.26 Broth microdilution method for antimicrobial activity of pure compounds

Compounds	Test microorganisms and MIC (µ/ml)					
	Gram positive bacteria		Gram negative bacteria		Yeasts	
	B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*S. serevisiae* TISTR 5169	*C. albicans* ATCC 10231
BH1	0.98	62.50	15.62	1,000	62.50	1,000
BE1	0.98	62.50	62.50	1,000	-	-
Penicillin G	1,000	-	15.62	-	ND	ND
Streptomycin	-	-	0.02	0.02	ND	ND
Cyclohexamide	ND	ND	ND	ND	<0.01	-
Ketokonazole	ND	ND	ND	ND	-	-

- = Inactive, ND = not determined

Table 4.27 Agar well diffusion method for antimicrobial activity of pure compounds

Compounds	Concentration (µg/ml)	Test microorganisms and inhibiton zones (mm)			
		Filamentous fungi			
		Dermatophytic Fungi		Plant phatogenic fungi	
		M. gypseam	*T. mentagrophytes*	*F. proliferatum*	*P. parasitica*
BH1	1000	-	+18	-	-
	500	-	-	-	-
	100	-	-	-	-
	50	-	-	-	-
	10	-	-	-	-
	1	-	-	-	-
BE1	1000	-	-	-	-
	500	-	-	-	-
	100	-	-	-	-
	50	-	-	-	-
	10	-	-	-	-
	1	-	-	-	-
Cyclohexamide	1000	-	-	+20.3	+36.1
	500	-	-	+18.7	+34.4
	100	-	-	+12.2	+32.5
	50	-	-	+9.2	+28.7
	10	-	-	-	+22.3
	1	-	-	-	-
Ketokonazole	1000	-	-	-	-
	500	-	-	-	-
	100	-	-	-	-
	50	-	-	-	-
	10	-	-	-	-
	1	-	-	-	-

- = Inactive, + = active

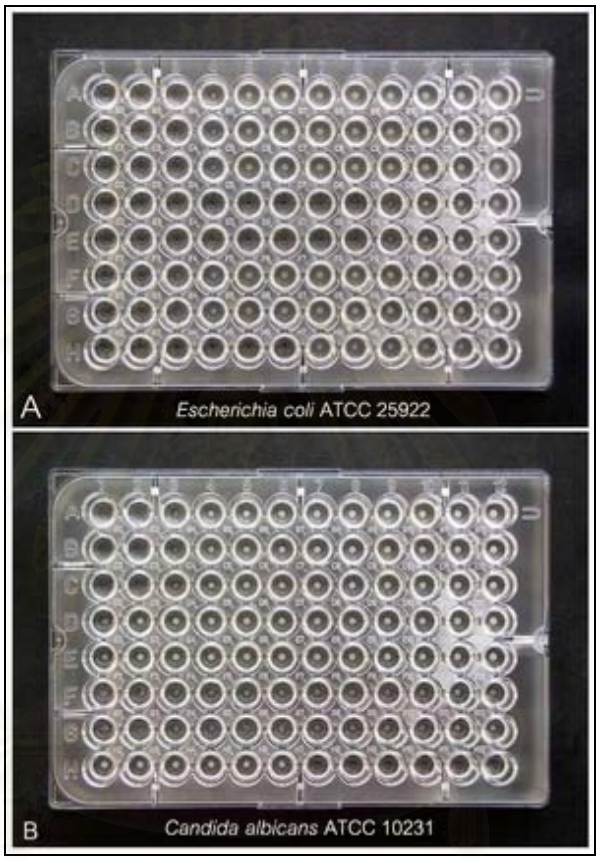

Figure 4.25 Broth microdilution methods for antimicrobial activity of isolated compound. A: Against *E. coli* ATCC 25922. B: Against *C. albicans* ATCC 10231. Clear or colorless wells indicate growth inhibition.

Antimicrobial activities of the pure compounds were tested. It was found that mixture BH1 was active against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. cerivisiae* TISTR 5169 and *C. albicans* ATCC 10231 at the concentraction 0.98, 62.50, 15.62, $1x10^3$, 62.50 and $1x10^3$ µg/ml, respectively and also active against *T. mentagrophytes* at the concentration $1x10^3$ µg/ml. Compound BE1 was active against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 at the concentraction 0.98, 62.50, 62.50 and $1x10^3$µ/ml, respectively

4.13.2 Cytotoxic activity

The *in vitro* activity of pure compounds from fungal isolate PcBr20 was tested against 5 cell lines including, HEP-G2 (hepatoma), SW620 (colon), CHAGO (lung), KATO-3 (gastric), BT474 (breast) and is reported in Table 4.28.

Table 4.28 Cytotoxic activities against cell line of pure compounds from endophytic fungus isolate PcBr20

Compounds	IC_{50} (µg/ml)				
	HEP-G2 (hepatoma)	SW620 (colon)	CHAGO (lung)	KATO-3 (gastric)	BT474 (breast)
BH1	>10	>10	>10	>10	>10
BE1	>10	>10	>10	>10	>10

IC_{50} was the minimum concentration of 50% inhibitory activity.

From the data in Table 4.28, the mixture BH1 and compound BE1 showed no activtity on cytotoxicity against 5 tumer cell lines.

An increase in the number of health problems caused by various cancers, drug-resistant bacteria, parasites, protozoa, and fungi is a cause of alarm. An intensive search for new and more effective agents to deal with these diseases is now under way and endophytes are a novel source of potentially useful medicinal compounds (Strobel, 2003). In this reserch, endophytic fungi were isolated from *C. oblonglifolius* which is Thai medicinal plant. Two pure compounds were isolated from culture broth of endophytic fungal isolate PcBr20, mixture BH1 which was a mixture of fusaric acid and dehydrofusaric acid. Compound BE1 which was a white amorphous solid (mp. 190-191 ^{o}C) and the structures are still under investigation.

The pure compounds showed antimicrobial activities. Mixture of fusaric acid and dehydrofusaric acid (Mixture BH1) exhibited activity against most test microorgisms such as gram positive bacteria, gram negative bacteria and some fungi. Mixture BH1 showed a broad spectrum of activity against test microorgamisms and no activity on cytotoxicity against cell lines. These results indicate that mixture BH1 may be developed to higher test with animal cell lines. In addition, compound BE1 exhibited against gram positive bacteria and gram negative bacteria.

Fusaric acid (5-butylpicolinic acid) was first discovered during the labaratory culture of *Fusarium hetesporum* by Yabuta et al. (1937). Fusaric acid is a fungal toxin with low to moderate toxicity synthesized by some *Fusarium* species such as *F. moniliforme* (sexual stagae *Gibberella fujikuroi*), *F. crookwellense*, *F. subglutinans*, *F. sambucinum*, *F. napiforme*, *F. heterosporum*, *F. oxysporum*, *F. solani*, and *F. proliferatum* (Wang and Ng, 1999; Bacon et al., 1996). Fusaric acid is a class of alkaloid natural products with important biological acitivities (Song and Yee, 2001). The *Fusarium* spp. mycotoxin, fusaric acid was tested for antimicrobial activity against *Ruminococcus albus* and *Methanobrevibacter ruminantium*. The growth of both organisms was inhibited by fusaric acid as low as 15 mg/ml (May, Wu, and Blake, 2000). Futhermore, the *in vitro* activity of fusaric acid exhibited activity against *Mycobacterium leprae* (Wang and Ng, 1999). In this research, mixture of fusaric acid and dehydrofusaric acid showed no activtity on cytotoxicity against 5 tumer cell lines such as HEP-G2 (hepatoma), SW620 (colon), CHAGO (lung), KATO-3 (gastric), and BT474 (breast). In

the other hand, fusaric acid exhibited marked antitumor activity on human colon adenocarcinoma cell lines LoVo, SW48, SW480, and SW742, as well as human mammary adenocarcinoma cell line MDA-MB-468 (Fernandez-Pol, Klos, and Hamilton, 1993). When assayed on tomato leaves and seedlings at $2.7x10^{-3}$ abd $2x10^{-4}$ M, respectively, fusaric acid and 9,10-dehydrofusaric acid showed wide chlorosis rapidly evolving into necrosis as well as a strong inhibition of root elongation, respectively. When asssayed at 10^{-4} M on brine shrimps (*Artemia salina*), fusaric acid, and 9,10-dehydrofusaric acid did not prove to be toxic (Capasso et. al., 1996).

CHAPTER V

CONCLUSION

Sixty two endophytic fungi were isolated from leaves, branches and bark of the Thai medicinal plant, *Croton oblongifolius.* Plant samples were collected from Kui Buri district, Prachuap Khiri Khan Province.

Endophytic fungi were isolated by using the surface sterilization method. A total of 62 isolates (100%) of endophytic fungi were identified. Twenty one isolates (34%) were identified as belonging to typical genera of endophytes such as *Phomopsis, Fusarium, Pestalotia, Cladosporium*, *Colletotrichum* and members of Xylariaceae. The remaining 34 isolates (55%) of endophytic fungi were mycelia sterilia. Meanwhile, 7 isolates (11%) of endophyte fungal isolates was unidentified.

The antimicrobial activities of these endophytes were tested by agar well diffusion method. Crude extracts were prepared from 6-8 weeks old culture. Culture broth extracts and mycelium extracts were tested against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Saccharomyces cerivisiae* TISTR 5169, *Candida albicans* ATCC 10231, *Microsporum gypseam*, and *Trichophyton mentagrophyte*. Thirty six (58%) of the extracts (culture broth extracts or/and mycelium extracts) displayed growth inhibition on at least one of the test microorganisms. For example, 5 isolates (8%) showed activity with culture broth extracts and mycelium extracts. In addition, 26 isolates (42%) showed activity with culture broth extracts and 5 isolates (8%) showed activity with mycelium extracts.

The metabolite profiles of culture broth extsracts and mycelium extracts was determined by thin layer chromatography. Sixty two isolates (100%) of culture broth extracts from endophytic fungi produced a various patterns of metabolite profiles while, 62 isolates (100%) of mycelium extracts from endophytic fungi produced a similar pattern of metabolite profiles.

Fungal isolate PcBr20 was chosen for further stydy for bioactive compounds because the culture broth extracts were active against the highest number of test microorganisms such as *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *S. cerevisiae* TISTR 5169*, C. albican* ATCC 10231 and *T. mentagrophytes.* Inaddition, the mycelia extracts also against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *C. albican* ATCC 10231 and *T. mentagrophytes*. Fungal isolate PcBr20 was identified as *Fusarium* sp. and *Gibberella sacchari* based on morphological features and nucleotide sequencing of ITS region, respectively.

Chromatographic and crystallization techniques were used to isolate bioacive compounds from culture broth extracts and mycelium extracts of endophytic fungi isolate PcBr20. The structures of pure compounds were elucidated by using their physical properties and spectroscopic techniques. Two compounds were isolated and investivated. The structure of mixture BH1 was a mixture of fusaric acid and dehydrofusaric acidls and compound BE1 has white amorphous solid (mp. 190-191 ^{o}C).

Antimicrobial activities and cytotoxicity of the pure compounds was tested. It was found that mixture BH1 was active against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. cerivisiae* TISTR 5169 and *C. albicans* ATCC 10231, and *T. mentagrophyte*s at the concentraction 0.98, 62.50, 15.62, 1x10^{3} , 62.50, 1x0^{3} and 1x10^{3} µ/ml, respectively, and no activity on cytotoxicity against 5 tumor cell lines. Compound BE1 was active against *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 at the concentraction 31.25, 250, 250 and 10^{3}µ/ml, respectively.

