ราเอนโดไฟต์ชนิดผลิตสารออกฤทธิ์ทางชีวภาพจากใบกระทุ่มนา

Mitragyna javanica KOORD & VAL

นางสาวธีรวัฒนา ภาระมาตย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

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BIOACTIVE COMPOUNDS-PRODUCING ENDOPHYTIC FUNGI FROM KRA THUM NA *Mitragyna javanica* KOORD & VAL. LEAVES

Miss Thirawatthana Pharamat

A Dissertation Submitted in Partial Fulfillment of the Requirements

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Thesis Title   BIOACTIVE COMPOUNDS-PRODUCING
ENDOPHYTIC FUNGI FROM KRA THUM NA
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ธีรวัฒนา ภาระมาตย์ : ราเอนโดไฟต์ชนิดผลิตสารออกฤทธิ์ทางชีวภาพจากใบกระทุ่ม
Mitragyna javanica KOORD & VAL. (BIOACTIVE COMPOUNDS-PRODUCING
ENDOPHYTIC FUNGI FROM Mitragyna javanica KOORD & VAL. LEAVES)
อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. ดร. ประกิตติ์สิน สีหนนทน์, 143 หน้า.

แยกราเอนโดไฟต์จากใบกระทุ่มนา Mitragyna javanica จากจังหวัดอยุธยา ปทุมธานี และ
อำนาจเจริญ ได้ราเอนโดไฟต์จำนวน 106 ไอโซเลต จำแนกชนิดราเอนโดไฟต์ได้ดังนี้ Colletotrichum
spp. Fusarium spp. Phomopsis spp. Phoma sp. Alternaria spp. Phyllostica spp. Xylaria spp.
Penicillium spp. Cladosporium sp.และราเอนโดไฟต์ชนิดไม่สร้างสปอร์ เมื่อนำสารสกัดจากน้ำหมัก
และเส้นใยของราเอนโดไฟต์ทั้ง 106 สายพันธุ์ทดสอบสารออกฤทธิ์ยับยั้งจุลินทรีย์ทดสอบและ
เซลล์มะเร็ง โดยทดสอบการการออกฤทธิ์ยับยั้งจุลินทรีย์ทดสอบมาตรฐาน 6 ชนิดได้แก่ Bacillus
subtilis ATCC633 Staphylococus aureus ATCC25923 Pseudomonas aeruginosa ATCC9027
Escherichia coli ATCC25922 Saccharomyces cerevisiae TISTR5169 และ Candida albicans
ATCC1023 โดยวิธี paper disc diffusion เทคนิค พบว่าราเอนโดไฟต์จำนวน 75.47% สามารถยับยั้ง
จุลินทรีย์ทดสอบได้อย่างน้อยหนึ่งชนิด 5.66 % สามารถออกฤทธิ์ยับยั้งจุลินทรีย์ทดสอบได้ทุกสายพันธุ์
12.15 % สามารถยับยั้งยีสต์ พบว่าราเอนโดไฟต์สายพันธุ์ PT11 มีฤทธิ์ยับยั้งจุลินทรีย์ทกสอบได้มาก
ที่สุด เมื่อทำการทดสอบการออกฤทธิ์ต้านเซลล์มะเร็ง 6 ชนิด ได้แก่ มะเร็งผิวหนัง (A375 ATCC no.
CRL-1619) มะเร็งกระเพาะอาหาร (KatoIII ATCC no. HTB-103) มะเร็งลำไส้ (SW620 ATCC no.
CCL-227) มะเร็งตับ (HepG2 ATCC no.HTB-8065) มะเร็งเต้านม (BT474 ATCC no. HTB-20) และ
มะเร็งเม็ดเลือดขาว (Jurkat ATCC no. CRL-2063) โดยวิธี MTT พบว่าราเอนโดนไฟต์สามารถยับยั้ง
มะเร็ง A375 Kato III SW620 BT474 HepG2 และ Jurkat 58.49% 60.38% 58.49% 45.28%
61.32% และ 31.13% ตามลำดับ โดยมีเปอร์เซนต์การอยู่รอดต่ำกว่า 40% ราเอนโดไฟต์สายพันธุ์
PT01มีความจำเพาะต่อเซลล์มะเร็งเม็ดเลือดขาว (Jurkat) ได้ 100% สามารถกระตุ้นการตายในมะเร็ง
เม็ดเลือดขาวแบบ apoptosis ถึง 90.04% เมื่อทำการจำแนกราเอนโดไฟต์ไอโซเลต PT11 และ PT01
โดยอาศัยลำดับนิวคลีโอไทด์ในบริเวณ ITS ของ rDNA พบว่ามีความสำคัญใกล้ชิดมากที่สุดกับ
Nodulisporium sp. และ Phoma herbarum เมื่อทำการแยกสารออกฤทธิ์ทางชีวภาพจากราเอนโดไฟต์
PT11 ได้สารบริสุทธิ์ 1 ที่มีกระตุ้นการตายของเซลล์มะเร็งเม็ดเลือดขาว 66.35±1.46% มีค่า IC_{50}
เท่ากับ 37.5±0.76 µg/ml.

สาขาวิชาเทคโนโลยีชีวภาพ	ลายมือชื่อนิสิต
ปีการศึกษา 2552	ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก

THIRAWATTHANA PHARAMAT: BIOACTIVE COMPOUNDS-PRODUCING ENDOPHYTIC FUNGI FROM KRA THUM NA *Mitragyna javanica* KOORD & VAL. LEAVES. THESIS ADVISOR: ASSOC. PROF. PRAKITSIN SIHANONTH,Ph.D., 143 pp.

One hundred and six endophytic fungal isolates were isolated from *Mitragyna javanica* leaves plants growing in Ayutthaya, Prathumthani and Amnatchareon provinces. These endophytic fungi were identified as *Colletotrichum* spp., *Fusarium* spp., *Phomopsis* spp., *Phoma* sp. *Alternaria* spp., *Phyllostica* spp. *Xylaria* spp., *Penicillium* spp., a *Cladosporium* sp. and the majority were mycelia sterilia. Crude extracts with ethyl acetate were examined for antimicrobial and anticancer activities. The antimicrobial activities were tested using paper disc diffusion assay technique against six reference microorganisms including *Bacillus subtilis* ATCC633, *Staphylococus aureus* ATCC25923, *Pseudomonas aeruginosa* ATCC9027, *Escherichia coli* ATCC25922, *Saccharomyces cerevisiae* TISTR5169 and *Candida albicans* ATCC1023. Seventy six percent showed antimicrobial activities against at least one tested microorganism with inhibition zones ranging from 7-40 mm.   Six percent displayed a broad antimicrobial spectrum and twenty two percent inhibited the pathogenic yeast *C. albicans*. Moreover, the endophytic fungus isolate PT11 exhibited the strongest antimicrobial activity against all test microorganisms. Anticancer activities were examined with six human cancer cell lines including A375 ATCC no. CRL-1619(malignant melanoma), KatoIII ATCC no. HTB-103(gastric cancer), SW620 ATCC no. CCL-227(colorectal cancer), HepG2 ATCC no.HTB-8065 (liver cancer), BT474 ATCC no. HTB-20 (breast cancer), and Jurkat ATCC no. CRL-2063 (T-cell leukemia)) by the MTT method. The crude extracts of the endophytic isolates exhibited 58.49%, 60.38%, 58.49%, 45.28%, 61.32% and 31.13% cytotoxicity (cell viability < 40%) against A375, Kato III, SW620, BT474, HepG2, and Jurkat, respectively. The endophytic isolate PT01 had the most specific activity against (0% cell viability) jurkat cells with potential induced 90.04% apoptosis cell death. The high potential endophytic fungus PT11 and PT01 were identified. Based on nucleotide sequencing of ITS region, they were closely related to 99% identity of *Nodulisporium* sp. and *Phoma herbarum*. The endophytic isolate PT11 showed strong antimicrobial and anticancer was chosen for further study of bioactive compounds. Isolation of culture both crude extract of the fungus isolate PT11 obtained pure compound 1 The compound 1 displayed apoptosis-inducing activities against the 66.35±1.46% Jurkat at with with IC_{50} 37.5±0.76 µg/ml.

Field of Study : _Biotechnology_   Student's Signature _Thirawatthana Pharamat_

Academic Year : _2009_   Advisor's Signature _Prakitsin Sihanonth_

ACKNOWLEGEMENTS

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LIST OF ABBREVIATIONS

ºC	degree Celsius
$CDCl_3$	deuterated chloroform
$CHCl_3$	Chloroform
cm	centimeter
cm^{-1}	reciprocated centimeter (unit of wave number)
^{13}C NMR	carbon-13 nuclear magnetic resource
COSY	Correlated Spectroscopy
d	doublet (NMR)
dd	doublet doublet (NMR)
ddd	doublet of doublet of doublet (NMR)
DEPT	Distortionless Enhancement by Polarization Transfer
EI	Electron impact
EtOAC	Ethyl acetate
h	hour
HMBC	Heteronuclear Multiple Bond Cerrelation
^{1}H NMR	Proton Nuclear Magnetic Resonace
Hz	Hertz
IR	Infrared
ITS	Internally transcribed Spacer
mm	millimeter

MEA	malt extract agar
MeOH	methanol
MHB	Mueller-Hinton broth
MHz	megahertz
mg	milligram
min	minute
ml	milliliter
MS	mass spectroscopy
m/z	mass to change ratio
NA	Nutrient agar
NB	Nutrient broth
nm	nanometer
NOESY	Nuclear Overhauser Enhancement Spectroscopy
PDA	Potato dextrose agar
ppm	part per million
PCR	poly chain reaction
SBA	Sabouraud agar
sp.	Species
spp.	Species
TLC	thin layer chromatography
TOCSY	Total Correlation Spectroscopy
UV	ultraviolet

YMA	Yeast malt extract agar
YMB	Yeast malt extract broth
μg	microgram
μl	microliter
	chemical shift

CHAPTER I

INTRODUCTION

In recent years, worldwide human health is being challenged by various bacteria, parasitic protozoans, fungi, the appearance of new life-threatening viruses and increasing diseases such as cancer, Aids, bird flu, swine flu, etc., accompanied with an increasing environment degradation and pollution. Moreover, global warming, which rising global temperature may result in the spread of, or returning of, diseases like malaria, dengue fever, plague fever, elephantiasis or diseases carried by insects, ticks and rodents. Therefore searching for new and effective bioactive compounds is needed and imported.

Natural products have traditionally provided a rich source of chemical diversity in the search for new biologically active molecules. Plants and microorganisms have been exploited as a source of antibiotics and pharmaceuticals. Endophytic fungi live internally, either intercellulary or intracellularly, and asymptomatically (without causing overt signs of tissue damage) (Saikonen et al., 1998) within plant tissues. They are ubiquitous and diverse (Saikkonen, 2007), which can be found in almost all plant species (Petrini, 1991; Huang et al., 2001) from the arctic to the tropics, and from agricultural fields to the most biologically diverse tropical forests (Arnold, 2007). Endophytes usually occur in above-ground plant tissues (within leaves, petioles, barks, and twigs), but also occasionally in roots, and are distinguished from mycorrhizae by lacking external hyphae or mantels (Saikonen et al, 1998). The symbiosis between plant and endophytic fungi has been ascertained: the plant protects and feeds the endophytic fungi, while endophytic fungi in turn produces bioactive substances (plant regulatory, antibacterial, antiviral, insecticidal substances) to enhance the growth and competitiveness of the host plant in nature (Caroll, 1998). Endophytic fungi can produce compounds similar or even identical to those produced by the host plant. Some of these microorganisms, isolated from the Pacific Yew tree (*Taxus brevifolia*), had shown ability to produce pactitaxel (Taxol[TM]), an anticancer diterpene formerly obtained from the host plant in artificial

culture medium (Stierle et al. 1995). Yin and company (2009) isolated endophytic fungi from *Gentiana macrophylla* a traditional Chinese medicinal plant

which produces the bioactive ingredient gentiopicrin. They found that two strains (QJ16 and QJ18) had a component with the same Rf value in TLC as that of authentic gentiopicrin and one ingredient of the QJ18 extract had a retention time identical with that of authentic gentiopicrin in HPLC. Li et al., (2005) also isolated endophytic fungi from 12 Chinese traditional medicinal plants, they found 9.2% of 130 isolates exhibited antitumor activity and 30% exhibitied antifungal activity and some of them exhibited broad-sprectum antifungal activity. Endophytic fungi, particularly from higher plants, had been an obviously rich and reliable source of bioactive and chemically novel compounds with huge medicinal and agricultural potential (Li et al, 2009; Tan & Zou, 2001; Strobel et al., 2004; Silva et al., 2006). They may have potential for therapeutic purposes and could be used as research tools (Tan and Zou, 2001).

Thailand is located in the tropical area and the Thai forests have a great biodiversity of plants (Wiyakrutta et al. 2004) and microorganisms (Tanticharean, 2003). It has been estimated that there may be over 1.5 million global fungal species. Thailand is estimated to have 7-10% (assume 100,000- 150,000 fungi) of the world total and about 1% has been described from Thailand too date (Tanticharean, 2003). Medicinal plants are reported to harbour endophytes (Strobel, 2002). **Thailand** is the home to a wide-range of herbal **plant** species. **Medicinal plants** and herbs have long been a part of everyday life in **Thailand**. Little has been reported about the endophytic fungi in many of Thai medicinal plants that means high opportunity to find new compounds and, for targeting natural products from interesting endophytic fungi from medicinal plants. *Mitragyna javanica* Koord & Val. (Rubiacea) is a traditional Thai medical plant, commonly known as "Krathum na". It is widespread in Thailand and could be a good source for endophytic fungi.

In these studies endophytic fungi were isolated from *M. javonica* leaves and screened for their abilities to produce bioactive compounds which have potential anticancer and antimicrobial activities. The *M. javanica* has been used as a Thai

medicinal plant for various pharmacological activities. Consequently, the Thai medicinal plant *M. javanica* may constitute another source of endophytic fungi with biological activity.

The objectives of these studies were

1. To isolate of endophytic fungi.
2. To screen of endophytic fungi for their antimicrobial and anticancer activities.
3. To identify of selected endophytic fungi.
4. To isolate and characterize metabolites from selected endophytic fungi.
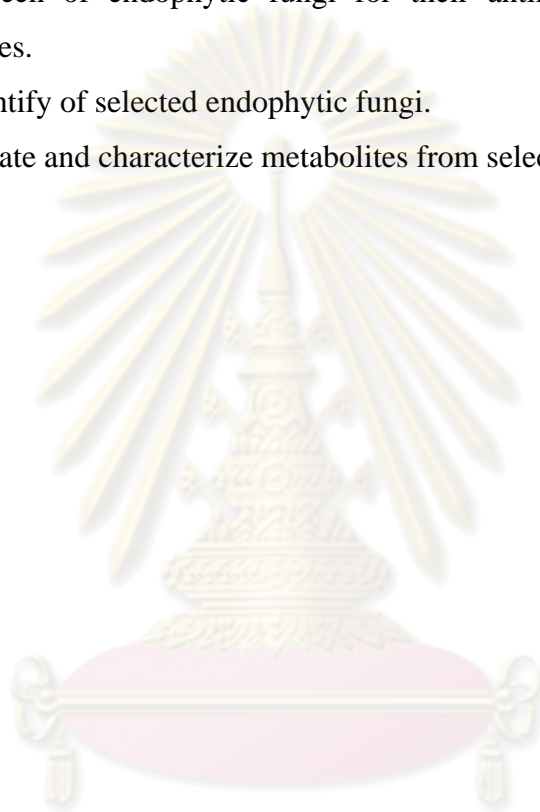
CHAPTER II

LITERATURES REVIEW

2.1 Endophtic fungi

Endophytes were recovered in Darnel, Germany, in 1904. One of the early publications on endophytic fungus was described by Freeman in 1904, and he referred to four other papers on endophytes that were published in 1898.   Freeman found the fungus in Persian darnel (*Lolium temulentum*) - an annual grass. In fact, grasses are probably the plants that have been most extensively studied as far as endophytes are concerned, and it was discovered that grasses with high endophyte content were often resistant to attack by certain insects. Darnel was apparently known to be toxic to some animals as far back as Roman times (Freeman, 1904). Since the recovery of endophytes in darnel in 1904, endophytes have been shown to be an outstanding as sources of bioactive natural products.