REFERRENCES

Thai

เต็ม สมิตินันท์. 2523. ชื่อพันธุ์ไม้แห่งประเทศไทย (ชื่อพฤกษศาสตร์-ชื่อพื้นเมือง). พิมพ์ครั้ง ที่ 2, กรุงเทพมหานคร: หจก. แฟนนี่พัลลิชชิ่ง.

โรงเรียนแพทย์แผนโบราณ วัดพระเชตุพน, สมาคม. 2521. ประมวลสรรพคุณยาไทย (ภาคสอง). กรุงเทพมหานคร: ไพศาลศิลป์การพิมพ์.

ลัดดาวัลย์ บุญรัตนกรกิจ. 2535. สมุนไพรน่าใช้ เล่ม 1. กรุงเทพมหานคร: แท่นทองปริ้นติ้ง เซอร์วิส.

ลีนา ผู้พัฒนพงศ์. 2530. สมุนไพรไทย ตอนที่ 5. กรุงเทพมหานคร: สำนักพิมพ์ฟันนีพับลิชชิ่ง.

เสงี่ยม พงษ์บุญรอด. 2502. ไม้เทศเมืองไทย. พิมพ์ครั้งที่ 1. กรุงเทพมหานคร: เขษมบรรณกิจ.

English

Abraham, w. and Hanssen, H. 1992. Fusoxysporone – a new type of deterpene from *Fusarium oxysporum*. Tetrahedron 48 (8): 10559-10562.

Bacon, C. W., Porter, J. K., Norred, W. P. and Leslie, J. F. 1996. Production of fusaric acid by Fusarium species. Applied and Enviromental Microbiology 62(11): 4039- 4043.

Bacon, C. W. and White, J. F., Jr. 2000. Microbial endophytes. New York: Marcel Dekker.

Barnett, H.L. and Hunter, B.B. 1998. Illustrated genera of imperfect fungi 4th. ed. Minnesota: APS Press.

Belesky, D.P., Devine, O.J., Pallas, Jr J.E. and Stringer, W.C. 1987. Photosynthetic activity of tall fescue as influenced by a fungal endophyte. Photosynthetica 21: 82-87.

Berny, P., Jaussaud, P., Durix, A., Ravel, C. and Bony, S. 1997. Rapid determination of the mycotoxin lolitrem B in endophyte-infected perennial ryegrass by high-performance thin-layer chromatography a validated assay. Journal of Chromatography A 769: 343-348.

Blodett, J.T., Swart, W.J., Louw, S. M. and Weeks, W.J. 2000. Species composition of endophytic fungi in *Amaranthus hybridus* leaves, petioles, stems, and roots. Mycologia 92(5): 853-859.

Brady, S.F. and Clardy, J. 2000. CR377, a new pentaketide antifungal agent isolated from an endophytic fungus. J. Nat. Prod. 63: 1447-1448.

Buzina, W., Braun, H., Freudenschuss, K., Lackner, A., Habermann, W. and Stammberger, H. 2003. Fungal biodiversity-as found in nasal mucus. Medical mycology 41: 149-161.

Capasso, R., Evidente, A., Cutignano, A., Vurro, M., Zonno, M.C., and Bottalico, A. 1996. Fusaric acid and 9,10-dehydrofusaric acids and their methylesters from *Fusarium nygamai*. Phytochemistry. 41(4): 1035-1039.

Carlile, M. J., Watkinson, S. C., and Gooday, G, W. 2001. The fungi. 2nd ed. Sandiego: Academic Press.

Carroll, G.C. 1986. The biology of endophytism in plants with particular reference to woody perennials. pp. 205-222. In Microbiology of the Phyllosphere (eds N.J. Fokkema and J. Van den Heuvel). Cambridge: Cambridge University Press.

Carroll, G.C. 1988. Fungal endophytes in stems and leaves: from latent pathogen to mutualistic symbiot. Ecology 69: 2-9.

Carroll, G.C. and Petrini, O. 1983. Patterns of substrate utilisation by some fungal endophytes from coniferous foliage. Mycologia 75: 53-63.

Cerkauskas, R.F. 1988. Latent colonization by *Colletotrichum* spp.: Epidemiolgical considerations and implications for mycoherbicides. Can. J. Plant Phthol. 10: 297-310.

Cerkauskas, R.F., Dhingra. O.D., Sinclair, J.B., and Asmus, G. 1983. *Amaranthus spinosus*, *Leonotis nepetaefolia*, and *Leonurus sibiricus*: New hosts of *Phomopsis* spp. in Brazil. Plant Dis. 67: 821-824.

Chen, G., Lin, Y., Wen, L., Vrijmoed, L.L.P. and Gareth Jones, E.B. 2003. Two new metabolites of a marine endophytic fungus (No. 1893) from an estuarine mangrove on the south china sea coast. Tetrahedron 59: 4907-4909.

Clay, K. 1991. Fungal endophytes, grasses, and herbivores. Pages 199-252 in: Microbial mediation of plant of plant-herbivore interactions. P.Barbosa, V.A. Krischik, and C.G. Jones, eds. New York: Jonh Wiley & Sons.

Clay, K. 1989. Clavicipitaceous endophytesof grasses: their potential as biocontrol agent. Mycol. Res. 92: 1-12.

Dombrowski, A.W., Bills, G.F., Sabinis, G., Koupal, L.R., Meyer, R., Ondeyka, J.G., Giacobbe, R. A., Monaghan, R.L. and Lingham, R.B. 1992. L-696,474, a novel cytochalasin as an inhibitor of HIV-1 protease. I. The producing organism ans its fermentation. J. Antibiot. 45: 671-678.

Ellis, M.B. 1971. Dematiaceous hyphomycetes, p. 308. Surrey: CAB internaltional.

Fernandez-Pol, J.A., Klos, D.J., and Hamilton, P.D. 1993. Cytotoxic activity of fusaric acid on human adenocarcinoma cells in tissue culture. Anticancer Res. 13(1): 57-64.

Freeman, S., and Rodriguez, R.J. 1993. Genetic conversion of a fungal plant pathogen to a nonpathogenic, endophytic mutualist. Science 260:75.

Ganer, G.B., Rottinghaus, G.E., Cornell, C.N. and Testereci, H. 1993. Chemistry of compounds associated with endophyte/grass interaction: ergovaline- and ergopeptine-related alkaloids. Agriculture, Ecosystems and Enviroment 44: 65-80.

Gardes, M. and Bruns, T.D. 1993. ITS primers with enhanced specificity for basidiomycetes: application to the identification of mycorhizae and rusts. Mol. Ecol. 2: 113-118

Gatenby, W.A., Munday-Finch, S.C., Wilkins, A.L. and Miles, C. O. 1999. Terpendole M, a novel indole-diterpenoid isolated from *Lolium perenne* infected with the endophytic fungus *Neotyphodium lolii*. J. Agric. Food Chem. 47: 1092-1097.

Harper, J.K., Arif, A.M., Ford, E.J., Strobe, G.A., Porco, J.A., Tomor, D.P., Oneill, K.L., Heider, E. M. and Grant, D. M. 2003. Pestacin: a 1,3-dihydro isobenzofuran from *Pestalotiopsis microspora* possesing antioxidant and antimycotic activities. Tetrahedron 59: 2471-2476.

Hartman, G.L., Manandhar, J.B., and Sinclair, J.B. 1986. Incidence of *Collectotrichum* spp. on soybeans and weeds in Illinois and pathogenicity of *Colletotrichum truncatum*. Plant Dis. 70: 780-782.

Hepperly, P.R., Kirkpatrick, B.L., and Sinclair, J.B. 1980. *Abutilon theophrasti*: Wild host for three fungal parasites of soybean. Phytopathology 70: 307-310.

Isaac, S. 1992. Fungal-plant interactions. London: Chapman & Hall.

Jayasuriya, H., Bills, G.F., Cascales, C., Zink, D.L., Goetz, M.A., Jenkins, R.G., Silverman, K.C., Lingham, R.B. and Singh, S.B. 1996. Oreganic acid: a potent novel inhibitor of ras farnesyl-protein transferase from an endophytic fungus. Bioorganic and Medicinal Chemistry Letters 6(17): 2081-2084.

Joseph, P.J., Dave, R.I. and Shah, N.P. 1998. Antagonism between yoghurt bacteria and probiotic bacteria isolated from commercial starter cultures, commercial yoghurts and a probiotic capsule. Food Australia 50(1): 20-23.

Ju, Y., Sacalis, J.N. and Still, C.C. 1998. Bioactive flavonoids from endophyte-infected blue Grass (*Poa ampla*). J. Agric. Food Chem. 46: 3785-3788.

Kanchaniprayudh, J., Zhou, Z., Yomyart, S., Sihanonth, P. and Hogetsu, T. 2003. Molecular phylogeny of ectomycorrhizal *Pisolithus* fungi associated with pine, dipterocarp, and eucalyptus trees in Thailand. Mycoscience 44: 287-294.

Konig, G.M., Wright, A.D., Aust, H., Draeger, S. and Schulz, B. 1999. Geniculol, a new biologically active diterpene from the endophytic fungus *Geniculosporium* sp. J. Nat. Prod. 62: 155-157.

Kongsaeree, P., Prabpai, S., Sriubolmass, N., Vongvein, C. and Wiyakrutta, S. 2003. Antimalarial dihydroisocoumarins produced by *Geotrichum* sp., an endophytic fungus of *Crassocephalum crepidiodes*. J. Nat. Prod. 66: 709-711.

Krohn, K., Florde, U., John, M., Root, N., Steingrover, K., Aust, H., Draeger, S., Schulz, B., Antus, S., Simonyi, M. and Zsila, F. 2001. Biologically active metabolites from fungi. Part 16: New preussomerins J, K and L from and endophytic fungus: structure elucidation, crystal structure analysis and

determination of absolute configuration by CD calculations. Tetrahedron 57: 4343-4348.

Kulik, M.M., 1984. Symptomless infection, persistence, and production of pycnidia in host and non-host plants by *Phomopsis batatae*, *Phomopsis phaseoli* and *Phomopsis sojae*, and the taxonomic implications. Mycologia 76: 274-291.

Lewis, D.H. 1973. Concept in fungal nutrition and the origin of biotrophy. Biol. Rev. 48:261-278.

Lewis, D.H. 1974. Micro-organisms and plants: The evolution of parasitism and mutualism. Pages367-392 in: 24th Symposium of the Society for General Microbiology. M.J. Carlise, and J.J. Skehel, eds. Cambridge: Cambridge University Press.

Li, J.Y., Harper, J.K., Grant, D.M., Tombe, B.O., Bashyal, B., Hess, W.M. and Strobel, G.A. 2001. Ambuic acid, a highly functionalized cyclohexenone with antifungal activity from *Pestalotiopsis* spp. and *Monochaetia* sp. Phytochemistry 56: 463-468.

Li, J., Sidhu, R.S., Bollon, A. and Strobel, G.A. 1998. Stimulation of taxol production in liquid cultures of *Pestalotiopsis microspora*. Mycol. Res. 102(4): 461-464.

Li, J., Sidhu, R.S., Ford, E.J., Long, D.M., Hess, W.M. and Strobel, G.A. 1998. The induction of taxol production in the endophytic fungus *Periconia* sp. from *Torreya grandifolia*. Journal of Industrial Microbiology and Biotechnology 20: 259-264.