2.1.1 Definitions of Endophytic Fungi

Originally, the term "endophyte" was introduced by De Barry (1886), that means "in the plant" (endon Gr. = within, phyton = plant). The usage of this word is assigned to all those microorganisms that colonize internal plant tissues. However, the definition of an endophyte is now broadened by many researchers and can include any organisms that live in plant tissue whether neutral, beneficial or detrimental (Sakora et al. 2007; Backman and Sikora, 2008). Caroll (1986) defined them as asymptomatic microorganisms living inside plants, while Petrini (1991) defined them as microorganisms that inhabit plant tissues at some stage in their life cycle without causing any apparent harm to their host. Wilson (1995) gave an additional definition, considering endophytes to cause unapparent and asymptomatic infections to the plant host. Hallman et al. (1997) described them, as those that may be isolated from surface sterilized plants or extracted from inner tissues and cause no damage to the host plant. Strobel and Long (1998) defined them as microorganisms that live in the intercellular spaces of stems, petioles, roots and leaves of plants causing no discernible manifestation of their presence and have typically gone unnoticed. Saikonen et al. (1998) referred them to fungi that live for all, or at least a significant part of their life

cycle internally and asymptotically in plant parts. They also include a wide range of fungi from fungal plant pathogens and saprophytes that have extended latency periods before disease or external signs of infection appear. Some even speak of the 'true endophytes' meaning those whose colonization never results in visible disease symptoms (Mostert et al., 2000). While, Hartley and Gange (2009) described the "true endophytes" as those fungi that are vertically transmitted via seed from one plant generation to the next. These fungi may never appear outside of their hosts. In contrast, the vast majority of endophytes infect plants by airborne spores, termed horizontal transmission. Tan and Zou (2001) described them, as a bacterial (inclunding actinomycetes) or fungal microorganism, which spend the whole or part of its life cycle colonizing inter-and/or intra-cellular inside the healthy tissues of the host plant. Schulz and Boyle (2005) described those fungi that can be detected at a particular moment within the tissues of apparently healthy plant hosts. Later, this term was expanded as "fungi and bacteria including actinomycetes, which spend the whole or at least a part of their life cycle colonizing inter- or intracellular, inside the healthy living tissue of the host plant, typically causing no apparent symptoms of disease (Verma et al., 2007).
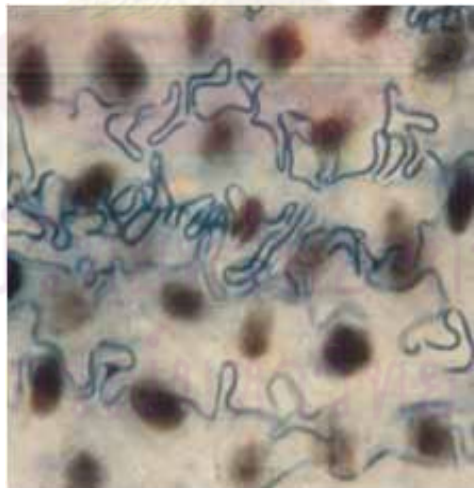
Figure 2-1: Endophyte fungus hyphae between the seed coat and the aleurone layer of a grass seed. (Aldrich-Markham et al., 2007)

2.1.2 The Plant and Endophyte relationship

All plant species are hosts to endophytic microorganisms. These include algae, mosses, ferns, orchids, lichens, grasses, and woody plants. Most endophytic

microorganisms are fungi. Most of these fungi belong to the Ascomycota and Deuteromycoter (Konig et al., 1999; Huang et al., 2001; Arnold and Lutzoni, 2007; Unterseher et al., 2007). Endophytes associated with plant tissues range from borderline pathogenic, to commensal, and to symbiotic (Strobel, 2003). Schulz et al. (1999) hypothesize that the host-endophyte interaction is a case of balanced antagonism: pathogens overcome the host's defenses to the extent that they cause visible damage, whereas endophytic virulence is only sufficient to be able to infect and colonize without causing visible damage. If the balance shifts, the endophyte may turn pathogen. Therefore, a mutualistic endophyte may become pathogenic and vice versa, depending on the environment such as soil, temperature and humidity etc., and the condition of the host plant. For this reason, the different types of symbiosis best describe an interaction at a particular point in time, rather than a species of microorganism (for most endophytes) (Bayman, 2007). In any of these situations, the plant is thought to provide nutrients to the endophytes, while the endophyte may produce compounds critical for the completion of its life cycle or essential for growth or self-defense (Strobel, 2003). The delicate equilibrium between host and endophyte seems to be controlled in part by chemical factors, for example, herbicidal natural products produced by fungi versus antifungal metabolites biosynthesized by the host plant (Huang et al., 2001).

2.1.3 Endophytic fungi transmission in host plant

Endophytes may be transmitted to host plants either vertically (directly from parent to offspring or seed born) or horizontally (from individual to unrelated individual) (Saikkonen, 2004). Vertically transmitted fungal endophytes are asexual or sexual spores and transmission of the systemically transmitted fungus passes from one generation to another through the seed. The fungus enters the seedling from the seed and spreads throughout the plant, entering new tissues as they arise. Since their reproductive fitness is intimately tied to that of their host plant, these fungi are often mutualistic. Conversely, horizontally transmitted fungal endophytes are sexual and transmit via spores that can be spread by wind, rain splashes, and/or insect vectors. Germinating spores invade the plant by cuticular penetration and entry through stomata or wounds (Saikkonen, 2007). Since they spread in a similar way to

pathogens, horizontally transmitted endophytes are often closely related to pathogenic fungi, though they are not pathogenic themselves.

2.1.4 Type of endophytic fungi

Two general groups of endophytic fungi are recognized, clavicipitaceous or grass endophyte are usually present in grasses, seed borne transmitted and non-clavicipitaceous or non-grass endophytes or woody-plant endophyte association (Petrini 1991). These consists of a diverse assemblage of organisms inhabit in woody plants (Schulz and Boyle, 2005; Sieber, 2007) that are based on host type. However, Rodriguez et al., (2009) suggested that non-grass endophytes represent three district functional classes based on host colonization patterns and mechanism of transmission between host generations (Table 2.1). All three classes have broad host ranges, Class 2 endophytes may grow in both above- and below-ground tissues, Whereas, Class 3 and 4 endophytes are restricted to above-ground tissues and roots, respectively. Class 3 endophytes form highly localized infections, while Class 2 and 4 endophytes are capable of extensive tissue colonization (Rodriguez et al., 2009).

Table2-1: Symbiotic criteria used to characterize fungal endophytic classes.

Criteria	Clavicipitaceae	Nonclavicitaceous		
	Class 1	Class 2	Class 3	Class 4
Host range	Narrow (Grasses)	Broad (Herbs, trees)	Broad (Herbs, trees)	Broad (Herbs, trees)
Tissue(s) colonized	Shoot and rhizome	Shoot, root and rhizome	Shoot	Root
In planta colonization	Extensive	Extensive	Limited	Extensive
In planta biodiversity	Low	Low	High	Unknown
Transmission	Vertical and horizontal	Vertical and horizontal	Horizontal	Horizontal
Fitness benefits*	NHA	NHA and HA	NHA	NHA

* Nonhabitat-adapted (NHA) benefits such as drought tolerance and growth enhancement. Habitat-adapted (HA) benefits result from habitat-specific selective pressures such as pH, temperature and salinity. (Rodriguez et al., 2009)

2.1.4.1 Grass endophytes

Grass endophytes may constitute a monophyletic clade with the fungal family clavicipitaceae. They belong to the Ascomycota, tribe Balansiae, which infect at least 80 genera and 300 species (Saikkonen et al, 1998), including genera *Atkinsonella*, *Balansia*, *Balansiopsis*, *Echinodothis*, *Epichloë*, *Myriogenospora*, *Neotyphodium* (the asexual form of Epichloë), and *Parepichloe¨* (Clay, 1988 and 1990; Clay and Schardl, 2002). Saikonen et al. (1998) described endophytic fungal *Neotyphodium* and its sexual or teleomorphic stage *Epichloë*, which are found almost exclusively in the grasses of the Pooidae. Most studies of endophyte-host grass interactions have involved *Neotyphodium* and *Epichloë*. Species of *Neotyphodium* and *Epichloë* tend to be host specific and often form genetically distinct races among populations of the same host species. Recent molecular evidence suggests that *Epichloë typhina*, considered ancestral to asexual clavicipitaceous endophytes, was a complex of diverse biological species, each with a high degree of host specificity.

Grass endophytes colonize their hosts systemically (except the roots). Several species are transmitted vertically by seeds to the next host generation and some species can be transmitted via asexual spores. Saikkonen (2004) stated that *Neotyphodium* endophytes are assumed to be strictly vertically transmitted. By contrast, *Epichloë* endophytes can also be transmitted either clonally or vertically (by growing into seeds) or sexually and horizontally (via spores) when the fungus forms external stromata with conidia (Saikkonen, 1998 and 2004).
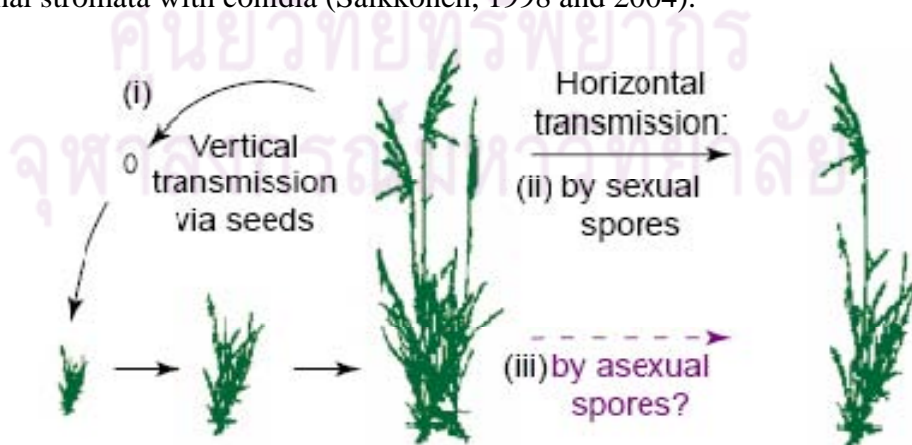
Figure 2-2: Life cycles of grass endophytes.
(Saikkonen et al., 2004)

Seed transmitted endophytes in families other than the Clavicipitaceae have been encountered in grasses. One group (p-endophytes; "P" for Phialophora) consists of closely related *Gliocladium*-like endophytes in perennial ryegrass and *Phialophora*-like endophytes in meadow fescue, and Arizona fescue (Malinowski and Belesky, 2000). The p-endophytes belong to the Eurotiales (Ascomycetes) (Siegel et al., 1995).

The vertically transmitted endophytes of some grasses can benefit their host via increased drought tolerance, protection against herbivory and pathogens (Clay and Schardl, 2002; Higgins et al., 2006). Rodriguez (2009) reported that most Clavicipitaceous endophytes enhance resistance of their host to insect feeding.

2.1.4.2 Non-Grass endophytes

In contrast to the grass endophytes, the non-grass endophytes are non-systemic and diverse both phylogenetically (polyphelectic clade) and with respect to life-history strategy (Schulze and Boyle, 2005; Rodriguez et al. 2009). They are harboured within all types of plants including woody gymnosperms and angiosperms (Saikkonen, 2007). Non-clavicipitaceous endophytes represent a broad range of species from several families of Ascomycota, Basidiomycota, Deuteromycota and a few Zygomycota (Petrini, 1991, Higgins et al., 2006; Sieber et al. 1988, Sieber, 2007). However, some endophytes genera such as *Phyllosticta*, *Phomopsis*, *Colletotrichum*, *Fusarium* and *Xylaria* occur in a wide variety of distantly related host plants (Suryanarayanan et al, 2003). Many non-systemic endophytes in woody plants are transmitted horizontally via asexual spores. In woody plants, newly flushed leaves are generally endophyte-free but soon become inhabited with fungal spores which are dispersed by air, rain splashes and animal vectors, from senescent and abscised previous season's leaves (Wilson and Carroll, 1994; Saikkonen, 2007; Herre et al., 2007) (Figure 2-3). However, endophytes of woody plants have been found in seeds and acorns. Therefore, endophytes of woody plants may also be transmitted vertically and maternally via seeds (Saikkonen et al., 1998).
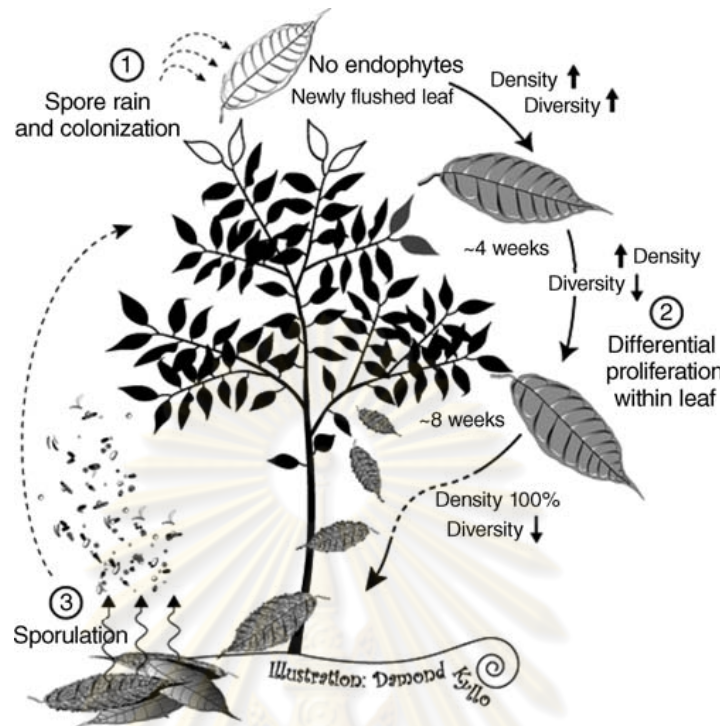
Figure 2-3: Proposed life cycle for tropical foliar endophytic fungi and their host plants. (Herre et al., 2007)

Saikkonen (2007) suggested that the role of endophytic fungi associated with woody perennials can be complex and labile both in ecological and evolutionary time. The size, complex architecture, and long age of sexual maturity of woody plants probably constrain the window for systemic growth, vertical transmission and the length of latency period of foliar fungi (Saikkonen et al., 2004). The fungus-plant interaction is along the antagonism-mutualism continuum. Saikkonen (2007) reasoned that endophytic fungi were unable to grow systemically through highly differentiated and hierarchically organized woody tissues to all above-ground parts of the tree, and that long age of maturity decreases opportunities for vertical transmission of the fungus *via* host seeds.

2.1.5 Diversity of Endophytes

The species diversity of endophyte communities in plant can be highly dependent on the number of plant taxa, the number of samples taken per plant, the choice of investigated plant tissue and artificial growth media and timing of the sampling activities (Unterseher et al., 2007). Some tree species host more than 100

species in one tissue type, but communities are usually dominated by few host specific species. Some studies show that endophyte communities are usually specific at the host species or family levels (Petrini 1986; Guo, et al., 2008), while endophytes are known to colonize multiple species of plants. Hartley and Gange (2008) reported that within each individual plant fungal diversity may be high, but it seems to vary from one location to another, ranging from a local scale of a few kilometers to an intercontinental scale. Diversity seems especially high in the tropics, and Arnold & Lutzoni (2007) had even suggested that fungi within tropical tree leaves may be hyperdiverse. Similar, Higgins et al. (2007) reported that fungi diversity is typically thought to increase with decreasing latitude associate with many other terrestrial organisms. Arnold et al. (2001) identified distinct host-related communities in tropical tree leaves based on endophyte quantitative analysis. Although few endophytic fungi were found to be entirely restricted to a particular plant species, significant differences were found in the frequency of infection of individual morphotaxa (Cannon and Simmons 2002). Endophytic fungi with some host and (or) tissue recurrence or specificity would have an important ecological significance in forest ecosystems (Guo et al, 2008).

The assemblages and colonization rates of endophytes are different in difference tissues, for example, bark, twigs, and leaves (Sun et al., 2008; Kumar and Hyde, 2004) and colonization frequency of the endophytes increased with the age of plant tissues (Taylor et al., 1999, Guo et al., 2008). For example, Suryanarayanan and Thennarasan (2004) showed the pattern of endophyte infection in the leaves of *Plumeria rubra*, a tropical deciduous tree. Older leaves were more densely colonized than the younger leaves. The senescent leaves had more endophytes in them than mature or young leaves. This increased colonization of old leaves was due to superinfection of the leaves over time by air-borne inoculum (Carroll et al., 1977; Suryanarayanan and Vijaykrishna, 2001; Suryanarayana and Thennarasan, 2004). Similar, Kumaresan and Suryanarayanan (2002) reported that the endophyte assemblage of senescent leaves of *R*hizophora *apiculata* was more diverse than that of the young leaves indicating that the susceptibility to endophytes fungi increases with leaf age. Sun et al., (2008) studied endophytes associated with medicinal plants and found that the colonization and isolation rate of endophytic fungi were higher in

twigs than in leaves. The colonization and isolation rate of endophytic fungi in twig increased with the age. They suggested that the composition and distribution of the endophytes are conspicuously affected by the types of plant tissues and plant species. Shankar Naik et al., (2008) investigated the colonization of endophytes in 15 medicinal shrubs during winter, monsoon and summer seasons and found that the greater number of isolates in winter and monsoon seasons than in summer season suggests that colonization by endophytes is correlated with climatic factors. Similarly, Saikkonen et al. (1998) proposed that colonization by sporulating tree and grass endophytes results in a seasonal accumulation of local, and perhaps independent, endophytes colonies. Therefore, this accumulation depends on the number of spore sources, seasonal and year climatic factor (humidity and rainfall). It may be summarized that the diversity of endophytic fungi in plants is effected by several factors, such as, type of plant tissue and species, size, number and age of samples, methodology, growth medium, geographical distribution and seasonal change.

2.1.6 The Effect of Endophytes Colonization on Plant Survival

Endophytes colonizing internal plant tissues usually obtained nutrition and protection from the host plant (Tan and Zou, 2001), and in response they produce some functional metabolites, which enhance the host fitness, anti-feedant activities, resistance against stresses as well as quality of the products. They indicated that endophytes play an important role in ecological communities. Endophyte-inflected grasses have been shown to have improved growth, reproduction and resistance/tolerance against different abiotic and biotic stress compared to endophyte free (E-). Thus, E+ plants are thought to be competitively superior compared to E- plants in grass populations and grassland communities. Hartley and Gange (2009) reported that endophytes also increase the biomass and competitive of their hosts. This effect was at least in part due to the endophytic production of phytohormones and other growth-promoting substances by the fungi (Petrini 1991, Schulze et al., 2002, Schulze and Boyle, 2005). Many plants infected by endophytes are also found to enhance uptake of phosphorus, another important element for plant growth (Guo et al., 2006) In addition, some endophytes can produce the plant hormone indole-3-acetic acid (IAA) which was isolated from cultures of *Acremonium coenophialum*,

Aureobasidium pullulans and *Epicoccum purpurascens*. (Tan and Zou, 2001). Endophytic *Fusarium* spp. from *Euphorbia. pekinensis* could effectively promote the growth of the host plantlets and suspending cells by producing IAA and gibberellin (GA) (Dai et al., 2008). Endophytes also improve the ecological adaptability of hosts by enhancing their tolerance to environmental stresses, and by producing antimicrobial metabolites resistance to phytopathogens and/or herbivores including some insects feeding on the host plant (Schulze et al. 2002, Liu et al. 2001, Schulze and Boyle, 2005). Grass endophytes enhance host fitness by the production of both alkaloids, that inhibited insect herbivory, and metabolites that stimulated plant growth. Saikkonen et al. (1998) and Caroll (1998) proposed that endophytes in leaves and stems of woody plants provide a defensive role for the host plant because they produced a wide array of mycotoxins and enzymes that can inhibit growth of microorganisms and invertebrate herbivores. Because non-grass endophytes are diverse and have shorter life cycles than their parential host plants (Saikkonen et al., 1998), Herre, et al. (2007) suggested that endophytic fungi can play a potentially important mutualistic role by augmenting host defensive responses against pathogens. Thus endophytes induce or increase the expression of intrinsic host defense mechanisms and providing additional sources of defense, extrinsic to those of the host (e.g., endophyte based chemical antibiosis). Furthermore, some endophytes were able to provide the host plant with protection against some nematodes, mammal and insect herbivores as well as bacterial and fungal pathogens. Some endophytes were capable of enhancing the hosts' allelopathic effects on other species co-growing nearby, usually being competitors for the nutrition and the space. This could be the reason why some plants with special endophytes were usually competitive enough to become dominant species in successional fields (Tan and Zou, 2001). Endophytes could also negatively affect their host plants, although they never caused apparent disease symptoms. A former beneficial interaction can be inverted if plants are exposed to stressful environments, for example, low water or nutrients supply (Unterseher, 2007).

Orchids were among the many plants that host endophytic fungi and mycorrhizal fungi. Orchid seeds are very small and may lack sufficient nutrients to sustain orchid embryo development. The endophytic fungus grows out of the seeds

after dispersal and enzymatically degrades the bark and other substrates to supply nutrients for the developing orchid embryo.

2.1.7 Endophytic fungi Isolation

Techniques for endophytes isolation and culture have been developed gradually over time. Following Schulze and Boyle (2005), there are three methods presently in use for detecting and identifying fungi in plant tissue: (1) histological observation; (2) surface sterilization of the host tissue and isolation of the emerging fungi onto appropriate growth media; and (3) detection by specific chemistry e.g. immunological methods or direct amplification of fungal DNA from colonized plant tissues. Results may depend on choice of technique; each has advantages and limitations, and each may bias results in favour of certain taxa.

The definitive way to detect endophytes and determine infection frequency is by direct microscopic observation. Light microscopy is also useful for screening proposes (Bayman, 2007) and SEM and TEM to visualize fungal structures within the plant tissue (Schulze and Boyle, 2005).   Tissues for light microscopy may be observed directly, preferably following vital staining to ascertain that the fungus is living or after fixation, clearing and staining. This method is best to distinguish the extent to which a fungus colonizes the host. However, histological observation has a disadvantage: identification of the fungi is often difficult because hyphae of many different taxa look alike. Other alternatives are direct identification by following germination as well as indirect identification by detection with taxon-specific probes or antibodies to allow to identification of certain target organisms (Bayman, 2007). Johnston et al. (2006) have visualized endophytic fungi within leaves by using a fluorescent labeling method to detect $(1/3)$-β-D-glucans in fungal cell wall through the use of a monoclonal antibody and observed them in thin sections under a compound microscope.

Isolation of endophytic fungi by surface sterilization of plant samples and incubation on culture media is the most common initial step in the studied of endophytes. In first stage the surface is sterilized in surfactant such as ethanol and/or tween 80, followed by a sterilizing agent, such as sodium hypochlorite (Schulze and Boyle, 2005) or hydrogen peroxide (Guo et al, 2005), and then with serial washing

in sterile water. The plant sample is then incubated on general perpose media, normally potato dextrose agar (PDA), Malt extract agar (MEA), Oat meal agar (OMA) and Water agar (WA). The fungal outgrowth from plant tissue is subcultured on to fresh medium for identification based on morphological examination by scrutinizing the fungal culture. These methods are simple, inexpensive, and allow detection of a wide range of fungi (Bayman, 2007). The procedure employed for surface sterilization has to be optimized for the host with regard to tissue sensitivity, age, and the organ being sterilized. However, the effectiveness of surface sterilization by checking for growth of epiphytes from surface sterilized samples is important. It is also important to check that the surface sterilization method has not damaged to endophytes by overly stringent sterilization procedures.

Fungal taxonomy is traditionally based on comparative morphological features. Isolates can be identified if they sporulate on culture media and fungal isolates that fail to sporulate, are categorized as mycelia sterilia (Huang et al., 2009) or morphotypes (Gou, 2003). Various techniques had been developed to promote endophyte sporulation, e.g. by growing them on different modification of artificial MEA, PDA, corn meal agar (CMA), potato carrot agar (PCA) and WA, as well as the inclusion of host tissue in plate cultures (Huang, et al., 2009) and under various incubation conditions (Guo, at al., 2003). However, numerous fungi still do not sporulate in culture. Molecular methods have been used for fungal taxonomy and identification of fungi that do not sporulate in culture medium and also for the classification of endophytic fungi within plant tissues. Furthermore, molecular techniques exhibit high sensitivity and specificity for identifying microorganisms and can be used for classifying microbial strains at diverse hierarchical taxonomic levels (Sette *et al.*, 2006, Huang et al., 2009). PCR assays have been used to detect and identify endophytic fungi. A variety of techniques are available, including DNA sequencing, RFLPs and variations DGGE and RAPDS. Most endophytic fungi were detected and identified by using specific PCR primer to amplify ribosomal DNA fragments, especially the internal transcribed spacer regions (ITS) of fungi (including ITS1, 5.8S, and ITS2) and comparative analyses of the rDNA sequences. The PCR amplification and subsequent restriction analysis of the ribosomal region spanning the ITS and the 5.8S rRNA was accepted as a powerful method for species identification

of fungi. Therefore in endophytic fungi, ITS1 and ITS2 were studied not only with regard to phylogenetics and taxonomy but also to detect endophytic fungi in host trees. For instance, Guo et al., (2005) monitored microbial communities inhabiting the stems of *Heterosmilax japonica* Kunth by light microscopy and cultivation, independent approaches, such as RFLP analysis and sequencing of rDNA ITS. Molecular phylogenetic analysis showed that a broad spectrum of fungi, including *Mycosphaerella*, *Phomopsis*, *Aureobasidium*, *Cladosporium*, *Glomerella*, *Botryosphaeria*, *Guignardia*, were able to colonize the plants internally. Schulze et al. (2005) concluded that in order to detect all the fungi associated with a host plant, it is strategic to: (1) always optimize surface sterilization ; and (2) not only use conventional methods of isolation onto culture media, but also to employ molecular methods. Another sequence that is sometimes useful in identification of fungal species is the 18S and D1/D2 region at the 5' terminal end of 28 rDNA (Unterseher et al., 2007; Okene et al., 2008). For example, Okane et al. (2008) studied the diversity and taxonomy of endophytes and the relationships between those endophytes and saprobic Xylariaceae in Thailand and found that of 224 newly isolated endophytic strains, 218 were confirmed as Xylariaceae by the sequence analyses and various groups of Xylariaceae in the phylogenetic tree based on 28S rDNA D1/D2 region sequences. There was less diversity in this sequence across species, especially among families, but in conjunction with ITS sequence data, 18S rDNA and 28S rDNA data could be helpful.

2.1.8 Importance of Endophytic fungi

Endophytes are chemical synthesizers inside plants. Many of them are capable of synthesizing bioactive compounds that can be used by plants for defense against pathogens and some of these compounds had been proved useful for novel drug discovery (Guo et al, 2008). Strobel & Daisy (2003) reported that the plants growing in unique environmental settings, having ethnobotanical uses, having extreme age or interesting endemic locations generally produce novel endophytic microorganisms, of which the secondary metabolites are usually unique and may ultimately be shown to have applicability in medicine.

2.2 Endophytic fungi and Bioactive compounds

Endophytes can produced certain phytochemicals originally characteristic of the host. This may be explained by horizontal gene transfer between the endophyte and the host and could occur during the evolutionary process. Studies carried out by Stierle et al., (1993) showed that the fungus *Taxomyces andreanae,* found as an endophytic fungus of *Taxus brevifolia* is able to produce a complex dipterpen, called "taxol", used as an anti-cancer. Futhermore, another endophytic fungal *Pestalotiopsis microspora* isolated from *T. wallachiana* also produced taxol (Strobel et al., 1996). Taxol was normally obtained from the host plant, but these findings showed that microorganisms could also produce this drug, possibly providing a new and more efficient method instead of using rare plants, these preserving the world's diminishing diversity. Some pharmaceutical substances produced by endophytic microorganisms having commercial value and are produced in low amounts by the host plant. Produced by endophytes, the metabolite pathway can be better studied and key genes involved with the synthesis of the compound may be found.

2.2.1 Secondary metabolites from Endophytic fungi

Several of bioactive compounds obtained from endophytic fungi include alkaloids, terpenoids, terpenoids, flavonoids, steroids, Xanthones, phenols, isocoumarines, pyrylene derivatives, quinines, furandiones, etc (Schulze et al., 2002, Schulze and Boyle, 2005). Up to now, most substances from endophytes exhibit a wide variety of biological activities as antibiotics, eg as antitumor, antiviral compounds, anti-inflammatory, antioxidant, antidiabetic, and immunomodulatory, biological control agents, and other bioactive compounds based on their different functional roles.

2.2.2 Antibiotics from Endophytic fingi

Antibiotics, defined as low-molecular-weight organic natural products produced by microorganisms that are active at low concentration against other microorganisms, were the most bioactive natural products isolated from endophytes (Guo et al., 2008). Secondary metabolites from endophytic fungi have been observed to inhibit or kill a variety of harmful disease-causing agents including, but not limited

to phytopathogens, as well as bacteria, fungi, viruses and protozoans that effect humans and animals. (Strobel and Daisy, 2003) Described below are some examples of antibiotic compounds from endophytic fungi.

Ambuic acid (Figure 2-4), an antifungal agent which has been recently described from several isolates of *Pestalotiopsis microspora,* found in many of the world's rainforest (Li et al., 2001)
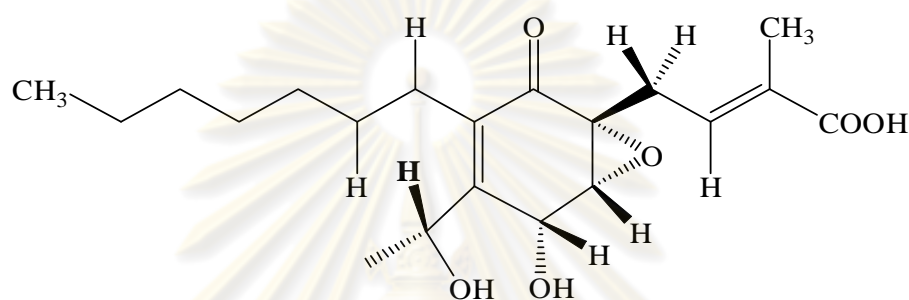
Figure 2-4: Chemical structure of ambuic acid, an antifungal agent produce by endophytic fungal *Pestalotiopsis microspora*

Cryptosporiopsis cf. *quercina* is the imperfect stage of *Pezicula cinnamomea*, a fungus commonly associated with hardwood species in Europe. It was isolated as an endophyte from *Tripterigeum wilfordii*, a medicinal plant native to Eurasia. A compound from *C. quercina* showed excellent antifungal activity against some important human pathogens, including *Candida albicans* and *Trichophyton* spp. A unique peptide antimycotic, termed "cryptocandin," was isolated and characterized. This compound contained a number of peculiar hydoxylated amino acids and a novel amino acid, 3-hydroxy-4-hydroxy methyl proline (Figure 2-5). The bioactive compound was related to known antimycotics-the echinocandins and pneumocandins. Cryptocandin was also active against a number of plant pathogenic fungi, including *Sclerotinia sclerotiorum* and *Botrytis cinerea*. Cryptocandin and its related compounds are currently being considered for use against a number of fungi causing diseases of the skin and nails.
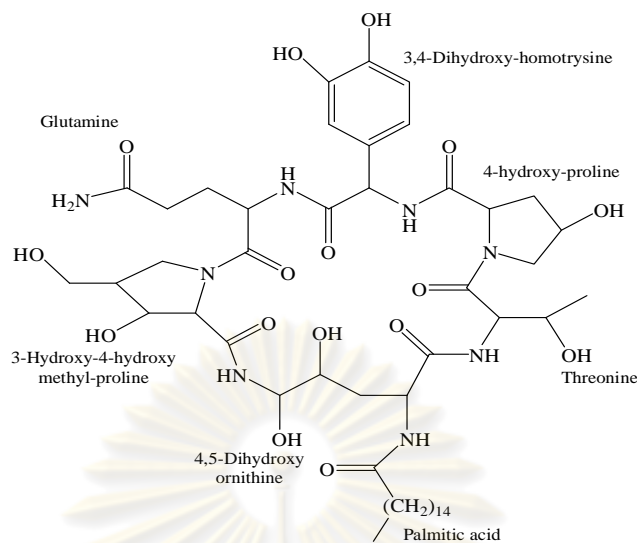
Figure 2-5: Chemical structure of cryptocandin A, an antifungal lipopeptide obtained from the endophytic fungus Cryptosporiopsis *quercina*

Cryptocine (Figure 2-6), a unique tetramic acid, was also produced by *C. quercina* (Figure 2.6). This compound possesses potential activity against *Pyricularia oryzae* as well as a number of plant-pathogenic fungi. The compound was generally ineffective against a general array of human-pathogenic fungi. Nevertheless, with MICs of this compound for *P. oryzae* being 0.39 μg/ml, this compound is being examined as a natural chemical control agent for rice blast and is being use as a base model to synthesize other antifungal compounds.