Li, J.Y. and Strobel, G.A. 2001. Jesterone and hydroxy-jesterone antioomycetes cyclohexenone epoxides from the endophytic fungus *Pestalotiopsis jesteri*. Phytochemistry 57: 261-265.

Li, J., Strobel, G., Sidhu, R. and Hess, W.M. 1996. Endophytic taxol-producing fungi from bald cypress, *Taxodium distichum*. Microbiology 142: 2223-2226.

Lu, H., Zou, W.X., Meag, J.C., Hu, J. and Tan, R.X. 2000. New bioactive metabolites produced by *Colletotrichum* sp., an endophytic fungus in *Artemisia annua*. Plant Science 151: 67-73.

May, H.D., Wu, Q., and Blake, C.K. 2000. Effects of the *Fusarium* spp. mycotoxins fusaric acid and deoxynivalenol on the growth of *Rumicoccus albus* and *Methanobrevibacter ruminantium*. Can. J. Microbiol. 46(8): 692-699.

Metz, A.M., Haddad, A. and Worapong, J. 2000. Induction of the sexual stage of *Pestalotiopsis microspora*, a taxol-producing fungus. Microbiology 146: 2079-2089.

McLean, K.S., and Roy, K.W. 1988. Incidence of *Colletotrichum dematium* on prickly sida, spotted spurge. And smooth pigweed and pathogenicity to soybean. Plant Dis. 72: 743-746.

Miller, J.D. 1986. Toxic metabolites of epiphytic and endophytic fungi of conifer needles. pp. 223-231. In Microbiology of the Phyllosphere (eds N.J. Fokkema and J. Van den Heuvel), Cambridge: Cambridge University Press.

Noble, H.M., Langley, D., Sidebottom, P.J., Lane, S.J. and Fisher, P.J. 1991. An echinocandin from an endophytic *Cryptosporiopsis* sp. and *Pezicula* sp. in Pinus sylvestris and Fagus sylvatica. Mycol. Res. 95: 1439-1440.

Ondeyka, J., Hensens, O.D., Zink, D., Ball, R., Lingham, R. B., Bills, G., Dombrowski, A. and Goetz, M. 1992. L-696,474, a novel cytochalasin as an inhibitor of HIV-1 protease. II. Isolation and structure. J. Antibiot. 45: 679-685.

Petrini, O. 1986. Taxnomy of endophytic fungi of aerial plant tissues. Pages 175-178 in: Microbiology of the phyllosphere. N.J. Foddema and J. van den Heuvel, eds. Cambridege: Cambridge University Press.

Polishook, J.D., Dombrowski, A.W., Tsou, N.N., Salituro, G.M. and Curotto, J.E. 1993. Preussomerin D from the endophyte *Hormonema dematioides*. Mycologia 85(1): 62-64.

Poter, J.K., Bacon, C.W., Cutler, H.G., Arrendale, R.F. and Robbins, J.D. 1985. *In vitro* auxin production by *Balansia epichloe*. Phytochemistry 24: 1429-1431.

Pulici, M., Sugawara, F., Koshino, H., Uzawa, J. and Yoshida, S. 1996. Pestalotiopsis A and B: New caryphyllenes from an endophytic fungus of Taxus brevifolia. J. Org. Chem. 61: 2122-2124.

Raid, R.N. and Pennypacker, S.P. 1987. Weeds as hosts for *Colletotrichum coccodes*. Plant Dis. 73: 643-646.

Ratnayake, A.S., Yoshida, W.Y., Mooberry, S.L. and Hemscheidt, T. 2001. The structure of microcarpalid, a microfilament disrupting agent from an endophytic fungus. Organic Letters 3(22): 3479-3481.

Ratnayake, A.S., Yoshida, W.Y., Mooberry, S.L. and Hemscheidt, T.K. 2001. Nomofungin: a new microfilament disrupting agent. J. Org. Chem. 66: 8717-8721.

Redlin, S.C. and Carris, L.M. 1996. Endophytic fungi in grasses and woody plants: systematic, ecology, and evolution. Minnesota: APS Press.

Rowan, D.D. 1993. Loitrems, peramine and paxilline: mycotoxins of the ryegrass/ endophyte interaction. Agriculture, Ecosystems and Environment 44: 103-122.

Santos, R.M.G. and Rodrigues-Fo, E. 2002. Meroterpenes from *Penicillium* sp. found in association with *Melia azedarach*. Phytochemistry 61: 907-912.

Sasaki, T., Takagi, M., Yaguchi, T., Miyadoh, S., Okada, T. and Koyama, M. 1992. A new antihelminthic cyclodepsipeptide, PF1022A. J. Antibiot. 45:692-687.

Schulz, B., Sucker, J., Aust, H.J., Krohn, K., Ludewig, K., Jones, P.G. and Doring, D. 1995. Biologically active secondary metabolites of endophytic *Pezicula* species. Mycol. Res. 99(8): 1007-1015.

Sherwood-Pike, M., Stone, J.K. and Carroll, G.C. 1986. Rhabdocline parkerii; a ubiquitous foliar endophyte of Douglas-Fir. Canadian Journal of Botany 64: 1849-1855.

Siegal, M.R., Latch, G.C.M., and Johnson, M.C., 1987. Fungal endophytes of grasses. Annu. Rev. Phytopathology 62:293-315.

Stierle, A.A., Stierle, D.B. Bugni, T. 1999. Sequoiatones A and B: Novel antitumor metabolites isolated from a redwood endophyte. J. Org. Chem. 64: 5479-5484.

Stierle, A. and Strobel, G. 1995. The search for a taxol-producing microorganism among the endophytic fungi of the Pacific Yew, *Taxus brevifolia*. Journal of Natural Products 58(9): 1315-1324.

Stierle, A., Strobel, G. and Stierle, D. 1993. Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific Yew. Science 260: 214- 216.

Stinson, M., Ezra, D., Hess, W.M., Sears, J. and Strobel, G. 2003. An endophytic *Gliocladium* sp. of *Eucryphia cordifolia* producing selective volatile antimicrobial compounds. Plant Science 165: 913-922.

Stone, J.K. 1987. Initiation and development of latent infections by *Rabdocline parkeri* on Douglas-fir. Canadian Journal of Botany 65: 2614-2621.

Stone, J.K. 1988. Fine structure of latent infections by *Rabdocline parkeri* on Douglas-fir, with observations on uninfected cells. Canadian Journal of Botany 66: 45-54.

Strobel, G. 2003. Endophytes as sources of bioactive products. Microbes and Infection 5: 535-544.

Strobel, G., Ford, E., Worapong, J., Harper, J.K., Arif, A.M., Grant, D.M., Fung, P.C.W. and Chau, R.M.W. 2002. Isopestacin, an isobenzofuranone from *Pestalotiopsis microspora*, possessing antifungal and antioxidant activities. Phytochemistry 60: 179-183.

Strobel, G., Hess, W.M., Baird, G., Ford, E., Li, J.Y. and Sidhu, R.S. 2001. *Stegolerium kukenani* gen. et sp. nov. an endophytic, taxol producing fungus from the Roraima and Kukenan tepuis of Venezuela. Mycotaxon LXXVIII: 353-361.

Strobel, G.A., Hess, W.M., Li, J., Ford, E. and Sears, J. 1997. *Pestalotiopsis guepinii*, a taxol-producing endophytic of the Wollemi Pine, *Wollemia nobilis*. Aust J. Bot. 45: 1073-1082.

Strobel, G.A., Miller, R.V., Martinez-Miller, C., Condron, M.M., Teplow, D.B. and Hess, W.M. 1999. Cryptocandin, a potent antimycotic from the endophytic fungus *Cryptosporiopsis* cf. *quercina*. Microbiology 145: 1919-1926.

Strobel, G. and Stierle, A. 1993. *Taxomyces andreanae*, a proposed new taxon for a bulbilliferous hyphomycete associated with Pacific Yew (*Taxus brevifolia*). Mycotaxon XLVII: 71-80.

Strobel, G.A., Torczynski, R. and Bollon, A. 1997. *Acremonium* sp. –a leucinostatin A producing endophyte of European yew (*Taxus baccata*). Plant Science 128: 97-108.

Strobel, G., Yang, X., Sears, J. and Kramer, R. 1996. Taxol from *Pestalotiopsis microspora*, an endophytic fungus of *Taxus wallachiana*. Microbiology 142: 435-440.

Subrananian, C.V. 1971. Hyphomycetes: An account of Indian species, except Cercosporae, pp. 657-658. New Delhi: The Indian Council of Agricultural Research.

Watcharadit, S. 2002. Secondary metabolites of endophytic fungus isolate ARE-1 from *Annona reticulata Leaf*. M.Sc's Thesis, Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

White, T., Bruns, T., Lee, S. and Taylor, J. 1990. Analysis of phylogenetic relationships by amplification and direct sequenceing of ribosomal RNA genes. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J., (eds) PCR protocols: a guide to methods and applications. Academic Press, New York, pp 315-322.

Song, J. J. and Yee, N. K. 2001. A concise synthesis of Fusaric acid and (*S*)-(+)-Fusarinolic acid. J. Org. Chem. 66: 605-608.

Sutton, B.C. 1980. The Coelomycetes: Fungi imperfecti with Pycnidia, Acervuli and Stroma. Surrey: Commonwealth Mycological Institute.

Tscherter, H. and Dreyfuss, M.M. 1982. New metabolites. Processes for their production and their use. International Patent Application PCT/EP8/00121:

Tscherter, H., Hofmann, H., Ewald, R. and Dreyfuss, M.M. 1988. Antibiotic lactone compound. U.S. Patent 4,753,959.

Wagenaar, M.M. and Clardy, J. 2001. Dicerandrols, new antibiotic and cytotoxic dimers produced by the fungus *Phomopsis longicolla* isolated from an Endangered Mint. J. Nat. Prod. 64: 1006-1009.

Wagenaar, M.M., Corwin, J., Strobel, G. and Clardy, J. 2000. Three new cytochalasins produced by an endophytic fungus in the Genus *Rhinocladiella*. J. Nat. Prod. 63: 1692-1695.

Wang, C., Wu, J. and Mei, X. 2001. Enhancement of taxol production and excretion in *Taxus chinensis* cell culture by fungal elicitation and medium renewal. Appl. Microbiol. Biotechnol. 55: 404-410.

Wang, J., Huang, Y., Fang, M., Zhang, Y., Zheng, Z., Zhao, Y. and Su, W. 2002. Brefeldin A, a cytotoxin produced by *Paecilomyces* sp. and *Aspergillus clavatus* isolated from *Taxus mairei* and *Torreya grandis*. FEMS Immunology and Medical Microbiology 34: 51-57.

Wang, J., Li, G., Lu, H., Zheng, Z., Huang, Y. and Su, W. 2000. Taxol from *Tubercularia* sp. strain TF5, an endophytic fungus of *Taxus mairei*. FEMS Microbiology Letters 193: 249-253.

Wang, H and Ng, T.B. 1999. Pharmacological activities of fusaric acid (5-butylpicolinic acid). Life Science 65 (9): 849-856.

Weaver, R. W., Angle, J. S. and Botlomley, P. S. 1994. Methods of Soil Analysis, Part 2. Microbiology and Biochemical Properties. pp. 389-393. Madison: Soil Science Society of America.

Webber, J. 1981. A natura control of Dutch elm disease. Nature 292: 499-451.

White, J.F. Jr and Cole, G.T. 1985. Endophyte-host associations in forage grasses. I: Distribution of fungal endophytes in some aspects of *Lolium* and *Festuca*. Mycologia 77: 323-327.