2-hydroxy-6-methylbenzoic acid was isolated from endophytic *Phoma* sp. and shown to be antibacterial. Also in 2004, two new fusicoccane diterpenes, namely pcriconicin A and B with antibacterial activities were isolated from *Periconia* sp., an endophytic fungus of *Taxus cuspidate* (Kim et al., 2004).
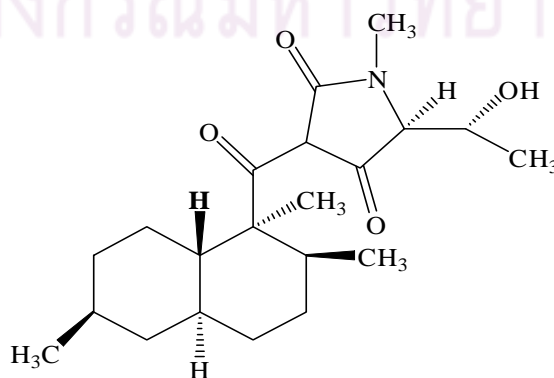
Figure 2-6: Chemical structure of cryptocine

Brefeldin A (Figure 2.7), a unique fungal metabolite of a 13-membered lactone ring, exhibited various biological activities, including antifungal, antitumor, antiviral, antimitotic and cytostatic activities (Wang et al, 2006). It has been isolated from a number of endophytic fungal species belonging to genera *Alternaria*, *Ascochyta*, *Pennicillium*, *Curvularia*, *Cerecospora*, *Phyllostica*, *Phoma Medicaginis* and *Cladosporium* (Wang et al, 2002; Weber et al, 2004; Wang et al, 2006)
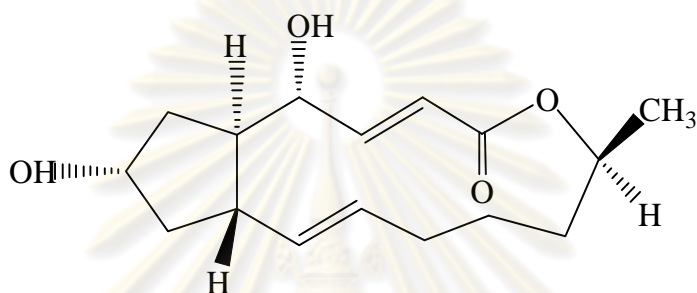
Figure 2-7: Chemical structure of brefeldin A, an bioactive compound was produced by several endophytic fungi

Rhizoctonic acid (Figure 2-8) is a bezophenone derivative. The compound obtained from *Rhizoctonia* sp. Cy064, an endophytic fungus in *Cynodon dactylon*. The Rhizoctonic acid had antibacterial activity against *Helicobacter pylori* with MIC of 25 mg/ml (Ma et al, 2004).Wang et al. (2008) reported that Rhizoctonic acid, asperfumoid, physcoin and 3,5-dichloro-ρ-anisic acid are produced by a *Penicillium* sp. an endophytic fungus residing in *Hopea hainanensi*s (Dipterocarpaceae), which exhibited the growth of *C. albicans* with MICs of 40.0, 20.0, 50,0 and 15.0 μg/ml, respectively. The 3, 5-dichloro-ρ-anisic acid was also showed growth inhibition against *A. niger* with MICs of 40.0 μg/ml. Moreover, Rhizoctonic acid, monomethylsulochrin, and 3, 5-dichloro-ρ-anisic acid all showed cytotoxic activity against KB cell line and HepG2 cell line.
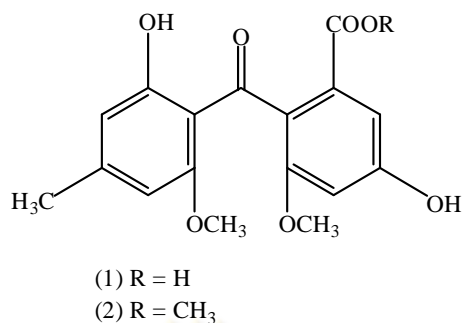
Figure 2-8: Chemical structure of (1) rhizoctonic acid (2) monomethylsulcochrin, an anti-*Helicobacter pylori* obtained from *Rhizoctonia* sp. Cy064, an endophytic fungus in *Cynodon dactylon*

Javanicin, an antibacterial naphthaquinone produced from an endophytic fungus of Neem, *Chloridium* sp. under liquid and solid media culture conditions (Figure 2-9). This highly functionalized naphthaquinone exhibited strong antibacterial activity against *Pseudomonas* spp., representing pathogens to both humans and plants (Kharwar et al, 2008).
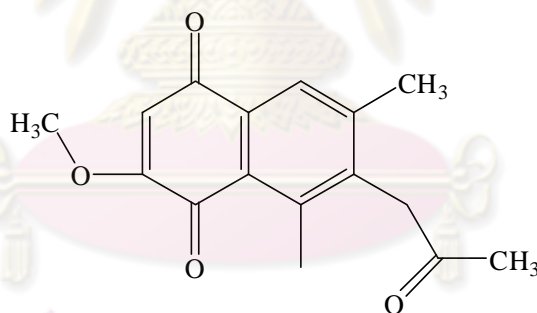
Figure 2-9: Chemical structure of javanicin, an antibacterial napthaquinone

Phomodione was isolated from culture broth of a *Phoma* species, discovered as an endophyte on a Guinea plant (*Saurauia scaberrinae*) (Figure 2-10). Phomodione exhibited a MIC of 1.6 μg/ml against *Staphylococcus aureus* using the disk diffusion assay, and was active against a representative oomycete, ascomycete and basidiomycete at between 3 and 8 μg/ml (Hoffmann et al, 2007).
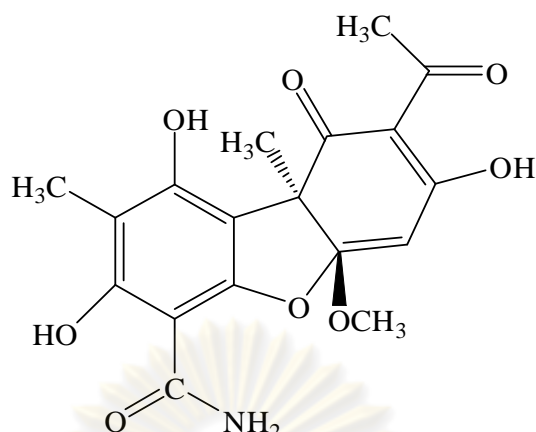
Figure 2-10: Chemical structure of phomodione, a bioactive compounds produced by an endophytic fungal *Phoma*

2.2.3 Anticancer Agents from Endophytic Fungi

The intensive chemical study of endophytic fungi began after the invention of taxol (paclitaxel), a billion dollar anticancer drug which was isolated for the first time from Facific Yew (*Taxus brevifola*) (Wani et al., 1971). More than 20 year later, Strobel isolated a novel taxol producing endophytic fungus *Taxomyces andrenae* from *T. brevifola* (Strobel, 2003). Paclitaxel is a complex dipterpere (Figure 2-11). The mode of action of paclitaxel was to preclude tubulin molecules from depolymerizing during the processes of cell division. Taxol is used to treat several kinds of tumors including ovarian, breast, and lung tumors and some head and neck carcinomas (Chen *et al*. 2008).
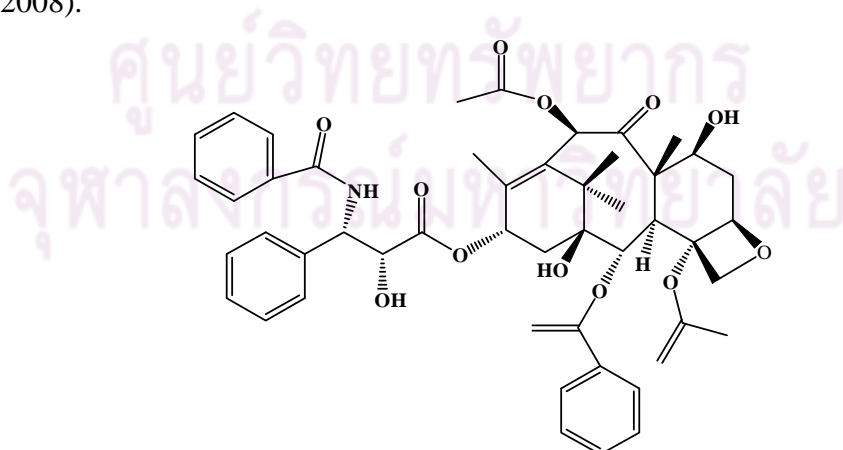
Figure 2-11: Chemical structure of paclitaxel, anticancer drug, was produced by many endophytic fungi. It too possesses outstanding antioomycete activity.

It had been reported that endophytic fungi *Pestalotia*, *Monochaetia*, *Alternaria*, *Pithomyces*, *Periconia* (Strobel, 2003), *Pestalotiopsis microspora* (Strobel et al., 1996), *P. pauciseta* (Gangadevi and Muthumary, 2008), *Turbercularis* sp. (TF-5) (Wang et al. 2000), *Fusarium solani* (Deng et al., 2001) and *Bartalania robillardoides* (Gangadevi and Muthumary, 2008) *Botryodiplodia theobromae* (Venkatachalam et al, 2008) have the ability to produce Taxol other than *Taxus* sp..

Neoplaether (Figure 2-12) was isolated from the culture of *Neoplacomema napellum* IFB-E016, an endophytic fungus isolated from healthy leaves of *Hopea hainanesis*. This compound exhibited significant cytotoxic activity against the human nasopharyngeal epidermoid tumor KB cell lines, with an IC_{50} value of 130 μg/ml. In addition, it showed antifungal activity against *Candida albicans*, with a minimal inhibitory concentration value of 6.2 μg/ml (Wang et al., 2006). Neoplaether had also been reported as secondary metabolites of fungi belonging to the genera *Pestalopsis*, *Xylaria*, *Phoma* and *Oospora*.
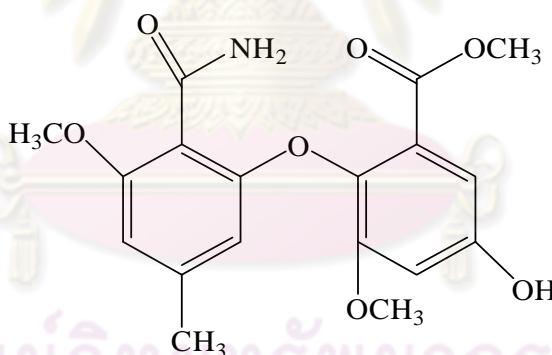
Figure 2-12: Chemical structure of Neoplaether

Trichodermamide C1 isolated from culture broth of the endophytic fungus *Eupenicillium* sp. and modified to a new dipeptide trichodermamide C (Figure 2-13). The trichodermamide C1 was shown by high content screening to display cytotoxicity towards the human colorectal carcinoma HCT116 and human lung carcinoma A549 with IC50 values of 0.68 and 4.28 μg/ml, respectively (Davis et al., 2008).
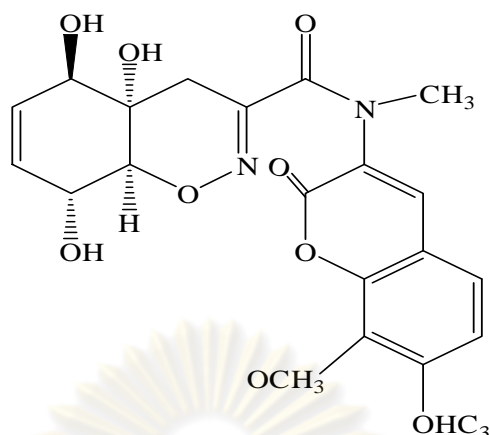
Figure 2-13: Chemical structure of trichodermamide C1

Wang et al. (2008) also reported that monomethylsulochrin, rhizoctonic acid, asperfumoid and 3,5-dichloro-ρ-anisic acid (Figure 2-14) were metabolites from a *Penicillium* sp. an endophytic fungus of *Hopea hainanensis,* showed cytotoxic activity against KB cell line with IC_{50} value of 30.0, 20.0, 20,0 and 5.0 μg/ml, respectively, and against HepG2 cell line with IC_{50} value of 30.0, 20.0, 15,0 and 10.0 μg/ml, respectively.

Asperfumoid   3,5-dichloro-ρ-anisic acid
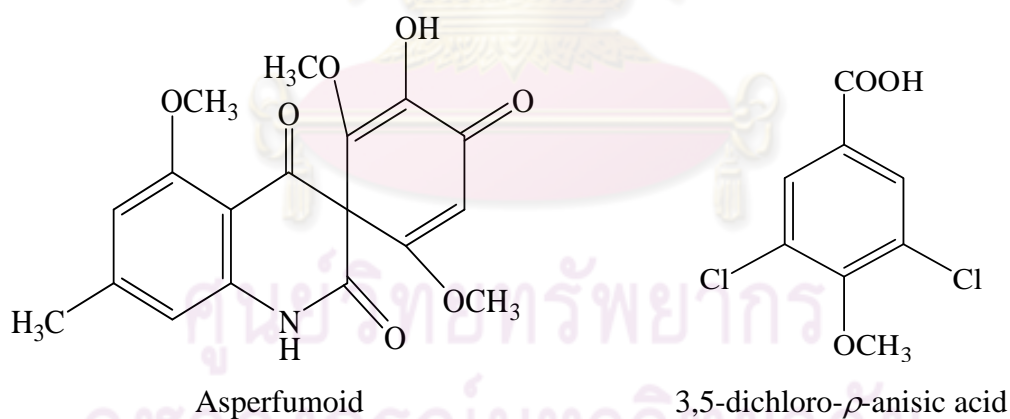
Figure 2-14: Asperfumoid and 3,5-dichloro-ρ-anisic acid are metabolites from *Penicillium* sp. an endophytic fungus of *Hopea. hainanensis*.

Camptothecin is a pentacyclic quinoline alkaloid (Figure 2-15), which showed excellent anticancer activity with a unique mechanism of action involving interference with eukaryotic DNA replication and transcription (Rehman et al, 2008). Campthothecin is known as a naturally occurring DNA topoisomerase I inhibitor (topomerases are the enzymes that wind and unwind the DNA that make up the

chromosome) (Potmersil et al, 1995). Camptothecin produced by *Entrophospora infrequens* (Puri, et al., 2005) and *Neurospora* sp. (Rehman et al, 2008) endophytic fungi of *Nothapodytes foetida*.
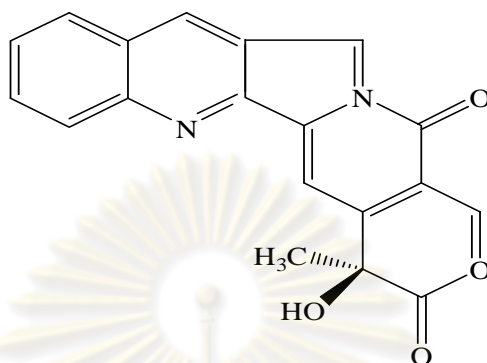
Figure 2-15: Camptothecin, an anticancer agent is produced by many endophytic fungi

Two new tetralone derivatives names xylariol A and B were produced by endophytic fungal *Xylaria hypoxylon* AT-28 from *Ligustrum lucidum*. They exhibited moderate cytotoxic activities against Hep G2 cells in the vitro cytotoxic assay with IC_{50} values of 22.3 and 21.3 μg/ml, respectively (Gu and Ding, 2008)

2.2.4 Antiviral Agents from Endophytic Fungi

Pestaloficoils A, B and D (Figure 2-16), cyclopropane derivatives produced by an endophytic fungus *Pestalotiopsis fici*. These compounds showed inhibitory effects on HIV-1 replication in C8166 cells (Liu et al., 2008). Azaphilones, named phomoeuphorbin A and C were isolated from cultures of *Phomopsis euphorbiae* and also activetly inhibited HIV-1 replication in C8166 cells (Yu et al. 2008)

Pestalficoils A

Pestalficoils B
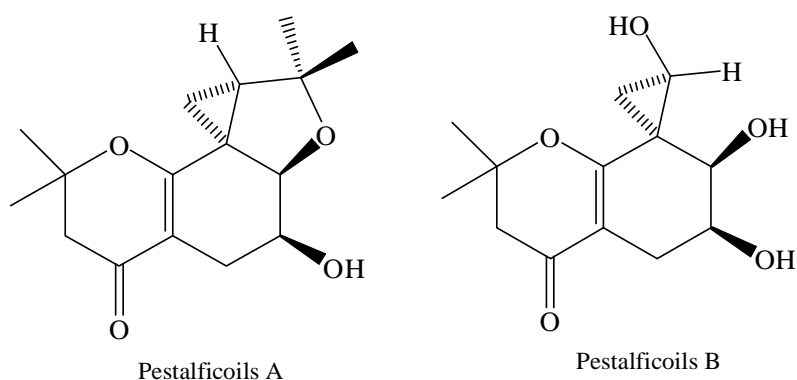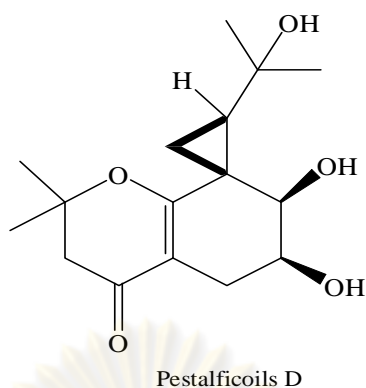
Pestalficoils D

Figure 2-16: Pestaloficoils A, B and D, cyclopropane derivatives produced by an endophytic fungus *Pestalotiopsis fici*

A cyclohexaepsipeptide, Pullularin A (Figure 2-17) was isolated from a fermentation broth of an endophytic fungus *Pullularia* sp., which exhibited activity against herpes simplex virus type I (HSV-1; IC_{50} 3.3 µg/ml). Moreover, Pullularin A was active against the parasite *Plasmodium falsiparum* K1 IC_{50} 3.6 µg/ml) and showed weak cytotoxicity to Vero cells (IC_{50} 36 µg/ml) (Isada et al, 2007).
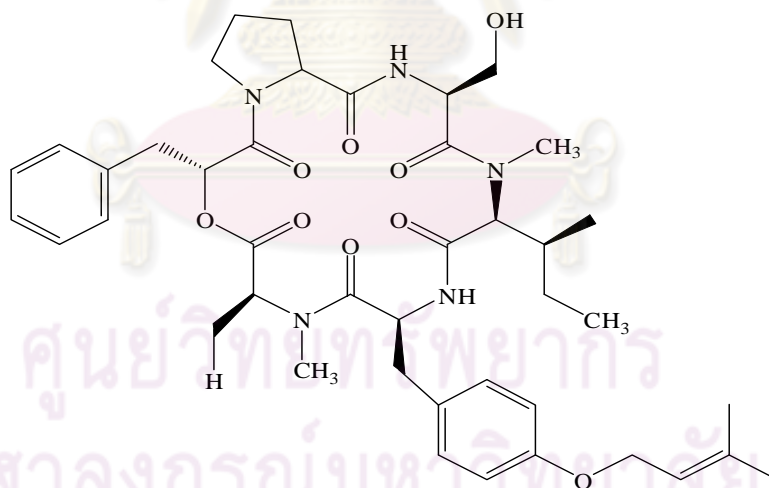
Figure 2-17: Antiviral cyclohexaepsipeptide, Pullularin A was isolated from fermentation broth of an endophytic fungus *Pullularia* sp.