White Jr., J.F., Drake, T.E. and Martin, T.I. 1996. Endophyte-host associations in grasses. XXIII. A study of two species of *Balansia* that form stromata on nodes of grasses. Mycologia 88(1): 89-97.

Yang, X., Strobel, G., Stierle, A., Hess, W.M., Lee, J. and Clardy, J. 1994. A fungal endophyte-tree relationship: *Phoma* sp. in *Taxus wallachiana*. Plant Science 102: 1-9.

Yue, Q., Miller, C.J., White, J.F. and Richardson, M.D. 2000. Isolation and characterization of fungal inhibitors from *Epichloe festucae*. J. Agric. Food Chem. 48: 4687-4692.

Zhou, Z., Miwa, M., and Hogetsu, T. 1999. Analysis of genetics structure of a *Suillus grevillei* population in a *Larix kaempferi* stand by polymorphism of inter-simple sequence repeat (ISSR). New Phytol. 144:55-63.

Zou, W.X., Meng, J.C., Lu, H., Chen, G.X., Shi, G.X., Zhang, T.Y. and Tan, R.X. 2000. Metabolites of *Colletrichum gloeosporioides*, and endophytic fungus in *Artemisia mongolica*. J. Nat. Prod. 63: 1529-1530.

APPENDICES

APPENDIX A

MEDIA

The media were prepared by sterilization in the autoclave at 121 ^{o}C for 15 minutes. pH was adjusted with NaOH or HCl before addition of agar and before sterilization.

1. Malt extract agar (MEA)

Malt extracts	20.0	g
Peptone	1.0	g
Glucose	20.0	g
Distilled water	1000	ml
Agar	15.0	g

2. Yeast-malt extract agar (YMA)

Glucose	10.0	g
Peptone	5.0	g
Yeast extracts	3.0	g
Malt extracts	3.0	g
Distilled water	1000	ml
Agar	15.0	g

3. Nutrient agar (NA)

Peptone	5.0	g
Beef extract	3.0	g
Distilled water	1000	ml
Agar	15.0	g

4. Sabouraud's dextrose agar (SDA)

Peptone	10.0	g
Dextrose	40.0	g
Distilled water	1000	ml
Agar	15.0	g

5. V8 agar

V8 Vegetable juice	200	ml
Calcium carbonate	4.0	g
Distilled water	1000	ml
Agar	20.0	g