Phomoeuphorbins A and C (Figure 2-18), azaphilones were isolated from cultures of *Phomopsis euphorbiae*, an endophytic fungus isolated from *Trewia nudiflora*. There compounds exhibited very weak inhibitory activities against HIV replication in C8166 cells *in vitro* (Yu et al. 2008).

Phomoeuphorbins A
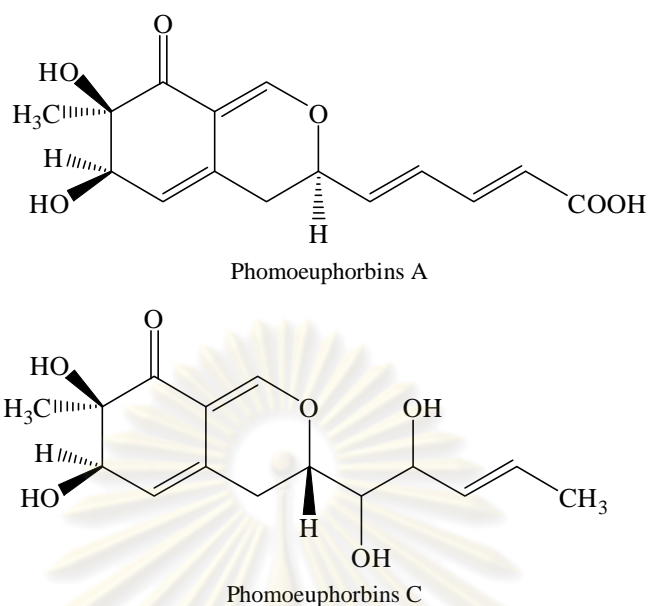
Phomoeuphorbins C

Figure 2-18: Chemical structure of phomoeuphorbins A and C

2.2.5 Immunosuppressive Agents from Endophytic Fungi

Endophytic fungi *Pestalopsis* species had been reported to produce immunosuppressive compounds in culture broth. For example, Pestalotiopsins A and B, immunosuppressive compounds produced from *Pestalotiopsis* sp., an endophytic fungus associated with the bark and leaves of *Taxus brevifolia*. (Pulici et al., 1996). *Pestalotia* sp. (Strain AB 1942R-114), isolated from a partridge pea plant (Cassia fistula), produces immunosuppressive Cytochalasin U (Burres et al., 1992). *Pestalotiopsis leucothes* HKUCC 10197, an endophytic fungus isolated from *Tripterygium wilfordii* has strong immunomodulatory effects especially in the suppression of various activities of peripheral blood mononuclear cells (PBMNC) (Kumar et al., 2005).

Fusarium subglutinans isolated from *T. wilfordii* produced immunosuppressive compounds Subglutinols A and B (Kumar et al., 2005) (Figure 2-19).
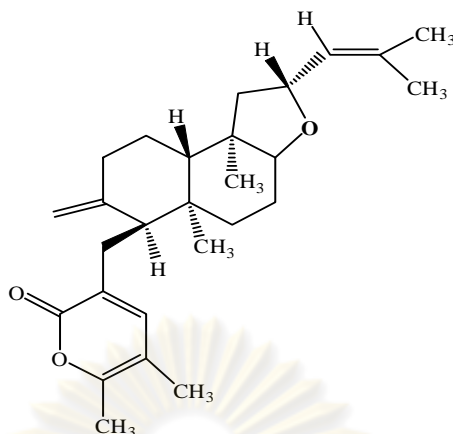
Figure 2-19: Chemical structure of subglutinol A

Colutellin A, an immunosuppressive peptide from *Colletotrichum dematium* – an endophytic fungus recovered from a *Pteromischum* sp. growing in a tropical forest in Costa Rica. Colutellin A inhibited CD4^{+} T-cell activation of interleukin 2 (IL-2) productions with an IC_{50} of 167.3±0.38 nM. Inhibition of IL-2 production by colutellin A at such a low concentration indicates the potential immunosuppressive activity of this compound (Ren et al, 2008).

2.2.6 Antioxidant Agents from Endophytic Fungi

Pestacin (Figure 2-10) and isopestacin obtained from *Pestalotiopsis microspora*, an endophytic fungi isolated from a combretaceaous plant, *Termina morbensis*, a native rainforest of Papua New Guinea. Both compounds display antimicrobial as well as antioxidant activity (Harper et al., 2003).
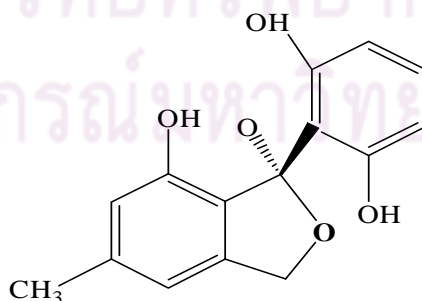
Figure 2-20: Chemical structure of pestacin

Liu et al. (2007) reported that the extracts from cultivated fruiting bodies of endophytic fungus *Xylaria* sp. YX-28 from *Ginko biloba*, especially the methanol

extract, are rich in phenolics and flavonoids. Both the phenolic and flavonoid components showed strong antioxidant activity by the 2, 2 diphenyl-1-picrylhydrazyl (DPPH) assay and β-carotene-linoleic acid assay.

2.2.7 Other Bioactive compounds from Endophytic Fungi

Four compounds (naphtho-γ- pyrones rubrofusarin B, fonsecinone A, asperpyrone B, and aurasperone A) were obtained from fractionation of the extract of *Aspergillus niger* IFB-E003, an endophyte in *Cyndon dactylon*. Rubrofusarin B was shown to be cytotoxic to the colon cancer cell line SW1116 (IC5O:4.5 μg/ml), and aurasperone A, inhibitory on xanthine oxidase (XO) (IC50: 10.9 μmol l^{-1}). Moreover, the four naphtho-γ-pyrones exhibited growth inhibitions against the five test microbes with minimal inhibitory concentrations (MICs) ranging in between 1.9 and 31.2 μg/ml (Song et al., 2004)

Podophyllotoxin, a well-known naturally occurring aryl tetralin lignan occurred in few plant species that is used as a precursor for the chemical synthesis of anticancer drugs like etoposide, teniposide and extopophose phosphate. The availiability of this lignan was becoming increasingly limited because of the scarce occurance of its natural sources and also because synthetic approaches for their productions are still commercially unacceptable. Podophyllotoxin was first reported to be produced by an endophytic fungus *Fusarium oxysporum* isolated from the medicinal plant *Juniperus recurva* (Kour et al., 2008). Also in 2006, Puri and co-workers reported that the endophytic fungus *Trametes hirsuta*, isolated from the rhizomes of *Podophyllum hexandrum*, was able to produced bioactive aryl tetralin lignans.

Several studies have shown that endophytic fungi produce volatile compounds, such as, a volatile antibiotic producing fungus, *Muscodor albus*, from *Cinnamomum zeylanicum* (Strobel et al., 2001)**.** This xylariaceaous fungus effectively inhibited and killed certain other fungi, and bacteria, by virtue of a mixture of volatile compounds that it produces. Strobel et al., 2008 found an endophytic fungus, *Gliocladiun roseum*, produced a series of volatile hydrocarbons and hydrocarbon derivatives on an oatmeal-based agar under microaerophilic conditions. This organism produced an extensive series of the acetic acid esters of straight-chained

alkanes including those of pentyl, hexyl, heptyl, octyl, sec-octyl and decyl alcohols. The hydrocarbon profile of *G. roseum* contains a number of compounds normally associated with diesel fuel and they suggested that the volatiles of this fungus have been dubbed 'myco-diesel'.

2.3 Secondary metabolites from Xylariaceae

The Xylariaceae is one the largest family in the Phylum Ascomycota (Stadler, 2009), with at least 85 genera and 1343 accepted species to date (Kirk et al. 2008). It is well known for the wide biological diversity in genera and species, demonstrating in worldwide and great biological diversity in tropics (Whalley and Edwards, 1998). Most of them occur on a wide range of living or dead angiosperms (more rarely on gymnosperms) (Peláez et al., 2008) and can also be found in litter, soil, dung, and associated with insects (Whalley, 1996; Peláez et al., 2008). However, they are predominant among both wood-decay and endophytes in plants (Rodrigues & Samuels, 1990; Petrini, 1995; Rodrigues, 1994, Stander, 2009). Ju and Rogers (1996) devided them into major lineages, according to the types of conidiogenous structures (*"Geniculosporium*-like" in *Xylaria* Hill ex Schrank and allies; "*Nodulisporium*-like" in *Hypoxylon* Bull. and relatives). Most of these endophytic xylariaceous fungi are usually isolated in the hypomycetous anamorph form as *Nodulisporium* and *Geniculosporium* species from a wide variety of plants, although telemorphs develop on a narrower range of hosts (Peláez et al., 2008). Morphological characters widely accepted to define the limits of the family Xylariaceae including the stromal form and colour, type of ostiole, structure of apical apparatus of asci, and the number, arrangement and morphology of ascospores, presence and absence of the germ slit, presence or absence of spore wall (Whalley and Edwards, 1998). However, most of these fungi do not form teleomorphs in culture and, thus, had not been identified (Rogers, 2000). Cultures of Xylariaceae had been shown to produce various unique or chemotaxonomocally significant secondary metabolites (Whalley and Edwards, 1998; Stadler and Hellwig, (2005); Stadler, (2009) and molecular techniques have been introduced to infer the relationships of taxa within the genus.

Xylariaceous fungi have been reported as producers of highly diversity secondary metabolites (Whalley and Edwards, 1998; Peláez et al., 2008, Stadler,

2009). Many of their secondary metabolites have biological activities. For example, several cytochalasins that bind to actin in muscle tissue, have been found in *Xylaria obovavata* (Abate et al., 1997) along with clonostachydiol, phaseolinon and xylobovatin, while cytochalasins have potent anti-tumor effects in various tumor cell lines, clonostachydiol is an anthelmintic and phaseolinon and xylobovatin show phytotoxic effects. Cytochalasins compounds have also been found in *X. hypoxylon* (Espada et al., 1997; Aldridge et al., 1972).
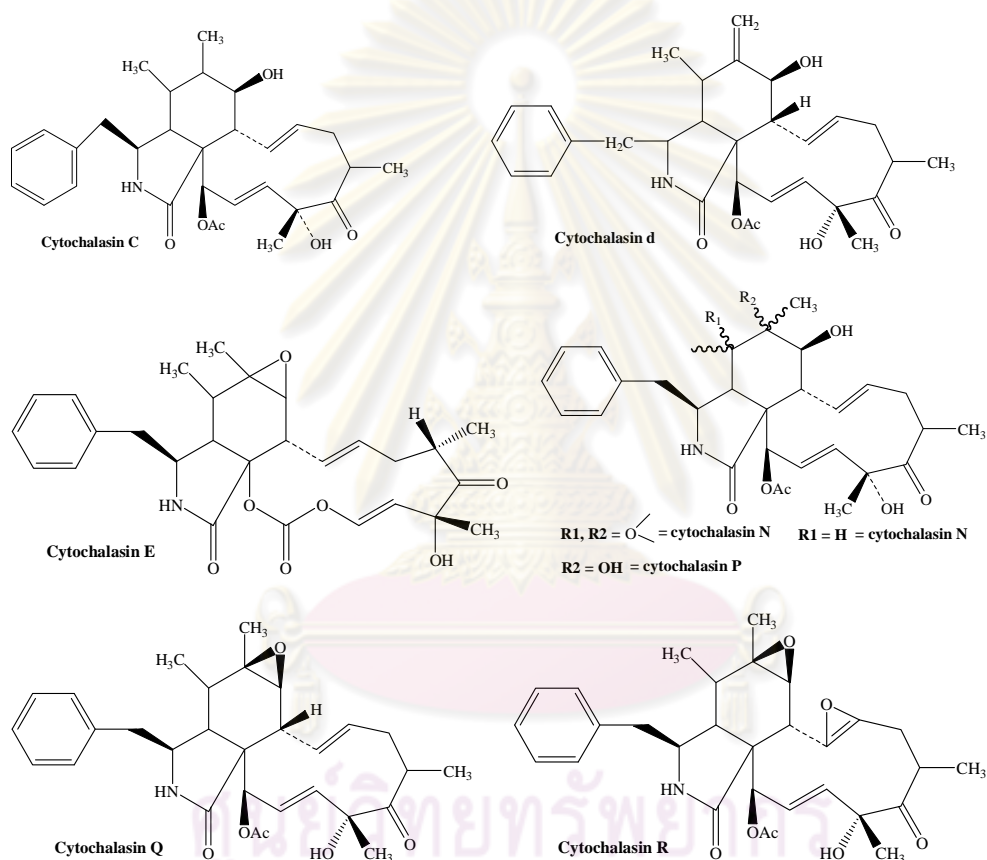
Figure 2-21: Cytochalasins, secondary metabolite structure from *Xylaria obovata* and *Xylaria hypoxylon*

Two tetralone derivatives named xylariol A and B were also isolated from *X. hypoxylon* AT-028. The compounds xylarial A and B both had moderate cytotoxic activity against the human hepatocellular carcinoma cell line Hep-G2 (Gu and Ding, 2008).
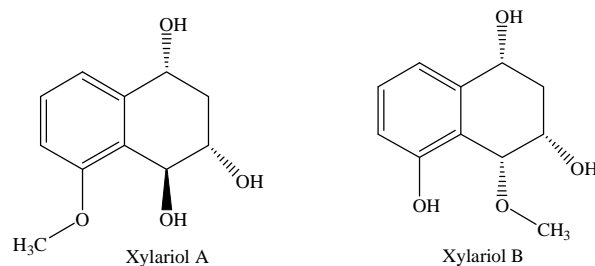
Figure 2-22: Xylariol A and B, bioactive compounds from *X. hypoxylon*

Xylarenal A and B, eremophilane sesquiterpenoids, were isolated form fermentatin broth of *X. persicaria* (Smith et al., 2000). These compounds were selected for neuropeptipe (neuropeptide is a polypeptide found in the peripheral and central nervous system) Y5 receptor but have only modest affinity.
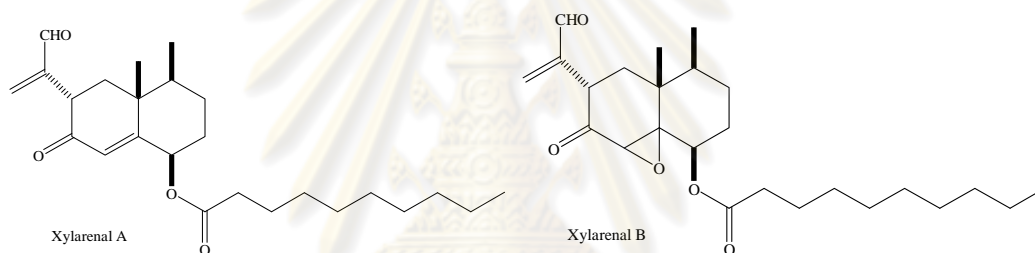
Figure 2-23: Xylareol A and B, bioactive compounds from *X. persicaria*