APPENDIX B

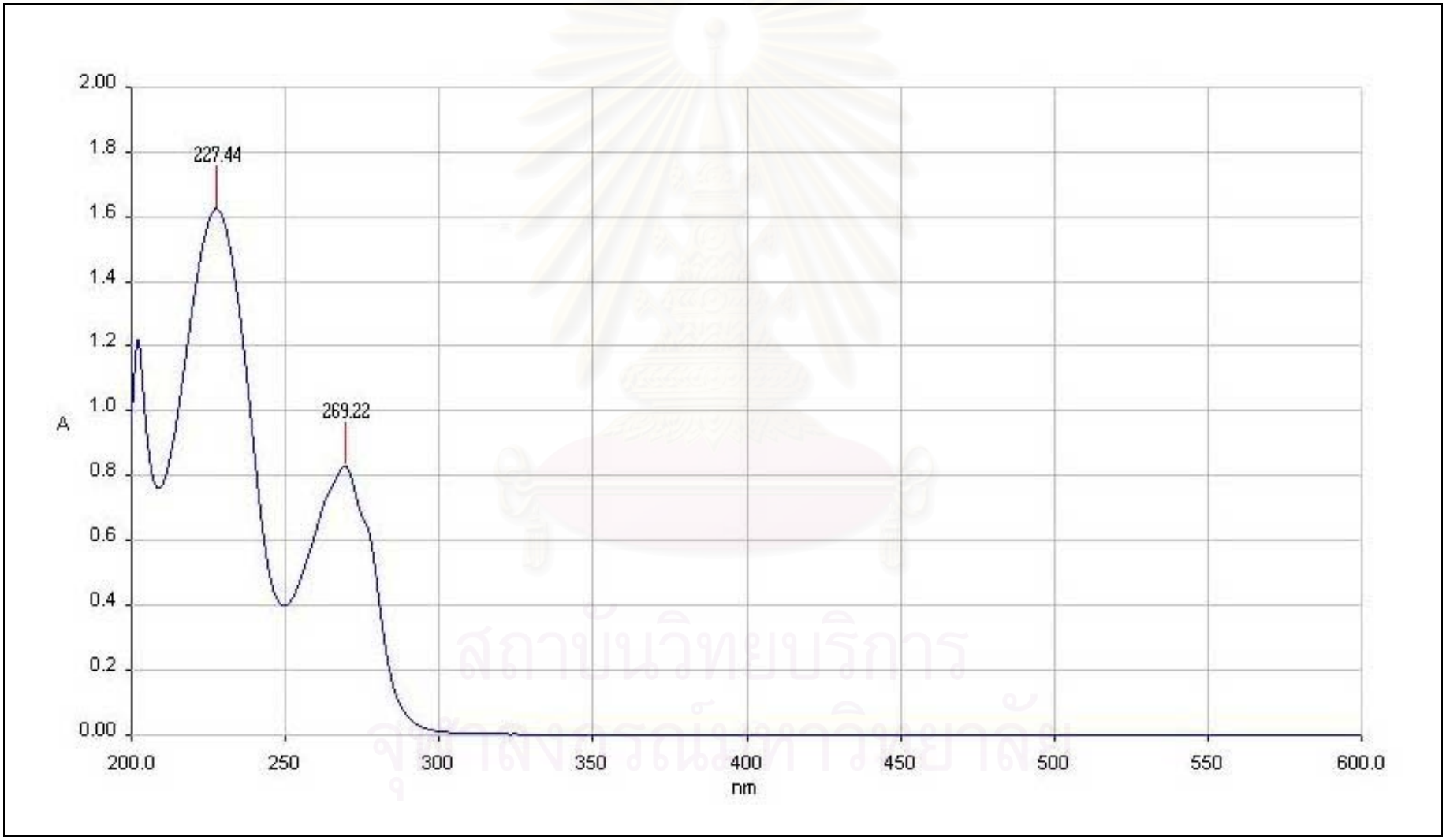

Figure 1 UV spectrum of mixture BH1

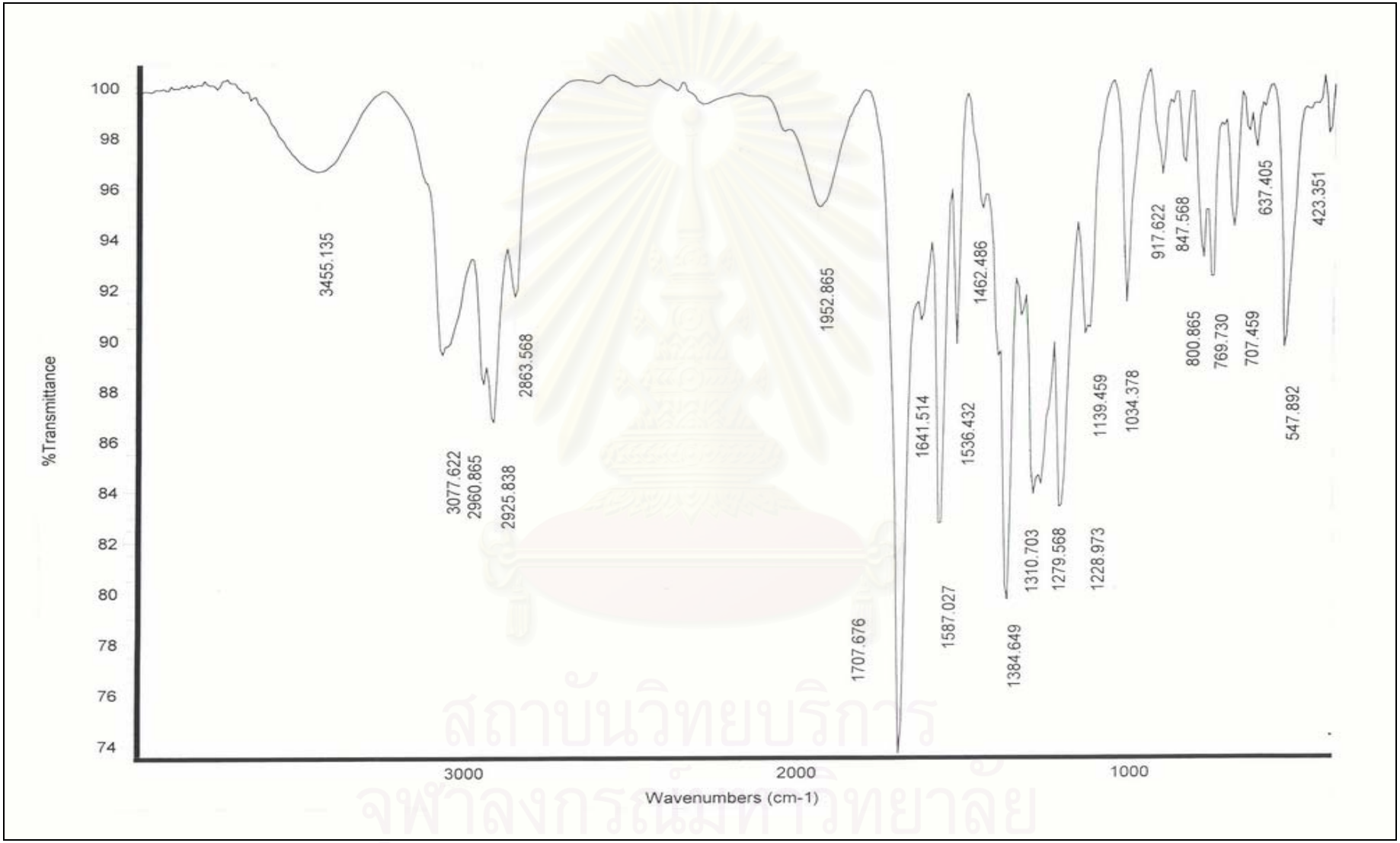

Figure 2 IR2 spectrum of mixture BH1

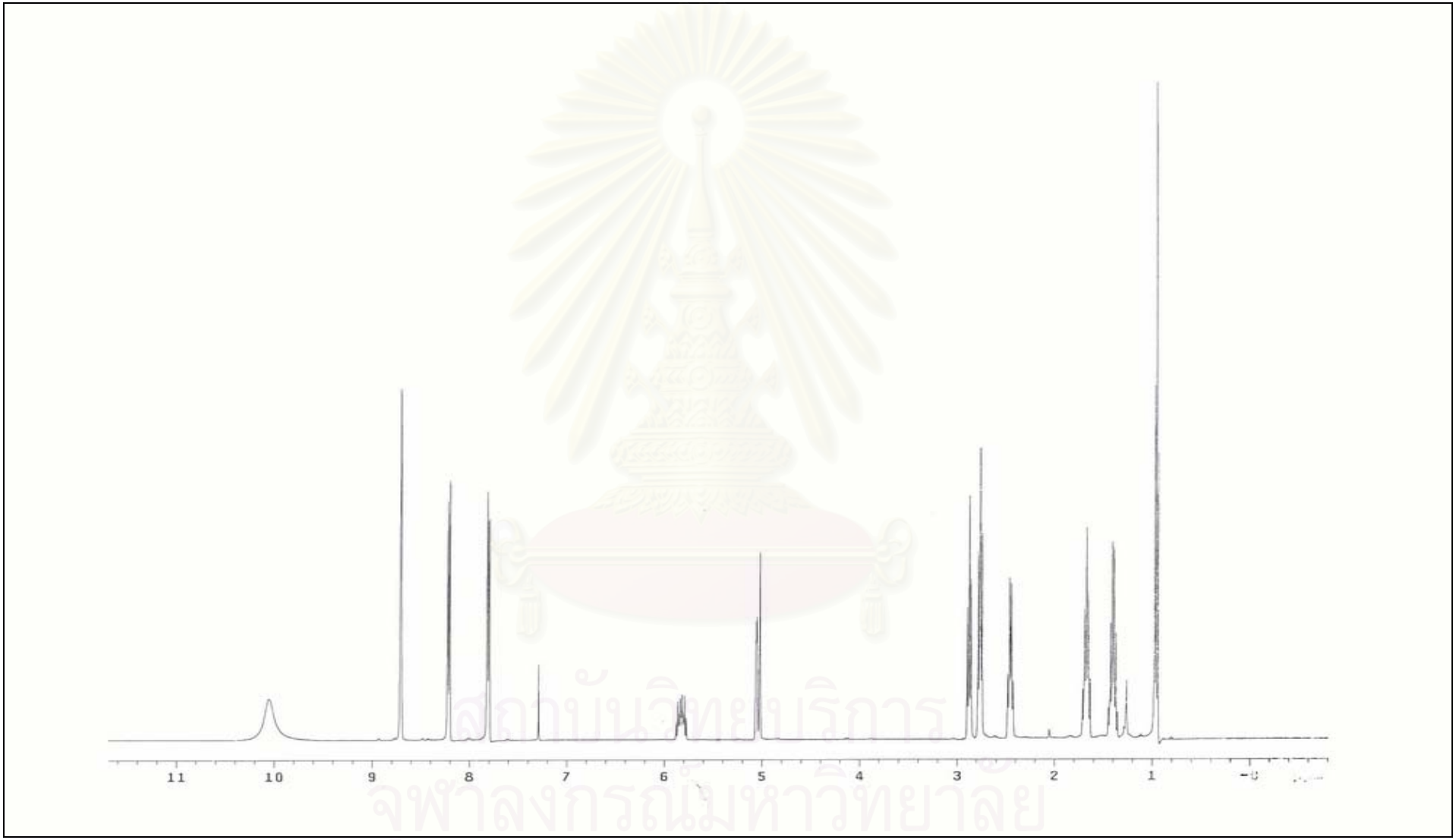

Figure 3 ^{1}H-NMR spectrum of mixture BH1

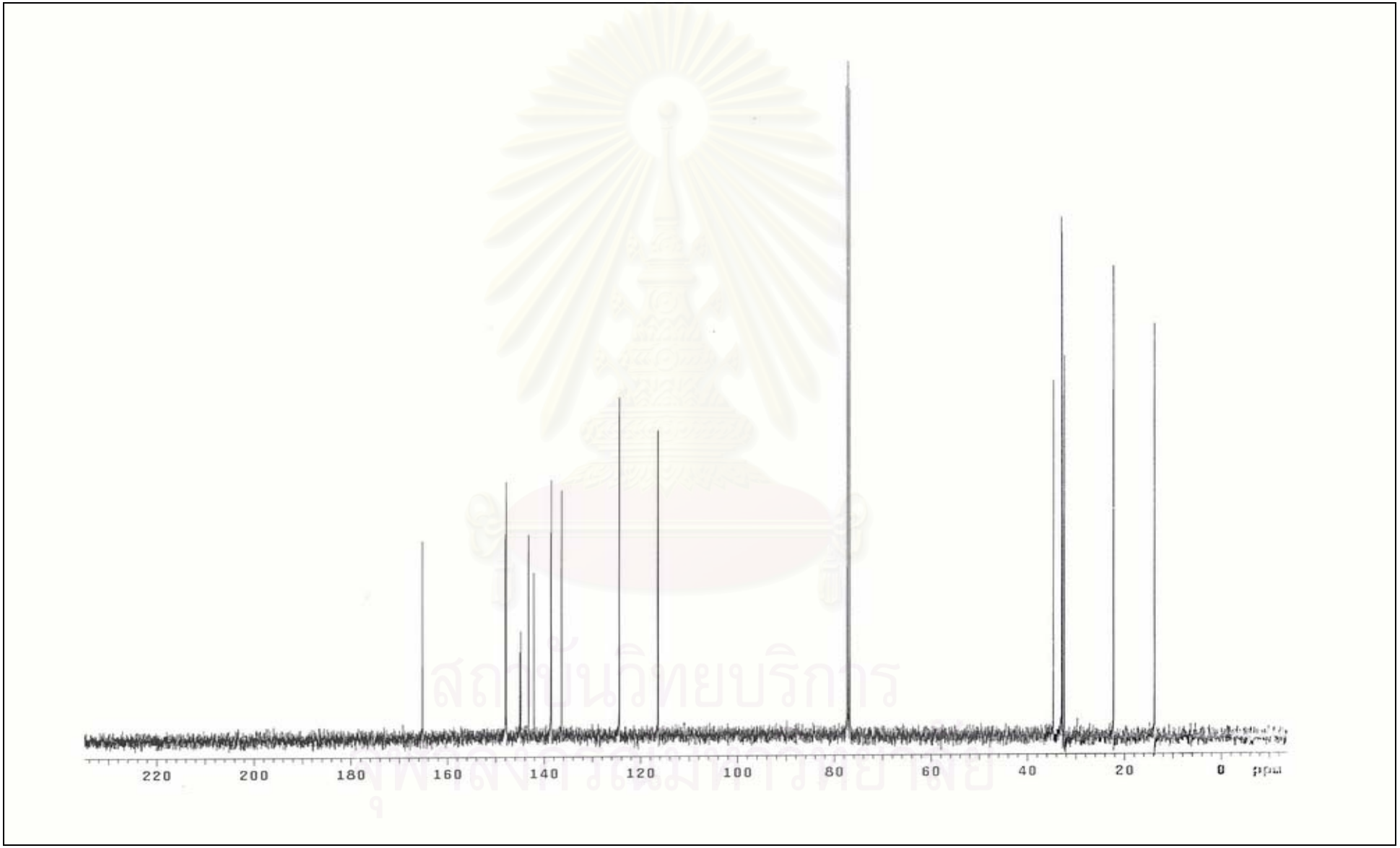

Figure 4 ^{13}C-NMR spectrum of mixture BH1

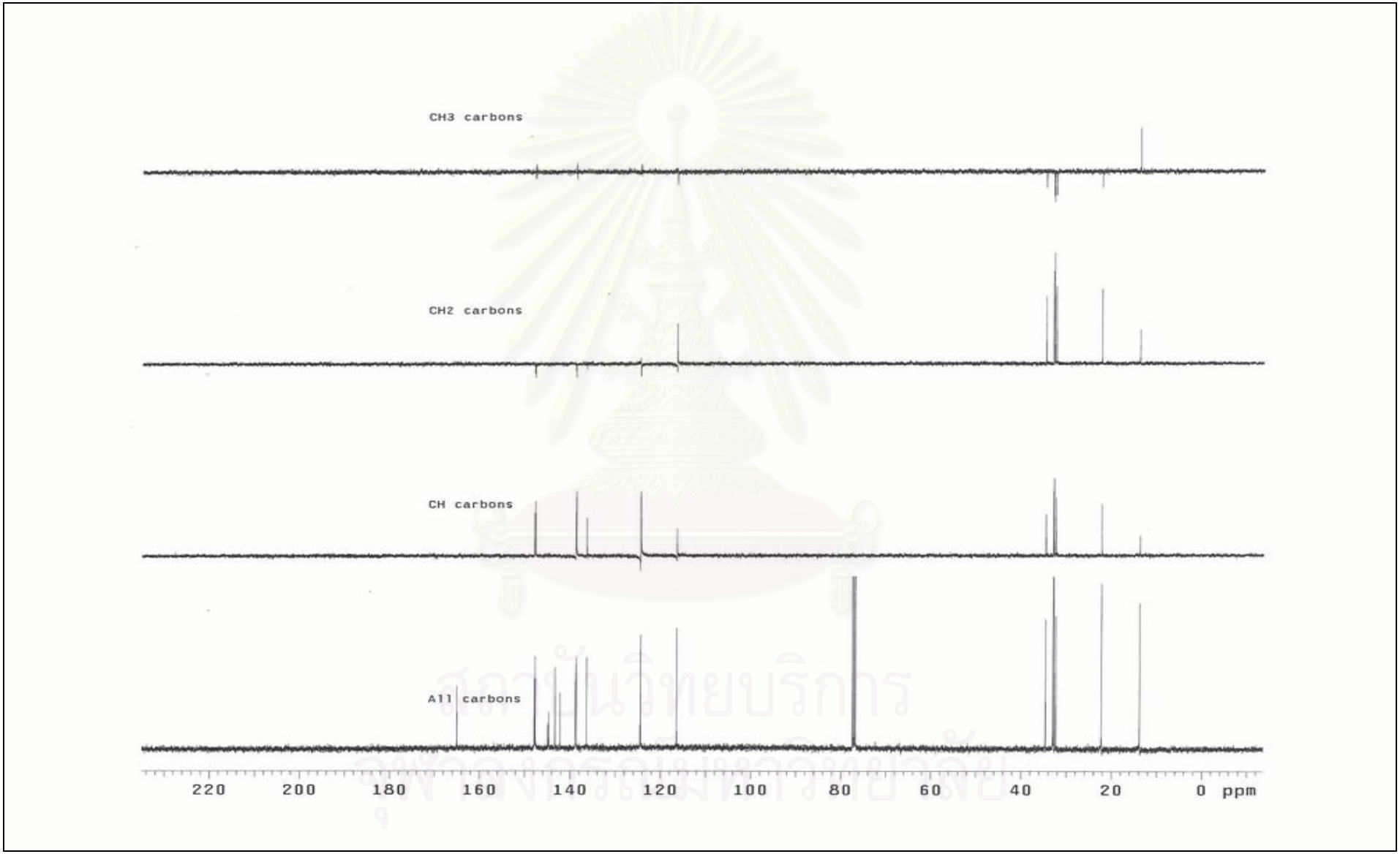

Figure 5 DEPT spectrum of mixture BH1

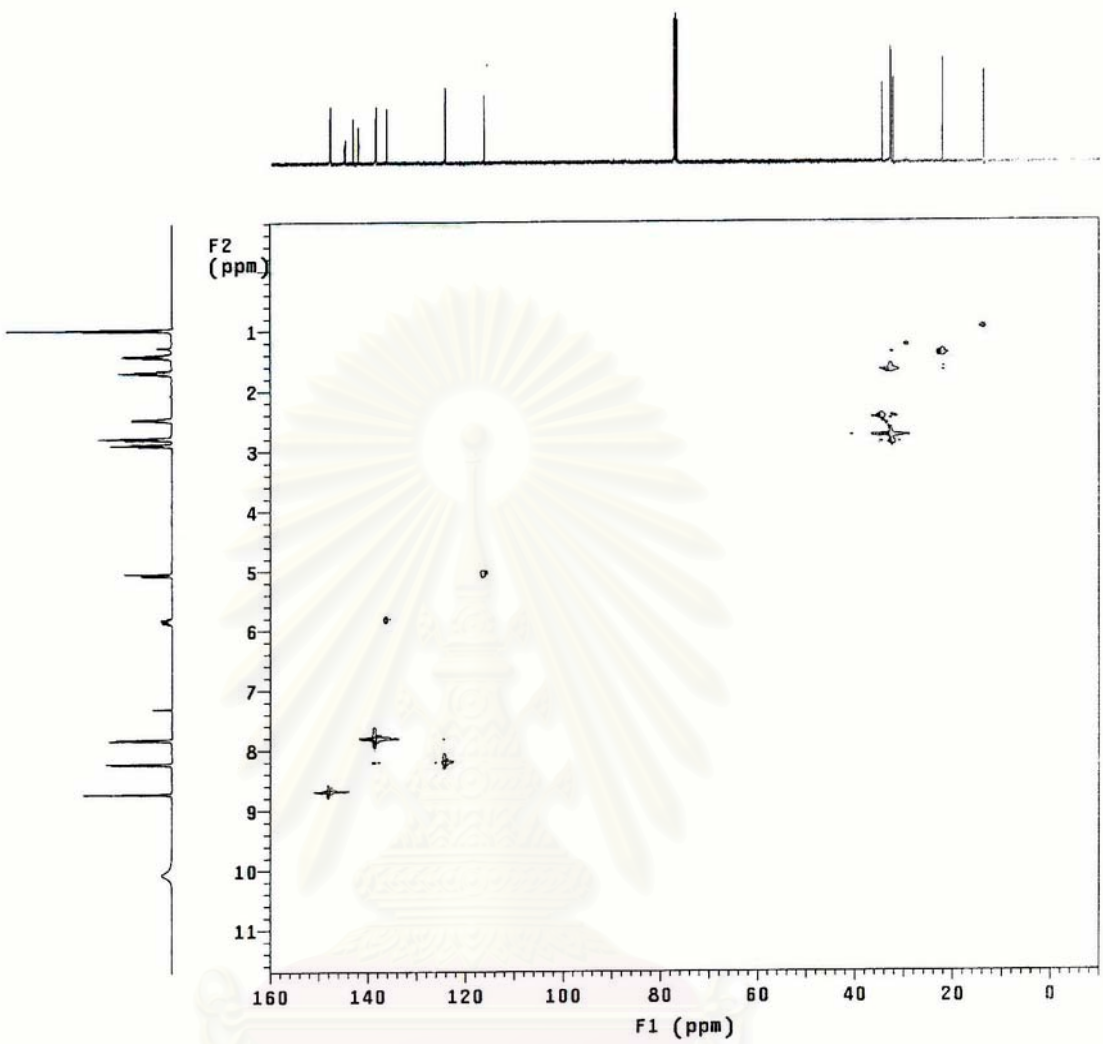

Figure 6 HSQC spectrum of mixture BH1

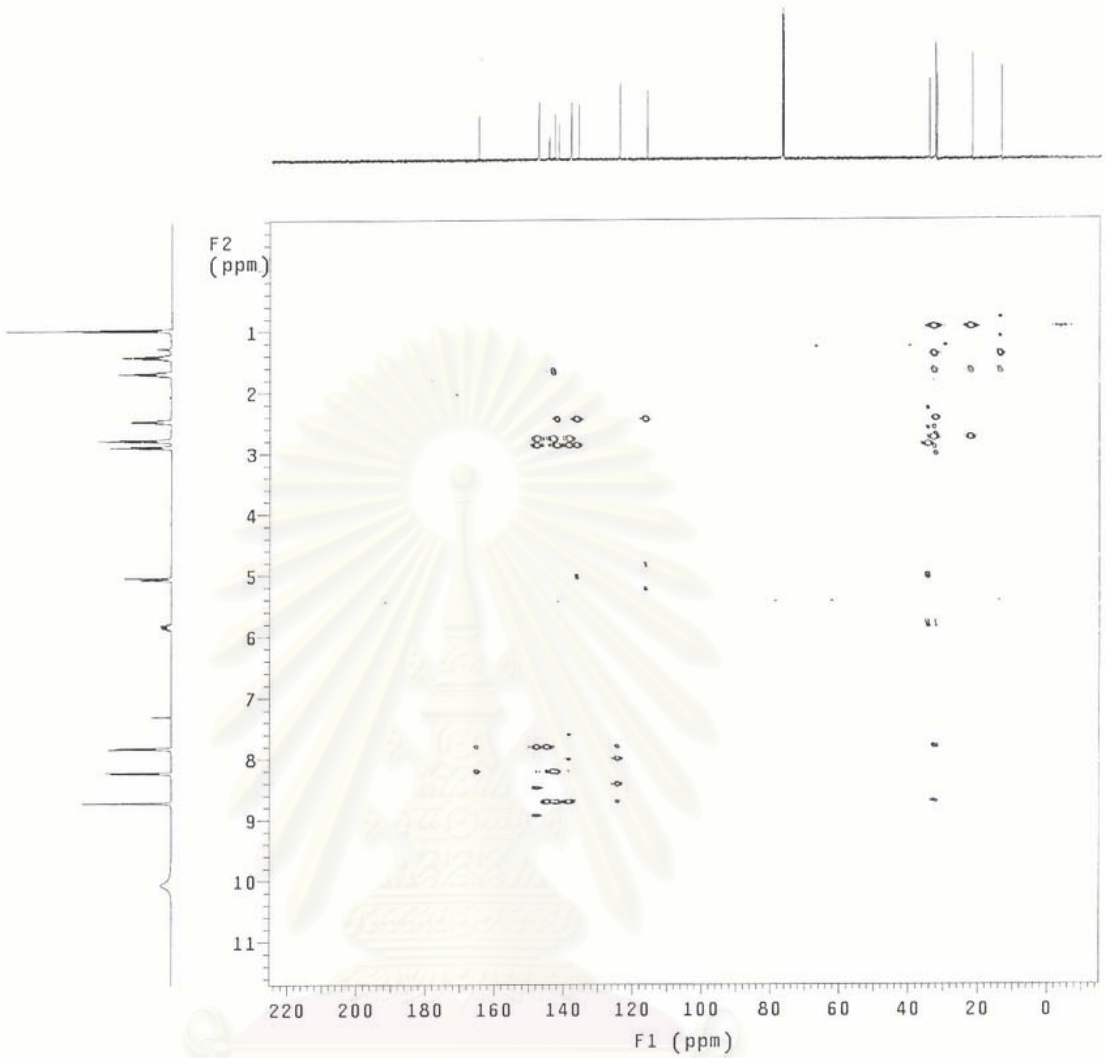

Figure 7 HMBC spectrum of mixture BH1

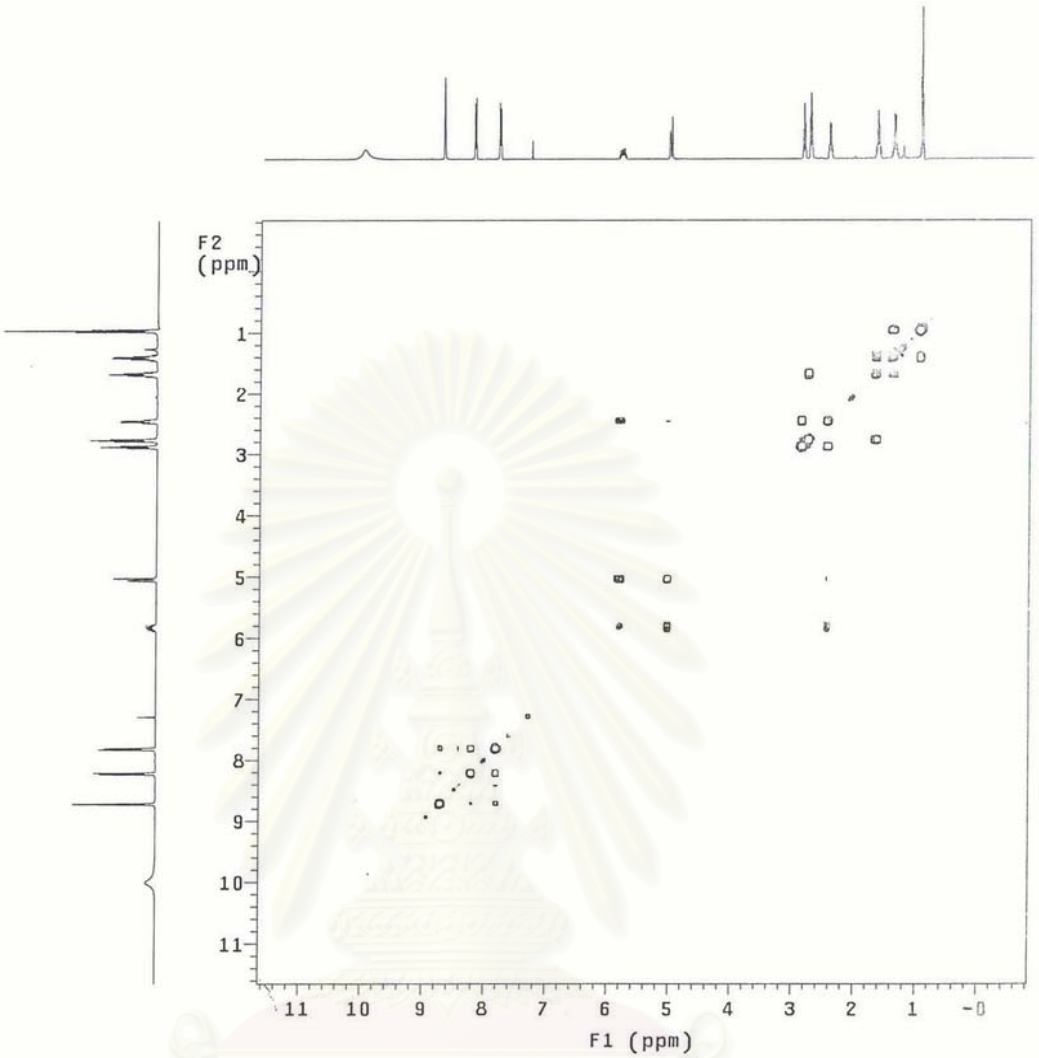

Figure 8 COSY spectrum of mixture BH1

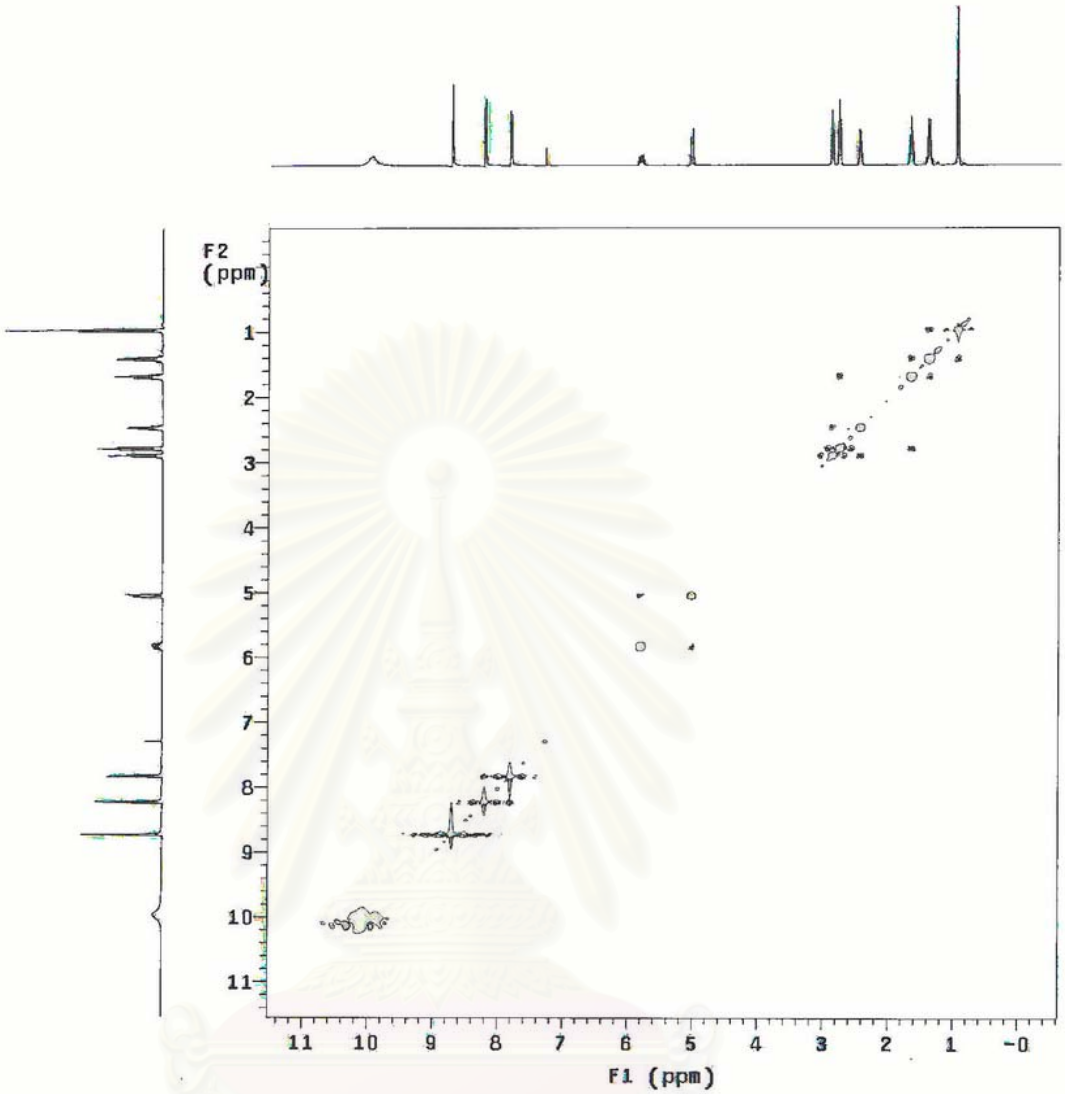

Figure 9 NOESY spectrum of mixture BH1