Several ketal compounds, Xytoketals have been isolated from the mangrove fungus *Xylaria* sp. (Liu et al., 2006; Yin et al., 2008, Xu et al., 2008), along with xyloester A, xyloallenoid B and dihydrobenzofuran which were also isolated from this fungus.
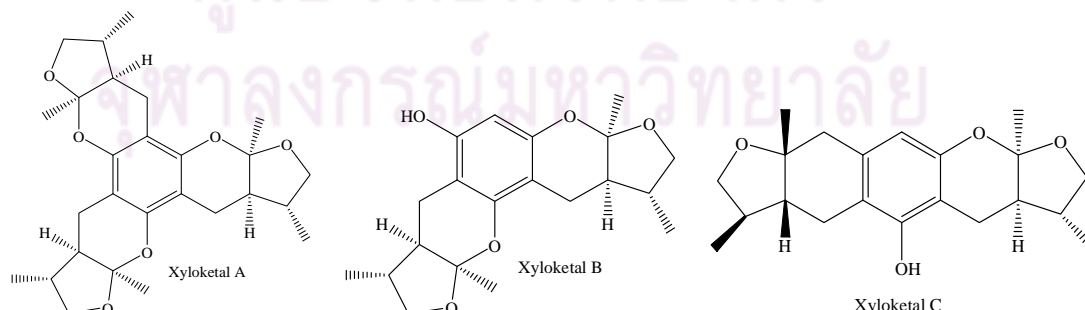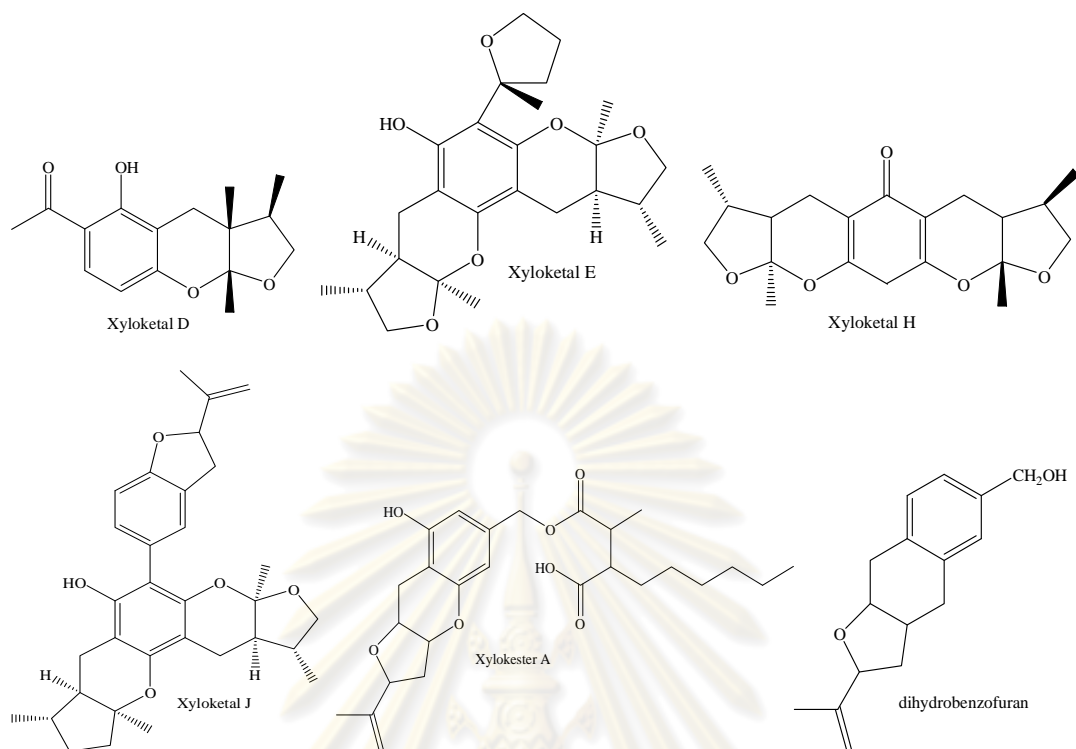
Figure 2-24: Xytoketals, the ketal compounds from mangrove fungus *Xylaria* sp.

Xanthones, 2-hydroxy-6-methyl-8-methoxy-9-oxo-9H-xanthene-1-carboxylic acid (1) and 2-hydroxy-6- hydroxymethyl-8-methoxy-9-oxo-9H-xanthene-1-carboxylic acid (2) and 2,8-dimethoxy-6-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ester (3) were found from enophytic fungus *Xylaria* sp. from Australian tree *Glochidion Ferdinadi* (Healy et al., 2004). The xanthones had been shown to display a wide range pharmaceutical, including anticancer, antimicrobial, antioxidant, anti-inflammatory and antivirol.

1: R_1 =H, R_2 = H, R_3 = CH_3, R_4 = CH_3
2: R_1 =H, R_2 = H, R_3 = CH_3, R_4 = CH_2OH
3: R_1 =CH_3, R_2 = CH_3, R_3 = CH_3, R_4 = CH_3
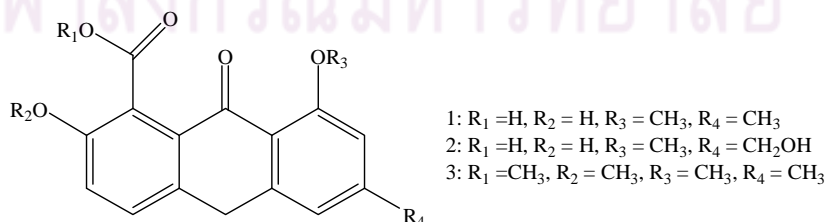
Figure 2-25: Xanthones, the bioactive compound from an endophytic *Xylaria* sp.

An antimicrobial compound, 7-amino-4-methylcoumarin was isolated from an endophytic *Xylaria* sp. YX-28 of *Ginkgo biloba* (Liu et al., 2008). It has broad-spectrum antimicrobial activity inhibiting the growth of the tested 13 microorganisms.
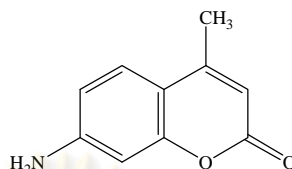
Figure 2-26: 7-amino-4-methylcoumarin, an antimicrobial compound from a *Xylaria* sp.

Two azaphilone derivatives named daldinins E and F have been found from fruit bodies of the *H. fuscum*, together with compounds daldinin C and 4,5,40,50-tetrahydroxy-1:10-binaphthyl (Quang et al., 2004). They showed antioxidative activities.
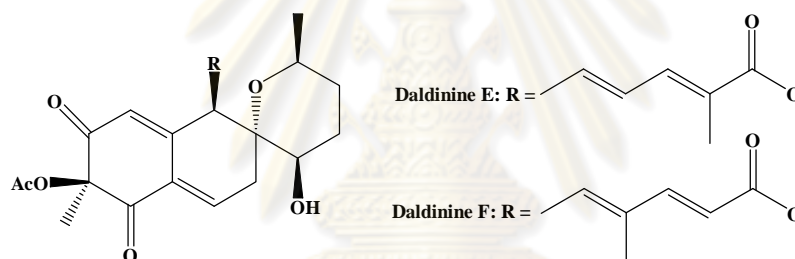
Figure 2-27: Daldinins E and F, an antioxidative from *H. fuscum*

Sordarins are an important family of antifungal agents. Sordarin was first isolated by Sigg and Stoll in 1967 from the fungus *Sordaria araneosa* (Sigg and Stoll,, 1967) and different compounds and derivative belonging to this class have subsequently been discovered. For example, In 1999 Darferner and colleagues isolated hypoxysordarin, a sordarin derivative from the fermentation broth of the facultative marine *Hypoxylon croceum* together with a gamma-lactone, hypoxylactone and sordarin. Both hypoxysordarin and sordarin exhibit high antifungal activities due to a specific inhibition of protein biosynthesis. Vincente et al. (2009) screened for sordarin-producing fungi in the family Xylariaceae, Amphisphaeriaceae and Diatrypaceae. They were found 22 new strains producing a number of different sordarin analogues, either known (sordarin, xylarin, zofimarin) or novel (isozofimarin and 4'-O-demethyl sordarin) and the sordarin and xylarin were the most frequently

found compounds in the class. The producing strains were more frequently associated with member of the Xylariales than another fungal order.
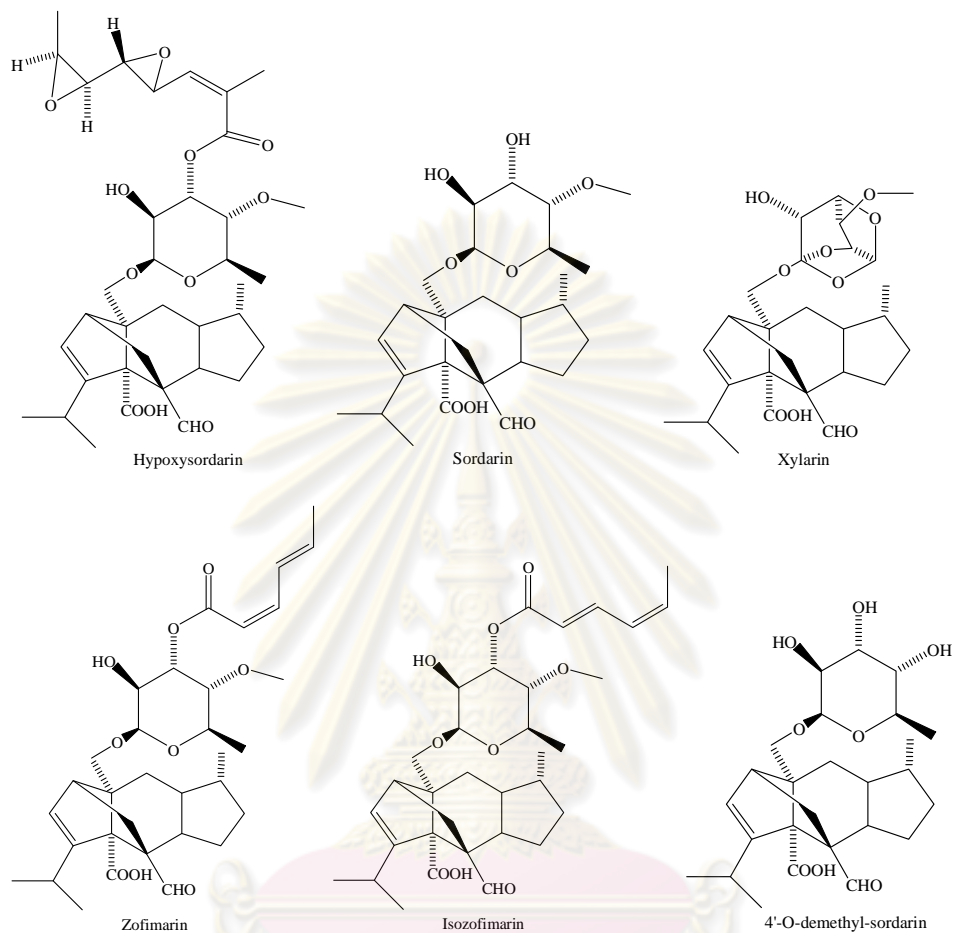
Figure 2-28: Sordarin and sordarin derivative produced by *Hypoxylon croceum* and *Xylaria* sp.

An azaphilone named hypomiltin was also isolated from the stromatal extract of the xylariaceous ascomycete *H. hypomiltum* (Hellwig et el., 2005).
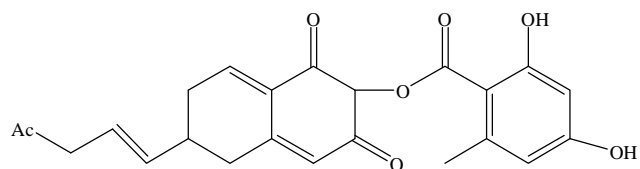
Figure 2-29: Hypomiltin, an azaphilone from *H. hypomiltum*

Xylactam, a nitrogen-containg copmpound and alkaloid, penochalasin B and neoechinulin A were extracted from fruiting bodies of *X. euglossa* (Wang et al., 2005). Penochalasin B was reported to have potential cytotoxicity against P338 cells.

Three Lactones, (+)-phomalactone, 6-(1-propenyl)-3,4,5,6-tetrahydro-5-hydroxy-4Hpyran-2-one and 5-hydroxymellein were isolated from an endophytic *Xylaria* sp. (Jiḿenez-Romero et el., 2008). The lactones have activity against a chloroquine-resistant *Plasmodium falciparum* strain

Other secondary metabolites from Xylariaceae have been reported such as cubensic acid, globoscinic acid, globoscin, beryeric acid. cameronic acid, malaysic acid and etc.
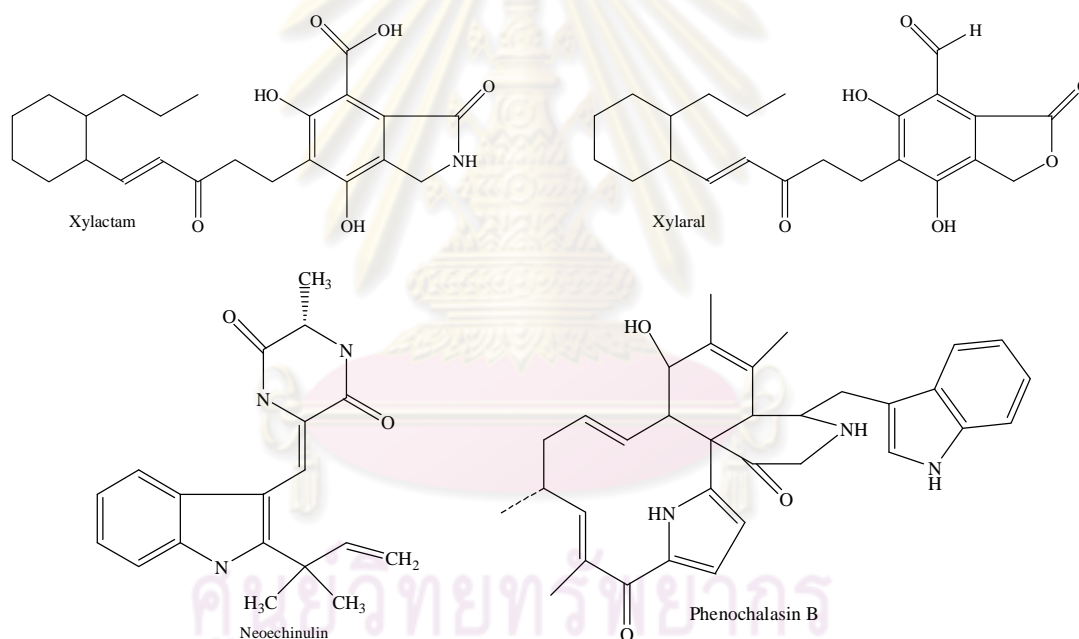
Figure 2-30: Xylactam , xylaral, neoechilulin A and phenochalasin B
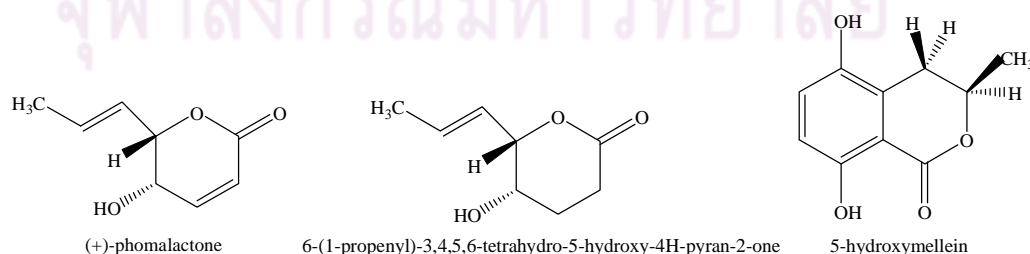
Figure 2-31: (+)-phomalactone, 6-(1-propenyl)-3,4,5,6-tetrahydro-5-hydroxy-4Hpyran-2-one and 5-hydroxymellein, lactones produce from endophytic fungus *Xylaria* sp.

Thailand had been considered as one of the areas containing a high percentage of unknown taxa of Xylariaceae (Rogers 2000; Okane, 2007). Several studies on endophytic xylariaceous fungi have been documented. For example, two novel benzoquinone metabolites, 2-chloro-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione and xylariaquinone isolated from endophytic *Xylaria* of *Sondoricum* leaves. These compounds showed in vitro active against *Plasmodium falciparum* and cytotoxicity against African green monkey kidney fibroblasts (Tansuwan et al., 2007). Glucoside derivetives, xylarosides A and B together with sordaricin and sordarins, isolated form fermentation broth of *Xylaria* sp. PSU-D14. Sordaricin and sordarins exhibited moderate antifungal activity against a wide range of fungal pathogens (Pongcharoen et al., 2008). In the same year, Pongcharoen et al. were isolated methyl aminobenzoate together with eleven known compounds such as Cytochalasin D, desavetylcytochalasin D, cytochalasin O, (*R*)-(-)-mellein methyl ether, 5-carboxymellein, (*R*)-(-)-5-hydroxymellein, 4-quinolinecarbonitrile, 4-quinolinecarboxaldehyde oxime , cyclo(L-Pro-L-Tyr), cerevisterol and uracil. Pongcharoen et al., (2008) reported that PSU-D14 produced glucoside derivetives, xylarosides A and B, sordaricin and sordarins. Additionally, Sordaricin and sordarins exhibited moderate antifungal activity against a wide range of fungal pathogens. Nodulisporacid A was isolated from a marine-derived fungus *Nodulisporium* sp. and exhibited moderate antiplasmodial activity (Kasettrathat et al., 2007). In 2002, Ondeyka and co-worker also isolated nodulisporic acid A, B, B_1, and B_2, potent insecticide from *Nodulisporium* sp. Secondary metabolites, 3-hydroxy-1-(2,6-dihydroxyphenyl)-butan-1-one, 1-(2-hydroxy-6-methoxyphenyl) butan-1- one , 2,3-dihydro-5-methoxy-2-methylchromen-4-one, dimeric naphthalenes nodulisporin A and B, dimeric indanone, nodulisporin C, and (4*E*,6*E*)-2,4,6-trimethylocta-4,6-dien-3-one were isolated from the culture extract of the endophytic fungus *Nodulisporium* sp. from *Juniperus cedre* from Gomera Island (Dai et al., 2006).
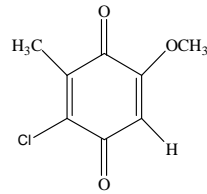
2, chloro-5-methoxy-3-methylcyclohexa-2,5-diene
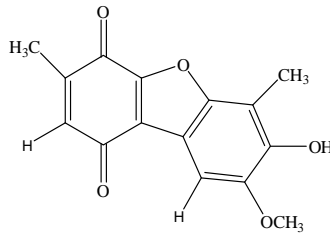
Xylariaquinone
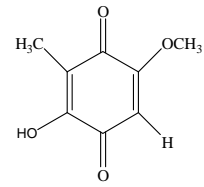
2, chloro-5-methoxy-3-methylcyclohexa-2,5-diene-1,4 dione
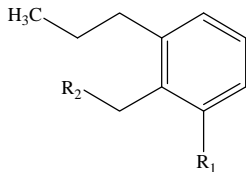
Xylarosides A: R₁ = OH, R₂ = glu
Xylarosides B: R₁ = glu, R₂ = OH
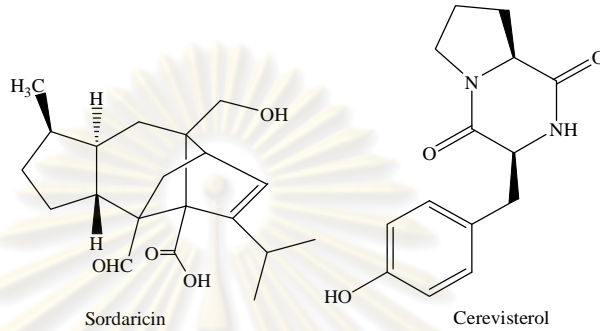
Sordaricin

Cerevisterol
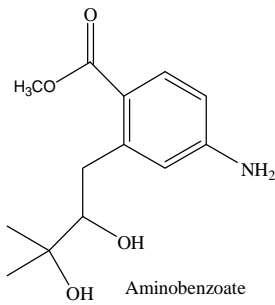
Aminobenzoate
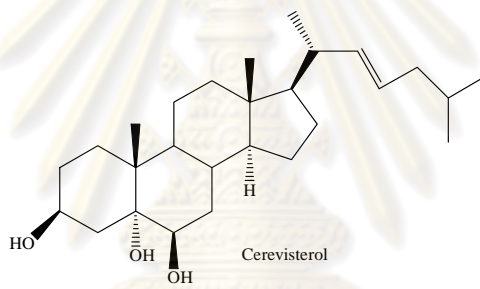
Cerevisterol
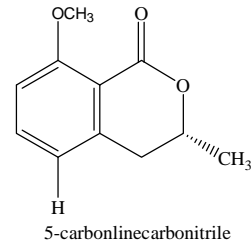
5-carbonlinecarbonitrile
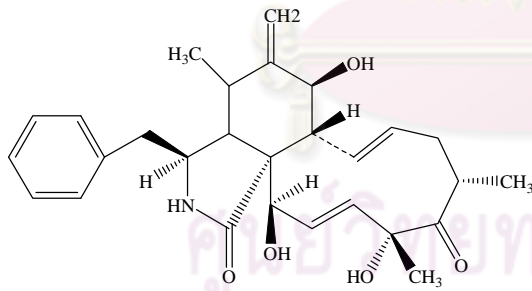
Desacetylcytochalasin D
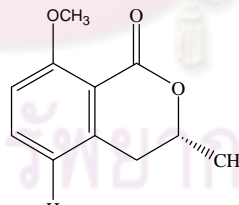
(R)-(-)-mellein methyl ether

(R)-(-)-5-hydroxymellein

4-quinolinecarbonitrile = R = CN
4-quinolinecarboxaldehyde oxime = R= CH=NOH

Cyclo(L-Pro-L-Tyr)

Nodulisporacid A

Nodulisporic acid A = R1 = O, R2 = H
Nodulisporic acid B = R1 = H, R = H
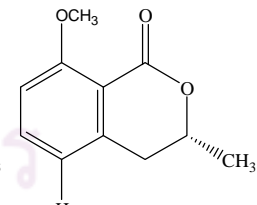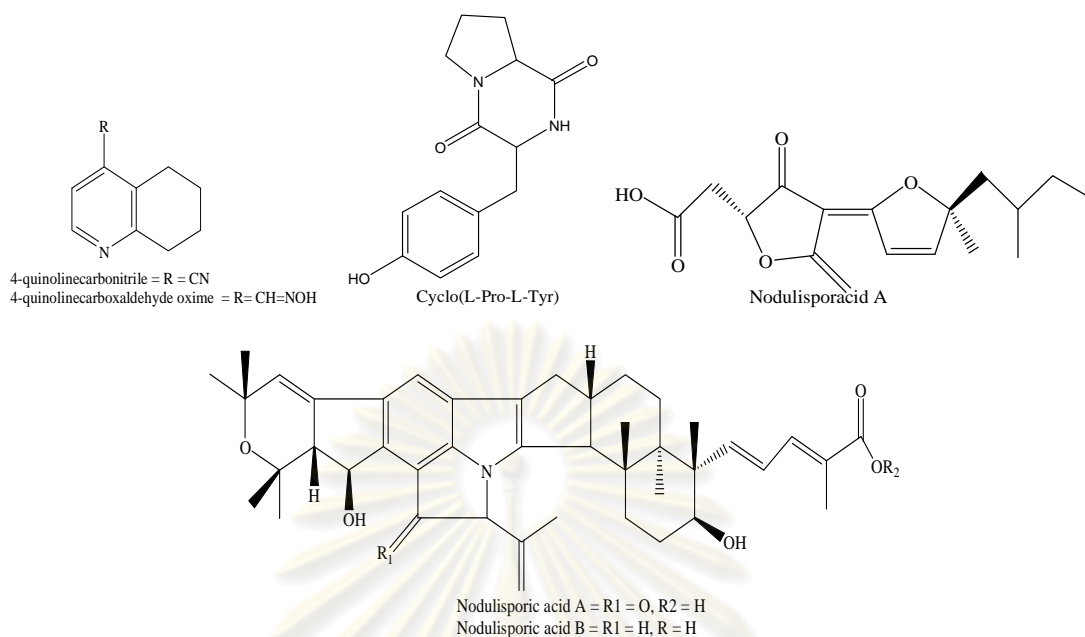
Figure 2-32: The secondary metabolite from endophytic Xylariaceous fungi in Thailand

2.4 Plant sample: Mitragyna Javanica Koord &Val

Mitragyna javanica Koord & Val known as "Krathum na" belonging to the family Rubiaceae, is found in tropical and sub-tropical regions of Asia. M. javanica has the synonym *M. diversifolia* (Wall. ex G. Don) Havil..   The *Mitragyna. javanica* is a deciduous tree; it is usually 8-15 ft in height. The stem is erect and branching. The bark is grey to dark grey-brown. Leaves are evergreen and are dark glossy green in colour, ovate-acuminate in shape, single leaf and leaves arranged opposite. Flowers are yellow, globular flowering head and inflorescence. Calyx glabrous, tubular below and with obtuse to rounded or broadly triangular lobes above, entire calyx c. 1.5 mm high. Corolla creamy white to (pale) yellow, often turning darker with age. Fruiting head is round 13-18 mm in diameter and seeded dehiscent with seed wing.

Figure 2-33: *Mitragyna javanica* trees

Figure 2-34: Leaves, flower and fruits of *Mitragyna javanica*

2.4.1 Taxonomy of the genus *Mitragyna*

According to the characteristics of morphology and genetic information, the genus *Mitragyna* belongs to the tribe Naucleeae of the family Rubiaceae. *Mitragyna* consists of approximately 10 species in the world (http://zipcodezoo.com/ Plants/M/Mitragyna_javanica/), 6 Asian species; *M. speciosa* (Korth.) Havil., *M. tubulosa* (Arn.) Havil., *M. parvifolia* (Roxb.) Korth., *M. hirsuta* HAVIL., *M. javanica* (Wall. ex G. Don) Havil., *M. rotundifolia* (Roxb.) O. Kuntze, (Sukrong et al., 2007 and 4 African species; *M. inermis* (Willd) O. Kuntze, *M. ciliate* (Aubrev. And Pellegr.), *M. stipulosa* (D.C.) O. Kuntze, *M. rubrostipulata* (Schum.) Havil (Srisiri, 2007). There are 4 species, *M. speciosa*, *M. hirsuta*, *M. javanica*, and *M. rotundifolia*,

commonly found in Thailand. Figure 2-35 shows taxonomic hierarchy of the genus *Mitragyna javanica*.

Kingdom   Plantae
　Subkingdom   Tracheobionta
　　Division   Magnoliopsida
　　　Subclass   Asteridae
　　　　Order   Gentianales
　　　　　Family   Rubiaceae
　　　　　　Subfamily   Cinchonoideae
　　　　　　　Tribe   Naucleeae
　　　　　　　　Genus   Mitrgina
　　　　　　　　　Specific epitet   javanica
　　　　　　　　　　Botanical name   *Mitragyna javanica*

Unambiguous Synonyms

1. *Mitragyna diversifolia*
2. *Nauclea adina* Blanco
3. *Nauclea diversifolia* Wall. Ex G. Don
4. *Stephegyne diversifolia* (Wall. Ex G. Don) Brandis
5. *Stephegyne parvifolia* Vidal
6. *Stephegyne tubulosa* Fern. -Vill.

Figure 2-35: Taxonomic hierarchy of the *Mitragyna javanica*

2.4.2 Phytochemical of *M. javanica*

The *M. javanica* has been used as a Thai medicinal plant having various pharmacological activities. The leaves have been used for treatment of diarrhea, dysentery and frambesia, and also the bark for treatment all types of skin disease, cancer and dysentery (Pongboonrod, 1979). Phytochemical studies revealed that the leaves containseveral alkaloids similar to those of *M. speciosa* (Kratom). The alkaloids have been reported to treated hypertension and showed effect on repression of nervous system and smooth muscle contraction in guinea-pig. Moreover, it has been reported that the leaves of *M. javanica* are sometimes chewed as a substitute for those of *M. speciosa*.

The leaves of *M. javinica* contained the alkaloid of the C(9)-H closed E ring normal-pseudo sequence and some alkaloids in the C(9) -OCH 3 closed E ring

normal-pseudo sequence. (Shellard et. al., 1967). Mitrajavine was the dominant alkaloid and in a recent TLC examination; this plant contained similar alkaloid content comparable to that of *M. Speciosa* devoid of Mitragynine. Often used as a replacement for Kratom in contains several corynanthedine alkaloids, and there is no evidence of the isomer, isomitrajavine while only the A isomer of the corresponding oxindole series was present. Pikaew et al., (2008) separated chemical components from branches of *Mitragyna diversifolia* Havil. Their structures were determined based on the spectroscopic methods (IR, ^{1}H NMR, ^{13}C NMR, 2D-NMR and MS) as well as comparison with literature data as sitost-4-en-3-one (1), 3®-acetoxy-12〈-hydroxylenan- 28,13®-olide (2), scopoletin (3), indole alkaloid (4) and 3,4-dihydroxybenzoic acid (5).
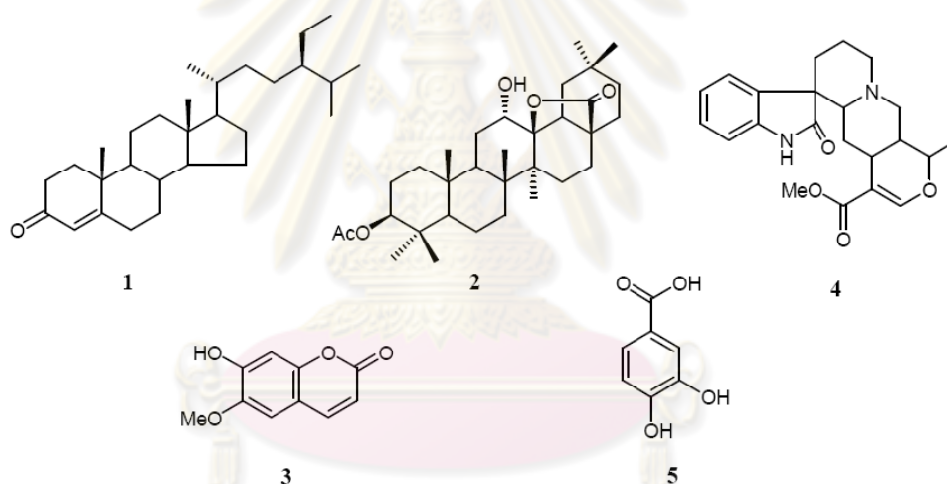
Figure 2-36: Chemical structure of *M. javanica* alkaloids; sitost-4-en-3-one (1), 3®-acetoxy-12〈-hydroxylenan- 28,13®-olide (2), scopoletin (3), indole alkaloid (4) and 3,4-dihydroxybenzoic acid (5)

CHAPTER III

MATERIALS AND METHODS

3.1 Reagents

- Ammonium hydrogen phosphate $(NH_4)_2HPO_4$ was used Merck KGaA, Germany.
- Potassium dihydrogen phosphate (KH_2PO_4) was used Merck KGaA, Germany.
- Trizma base, minimum 99.9% titration was used Sigma-Aldrich Co., Inc., Singapore.
- Hexane
- Ethyl acetate
- MeOH
- Chloroform
- Ethylenediaminetetraacetic Acid (EDTA) was used Sigma-Aldrich Co., Inc., Singapore.
- Ethanol absolute, Analytical grade, ACS. was used Scharlau Chemie S.A., Spain.
- Sodium hydroxide (NaOH) was used Merck KGaA, Germany.
- 100 bp Ladder Sharp DNA Marker was used Fermentas International Inc., Canada.