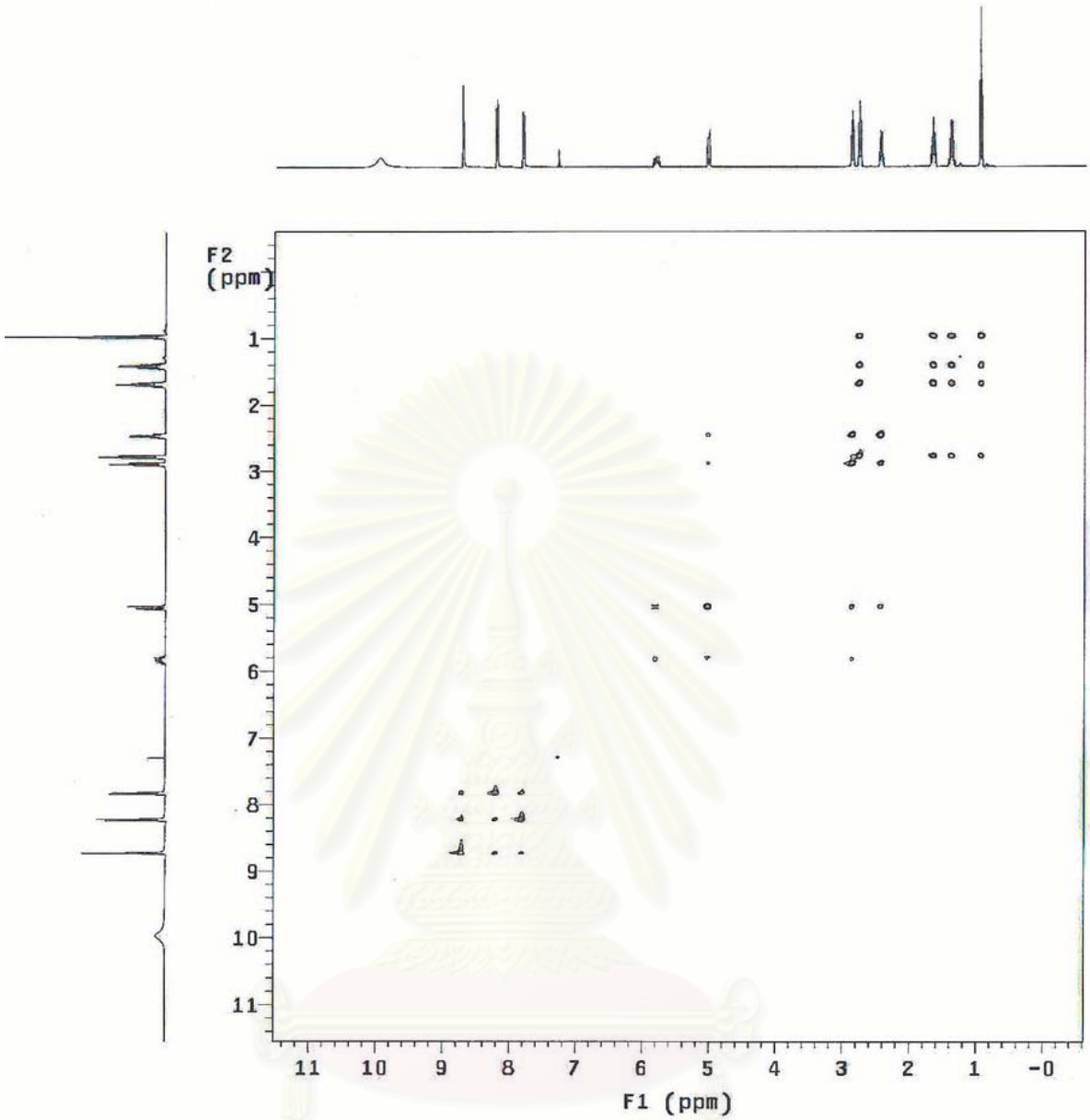

Figure 10 TOCSY spectrum of mixture BH1

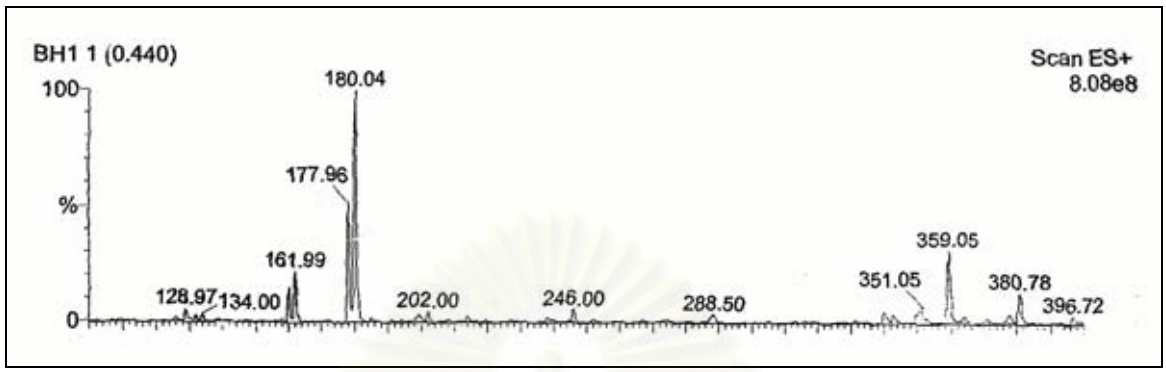

Figure 11 LC-MS of mixture BH1

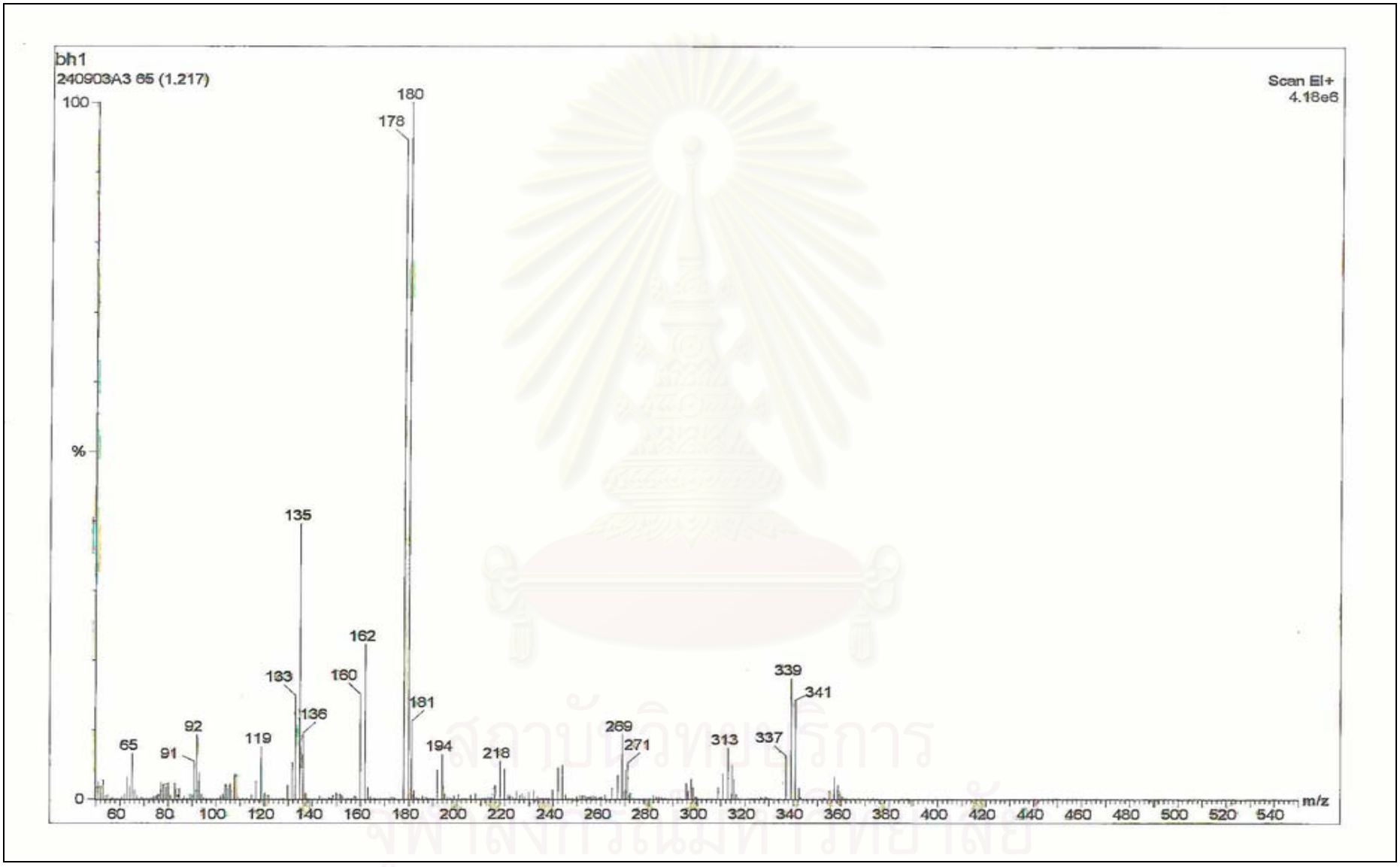

Figure 12 EI-MS spectrum of mixture BH1

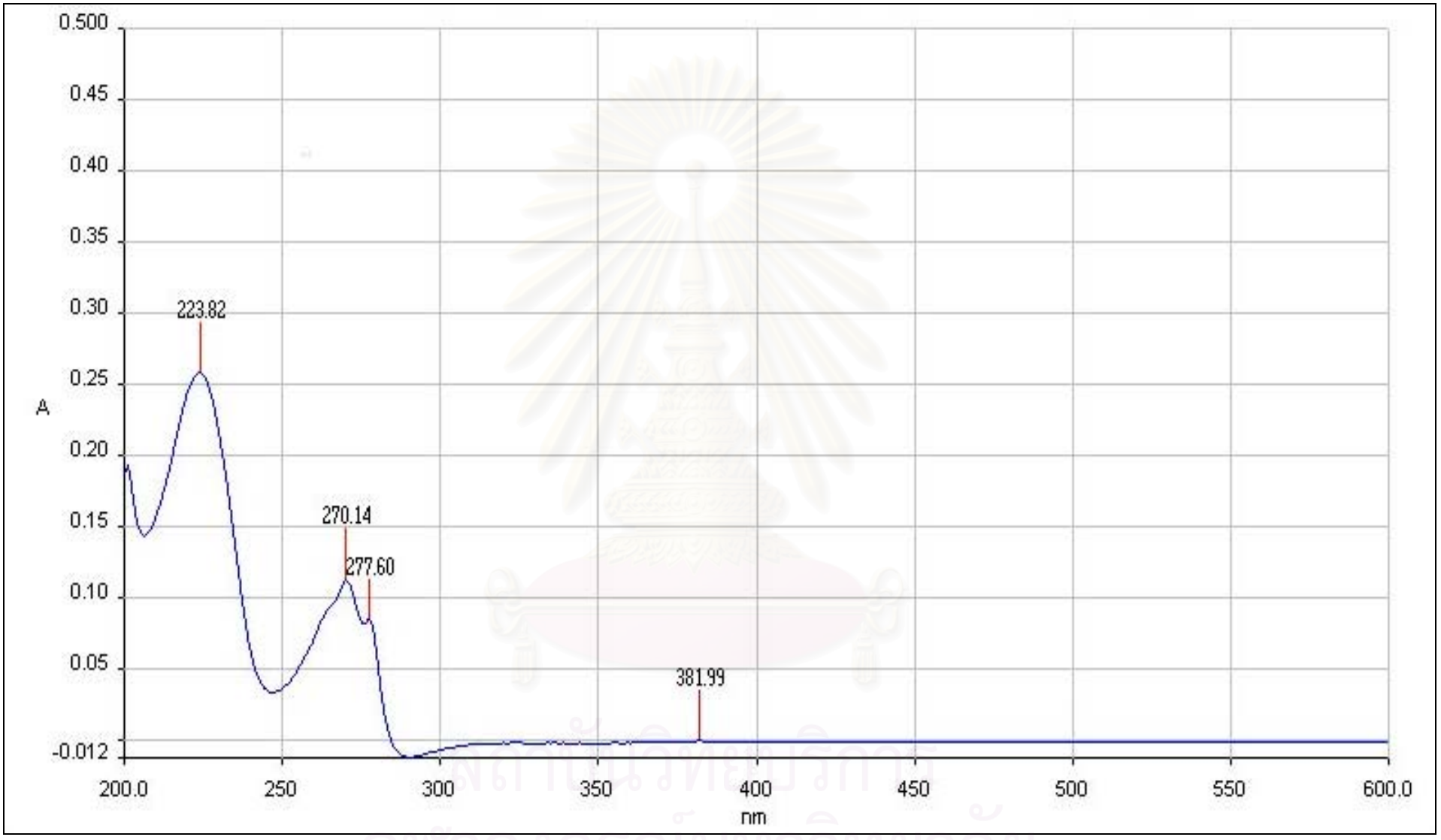

Figure 13 UV spectrum of compound BE1

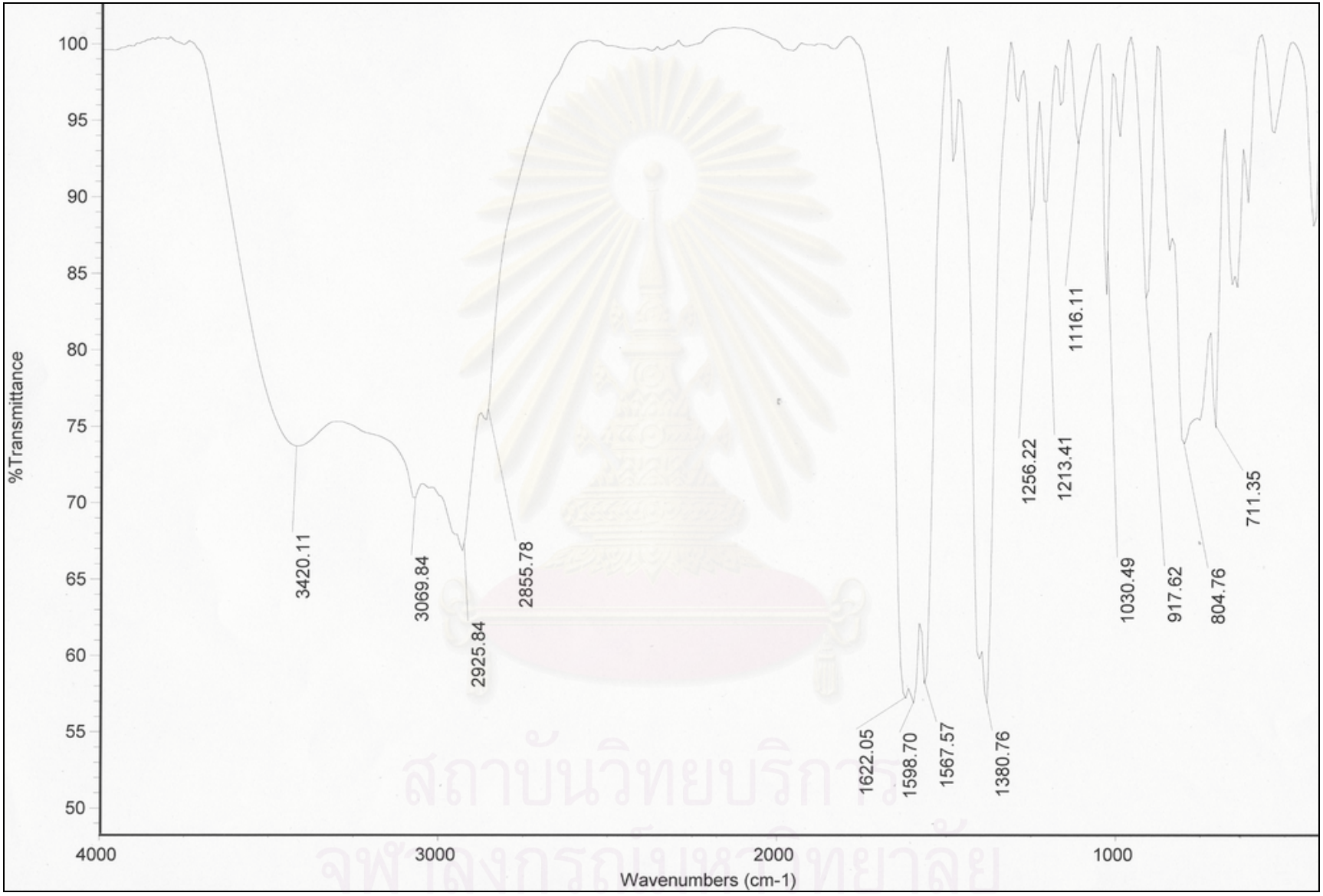

Figure 14 IR2 spectrum of compound BE1

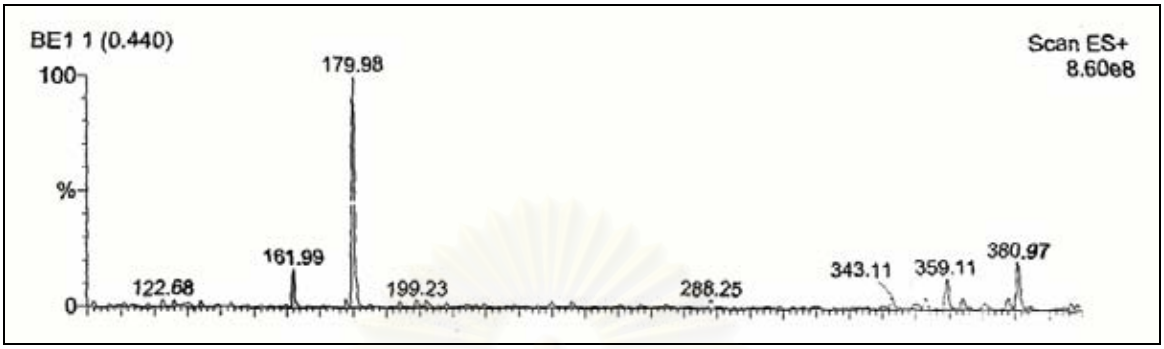

Figure 15 LC-MS of compound BE1

Appendix C

Table 1 Raw data (average) of mycelium dry weight, pH, and inhibition zone of endophytic fungus isolate PcBr20

Time (days)	Mycelium dry weight (g/l)	pH	Test microorganisms and inhibition zone (mm)					
			Gram positive bacteria		Gram negative bacteria		Yeast	
			B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 2592	*P. aeruginosa* ATCC 27853	*S. cerevisiae* TISTR 5169	*C. albicans* ATCC 10231
0	0.0163	5.36	-	-	-	-	-	-
1	2.5330	4.18	-	-	-	-	-	-
2	3.2280	4.03	34.00	25.00	22.00	-	17.50	-
4	3.7350	3.89	37.33	29.33	28.67	-	23.67	-
6	3.8430	3.96	37.00	28.00	30.33	-	25.33	-
8	3.7600	3.96	38.33	28.67	33.00	-	23.67	-
10	3.7740	3.69	37.00	29.67	32.33	-	25.00	-
12	3.4800	3.92	37.67	30.33	33.67	-	26.17	-
14	2.9580	5.14	37.33	29.67	34.33	-	25.00	-

- = Inactive

BIOGRAPHY

Mr. Bumrungsak Puriso was born on June 28, 1979 in Buriram province, Thailand. He graduated with a Bachelor Degree of Science in Biotechnology from the Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, Thailand in 2000. He has been studying for a Master Degree of Science in Industrial Microbiology, Faculty of Science, Chulalongkorn University, Thailand since 2001.

Presentation at Scientific Conference

Bumrungsak Puriso, Surachai Ponpakakul, Ek Sangvichien, Surang Thienhirun, Nattaya Ngamrojvanich, Jittra Kanchanaprayudh, Anthony J.S. Walley, and Prakitsin Sihanonth. **Antimicrobial compounds of endophytic fungi isolated from *Croton oblongifolius* in Prachuap Khiri Khan**. Abstract Book, The 15th Annual Meeting of the Thai Society for Biotechnology "Sustainable Development of SMEs Through Biotechnology and JSPS-NRCT Symposium" The Forefront of Bioinformatics Application. February 3-6, 2004. Chiang Mai, Thailand. p. 114.