3.2 Instruments

- Incubator (Model 800, Memmert GmbH and Co. KG., Western Germany)
- Incubator shaker (Model SK-737, Amerex Instruments, Inc., USA)
- CO_2 Incubator (Thermo electron corporation USA)
- Autoclave (Model Autoclave ES-315, Tomy Seiko Co., Ltd., Tokyo, Japan)
- Hot air oven (Model UC 30, Memmert GmbH and Co. KG., Western Germany)
- Spectrophotometer (Genesys 20 Model 4001/4, ThermoSpectronic, Rochester., New York, USA)
- Laminar flow 'clean' (Model V6, Lab Service Ltd., Part)
- Cold room (Model Compakt 880(B)H, Foster Refrigerator (U.K) Ltd., U.K)

- 4-Digit precision weighting balance (Model AG 204, Mettler Toledo, Switzerland)
- Microwave (Model 000502174, Thai Cityelectric Co. Ltd.,Thailand and National model NE-E72 High power 700 w, Tokyo, Japan)
- Hot plate stirrer (Model C-MAG HS 10, Becthai Bangkok Equipment & Chemical Co., Ltd., Thailand)
- Vortex mixer (Model G-560E, Scientific Industries, Inc., Bohemia. N.Y., 11716, USA)
- Nano Spin (Model NS-060, Nippon Genetic Co., Ltd., Japan)
- Water bath (Model WB14, Becthai Bangkok Equipment & Chemical Co., Ltd., Thailand)
- pH meter (Mettler-Toledo International Inc., New York, U.S.A.)
- Gel Documentation system (Bio-Rad Laboratories Gel Doc TM XR, California, U.S.A.)
- Electrophoresis chamber set (Mupid-ex, Bruker BioSpin Inc., Fällanden, Switzerland)
- High Speed Refrigerated Centrifuge (Beckman Coulter TM Avanti J-30I, Palo Alto, California, U.S.A.)
- Microscope (Model CH 30RF200, Olympus Optical Co., Ltd., Japan)
- Inverted microscopy (Model CK2 Olympus Optical Co., Ltd., Japan)
- Kubota Refrigerated Microcentrifuge 6500 (Kubota Corporation, Tokyo, Japan)
- UV-VIS spectrometer model V-530 (PC) (PerkinElmer instruments Lambda 25, Massachusetts, U.S.A.)
- Takara Thermal Cycler Dice Standard TP-65 (TaKaRa Bio Inc., Otsu, Shiga, Japan)
- Microhomogenizer Tomy Micro Smash^{TM} (Model MS-100 Serial no. MS031134 Active DNA^{TM}, Tomy Digital Biology, Tokyo, Japan)
- Dry Thermo Unit (Model DTC-1B, Bio-Version, Taitec copporation, Japan)
- Liquid N_2 tank (34HC, USA)
- Microplate reader (ELX 800, Biotek Instruments Inc)
- Water bath (Memmert, Germany)

- Rotary evaporator (Eyela N-1000, Japan)

3.3 Plant Samples Collection

Healthy leaves of *M. javanica* were collected from Pathumthani, Ayuthaya and Amnatcharean provinces. The leaf samples were kept in plastic bags and stored at 4°C in a refrigerator prior to isolation of endophytic fungi.

3.4 Culture Media

Culture media used for isolation, cultivation, morphological observation of endophytic fungi were Malt Extract Agar (MEA) and Broth (MEB). Potato Dextrose Agar (PDA) and Sabouraud Dextrose Agar (SDA) were used to study macroscopic morphology. The medium for cultivation for metabolites of bioactive compounds was Malt Extract Agar and Broth. Media for growing bacteria were Nutrient agar and broth. Yeast Malt Extract Glucose Agar (YMA) and Broth (YMB) were used for growing yeast. The formula of the media composition is given in Appendix A.

3.5 Isolation of Endophytic Fungi

The leaf samples were washed under running tap water for 10 minutes and dried before endophytic fungi were isolated by using a surface sterilization method. Four disks (6 mm in diameter) were cut from each leaf. The leaf disks were surface sterilized by immersion in 95% ethanol for 1 minute, followed by 3.5 % sodium hypochlorite solution for 3 minutes and finally 70% ethanol for 30 seconds. The surface sterilized samples were washed twice in sterile distilled water and allowed to surface dry on filter papers under sterile condition. The leaf disks were placed on the surface of MEA plates supplemented with 1% (w/v) of streptomycin to prevent bacterial growth. The efficiency of surface sterilization was confirmed by pressing the sterilized leaf disks on to the surface of MEA medium. All petri dishes were then incubated at room temperature (30-37 °C) and examined everyday for fungal mycelium under a stereomicroscope. The fungal mycelium growing out from leaf disks were subsequently transferred to fresh MEA plates by hyphal tip transfers. (Strobel *et al.* 1996) and incubated at room temperature for 7-14 days. The purity of isolated endophytic fungi was determined by colony morphology by observation

under stereomicroscope. The endophytic fungal isolates were maintained in MEA for future studies.

3.6 Screening of Endophytic Fungi for their Antimicrobial Activities and Anticancer activities

3.6.1 Fermentation and Extraction

The endophytic fungal isolates were grown on MEA plates at room temperature for 7-14 days depending on their growth rate. Ten disks (7 mm diameter) of the grown culture were from the edge of the colony inoculated into a 500 ml Erlenmeyer flask containing 150 ml of MEB, incubated at room temperature for 30 days under static condition.

The cultures were filtered through No. 4 filter paper. The filtered broths were extracted exhaustively with ethyl acetate. The mycelia were frozen at -20°C and grounded with mortal before extracted exhaustively with ethyl acetate. The solvent phase from culture broth and mycelium were pooled and evaporated to dryness under reduced pressure using rotary evaporator.

The crude extracts were dissolved in 3 ml of 10% dimethylsulphoxide (DMSO) in sterile distilled water and kept at -20°C prior to testing for their antimicrobial and anticancer activities.

3.6.2 Screening of Endophytic Fungi for their Antimicrobial Activities

Screenings of endophytic fungi for their antimicrobial activities were tested by dual-culture diffusion method.

3.6.2.1 Tested Microorganisms

The *in vitro* antimicrobial susceptibility test was performed by using standard microorganisms as shown in table 3-1.

Table 3-1: Tested microorganisms for antimicrobial assay

Microorganisms		Reference strains
Bacteria	Gram positive, rod	*Bacillus subtilis* ATCC 6633
	Gram positive, coccus	*Staphylococcus aureus* ATCC 25923
	Gram negative, rod	*Pseudomonas aeruginosa* ATCC 9027
	Gram negative, rod	*Escherichia coli* ATCC 25922
Fungi	Yeast form	*Saccharomyces cerevisiae* TISTR 5169
		Candida albicans ATCC 1023

3.6.2.2 Preparation of Bacterial Inoculum

Tested bacteria were grown on Nutrient Agar (NA) for 24 h at 37°C. Four or five colonies were inoculated into 5 ml of Nutrient Broth (NB) and incubated at 37°C for 2-6 h, depending on growth rate. The turbidity of the bacterial suspension was adjusted with NB to match the turbidity of 0.5 McFarland standard (OD 0.1 at 625 nm).

3.6.2.3 Preparation of Yeast Inoculum

Yeasts were grown on Yeast-Malt Extract Agar (YMA) for 24 h at room temperature. Selected 4-5 colonies were inoculated into 5 ml of Yeast-Malt Extract Broth (YMB). The turbidity of the yeast suspension was adjusted with YMB to match the turbidity of 0.5 McFarland standard.

3.6.2.4 Inoculation of the Test Plates

Within 15 minutes after adjusting the turbidity of the inoculum suspension a sterile swab were dipped into the inoculum suspension. The swab was pressed firmly against the inside wall of the tube above the fluid level to remove excess inoculum from the swab. The dried surface of NA plates for bacteria and YMA plates for yeast were inoculated by streaking the swab over the entire surface of the medium three times, rotating the plate approximately 60 degree after each application to ensure an even distribution of the inoculum. The surface of the medium was allowed to dry for 3-5 minutes.

3.6.2.5 Application of Crude Extracts

The sterile filter paper discs (6 mm diameter) were impregnated with crude extract solutions and placed on inoculated plates. Bacteria and yeast plates were incubated at 37°C and room temperature, respectively for 24 h. The diameter of the inhibition zones were measured in millimeter with a ruler.

3.6.3 Screening of Endophytic Fungi for their Anticancer Activities

Screening of endophytic fungi for their anticancer activities was undertaken by the MTT (3-4, 5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide) colorimetric method. The assay is based on the reduction of MTT by enzymes of the mitochondrial electron transport assembly, which produces blue formazan crystals. The reduction is proportional to the viability of the cell. The absorption of formazans can be measured with a microplate reader at 540 nm. The MTT assay protocol was described by Palaga et al. (1996).

3.6.3.1 Cell Lines

The crude extracts were against 6 human cancer cell lines as showed in table 3-2

Table 3-2: Cancer cell lines for MTT assay

	Type of cancer	Reference cell line
A375	Human malignant melanoma	ATCC no.CRL-1619
BT474	Human ductal carcinoma	ATCC no.HTB-20
HepG2	Human liver hepatoblastoma	ATCC no. HTB-8065
Jurkat	Human acute T-cell leukemia	ATCC no. CRL-2063
KatoIII	Human gastric carcinoma	ATCC no. HTB-103
SW620	Human colorectal adenocarcinoma	ATCC no. CCL-227

3.6.3.2 Cell Lines Culture

Experimental cell line cultures were prepared from deep-frozen stock vials and always kept in a subconfluent state. They were cultured in RPMI 1640 in T-

$75cm^2$ flak, supplemented with 10% heat-activated fetal bovine serum (FBS; Hyclone Laboratories, Inc.; Logan, UT), 1mM sodium pyruvate, 1% HEPES, 10 U/ml penicillin G and 50 U/ml streptomycin sulfate incubated at 37°C, 5% CO_2, 95% humidity. Cell lines were observed morphological everyday under reverse-microscopy and the medium changed every 2-3 days.

3.6.3.3 Trypsinizing Adhesive Cell Lines (A375, BT474, HepG2, Kato III, and SW620)

The monolayers of human cancer cells were harvested by trypsinization. Medium culture was removed by using a serological pipette. Cells were washed with 5 ml of PBS and PBS removed and then 2.5 ml of 0.25% trypsin-EDTA added for 3-5 min followed by 5 ml of pre-warmed complete RPMI 1640. Cell suspensions were centrifuged at 1000 rpm for 5 min and the supernatant discharged. Harvested cells were suspension with pre-warmed complete RPMI 1640 medium.

3.6.3.4 Harvest Non-adhesive Cell lines (Jurkat)

Cell suspensions were transferred to centrifuge tubes and centrifuged at 1000 rpm for 5 min. Cells were re-suspended in pre-warmed complete RPMI 1640 medium. After determination of the cell density, the appropriated concentration was adjusted by dilution in pre-warmed RPMI 1640 complete medium.

3.6.3.5 Determination of Cell Concentration

For the determination of the cell concentration 20 µl of cell suspension was transferred to an Eppendorf tube and stained with 20 µl of trypan blue solution. Cells were determined using Haemacytometer under reverse-microscopy.

3.6.3.6 *In Vitro* Cytotoxicity Assay

The human cancer cells were harvested from exponential phase maintenance culture (T-75cm^2) in RPMI 1640 Medium, counted by trypan blue exclusion, and diluted to 5×10^4cells/ml with pre-warmed complete RPMI 1640 medium. The cell suspensions were seeded in 96 well microculture plates, 100 µl/well and incubated for 24 h at 37°C, 5% CO_2, 95% relative humidity before crude extract

addition. A 5 μl of crude extract was added into each well of 96 well microculture plates and incubated at 37°C, 5% CO_2 for 24 h. (5 μl of 10% DMSO used as control for zero inhibition) Each sample was tested in triple wells. At the end of exposure, 10 μl of 5 mg/ml (w/v) of MTT solution was added to each well and incubated for 24 h at 37°C, 5% CO_2 and then the supernatant was removed and added 100 μl of isopropanol containing 0.04 N HCl to solubilized the MTT-formazan crystal was added. The optical densities of the wells were measured with a microplate reader at 540 nm. Cells viability were calculated by the following equation

$$\text{Viabilty}(\%) = \frac{\text{OD test average} - \text{OD blank average}}{\text{OD control cell average} - \text{OD blank average}} \times 100$$

OD test average   = Average absorbance of cells when incubated with crude extract

OD control cell average   = Average absorbance of cells when incubated with 10% DMSO

OD blank average   = Average absorbance of PRMI-1640 medium

3.7 Apoptosis Test

Morphological changes of apoptotic cells induced by crude extract such as shrinking, blebbing and formation of apoptotic bodies (DNA fragmentation) can be detected by staining of DNA with Hoechst 33342 fluorescence dye and observed under the fluorescence microscope. The dye binds with AT pairs in the DNA. Healthy cells emit only weak blue fluorescence since the DNA is distributed evenly in nucleus. The nucleus of apoptosis cells is of smaller size and due to condensed DNA shows a strong blue signal. Apoptotic bodies are visible as small clumped dots.

3.7.1 Apoptosis Test for Adhesive Cells (A375, BT474, HepG2, KATO III, and SW620)

Cell suspensions at 1×10^6 cells/ml were cultured on 35 mm coverslips in petridishs and incubated at 37°C, 5% CO_2 for 24 h. Subsequently, the cells were treated with 50 μl of crude extract. Negative control cells were treated with 0.1%

DMSO. Positive control cells were treated with100 μg of etoposide. After that, cells were washed with phosphate buffered saline (PBS) at pH 7.4 and fixed with 1% glutaraldehyde in PBS for 2 h at room temperature in dark condition. Finally, the cells were washed with PBS and the nuclei were stained with Hoechst 33342 (1μg/ml in PBS) fluorescence dye and covered with a glass slide. Cell apoptosis was observed under fluorescence microscopy and the fluorescence images were recorded using a digital camera. In all, three different fields were randomly selected for counting. The percentage of apoptotic cells were calculated by dividing the average number of apoptotic cells by the total number of cells $\times$ 100.

3.7.2 Apoptosis Test for Non-adhesive Cells (Jurkat)

Jurkat cells at concentration of 1×10^{6} were cultured in 35 mm petridishes and incubated at 37°C, 5% CO_2 for 24 h. The cells were treated with 50 μl of crude extract, 50 μl of 10% DMSO as negative control and 10 μM of etoposide as positive control incubated for 18 h. After incubation, cells were harvested by centrifugation at 1000 rpm for 5 min and washed with PBS three times. Then, cells were fixed with 1% glutaraldehyde in PBS for 2 h at room temperature in dark conditions. Finally, the cells were washed with PBS and the nuclei were stained with Hoechst 33342 (1μg/ml in PBS) fluorescence dye and covered with a glass slide. The fluorescence images were recorded using a digital camera. Cell apoptosis were observed under fluorescence microscopy. The percentage of apoptotic cells in apoptosis were calculated by dividing the number of apoptotic cells by total number of cells $\times$ 100.

3.8 Identification of Selected Endophytic Fungi by Morphological Characteristics and Molecular Technique

3.8.1 Morphological Identification of Selected Endophytic Fungi

The selected endophytic fungi which showed the best antimicrobial and anticancer activities were identified by morphological characteristics using the microscopical features of colony and conidia morphology. Species identifications were done with reference to Barnett and Hunter (1998) and Von Arx (1978).

3.8.2 Molecular Identification of Selected Endophytic Fungi

The selected bioactive endophytic fungi were identified based on analysis of nucleotide sequences of the internal transcribe spacer (ITS) regions of rDNA (Figure 3-1).
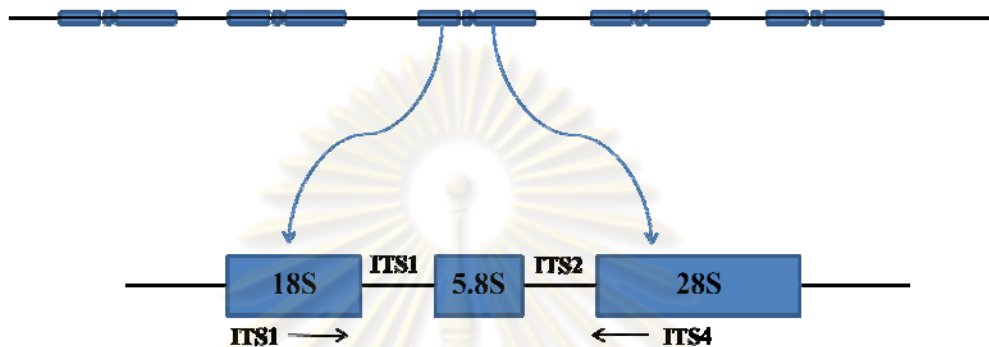
Figure 3-1: Internal transcribe spacer (ITS) regions of rDNA

3.8.2.1 DNA Preparation

The selected endophytic fungi were cultured into 100 ml MEB at room temperature for 7 days, and their mycelia were harvested and put into 2-ml extraction tubes to fill about one third of each tube with zirconia balls about 3-5 beads tube. DNA was extracted. Briefly, 700 μl of 2X CTAB lysis buffer [2% cetyltrimethylammonium bromide, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 0.5% b-mercaptoethanol] was added to homogenize. After incubation in a block heater at 65ºC for 1 h, 700 μl chloroform: isoamyl alcohol mixture (24:1,v/v) was added to the tube and then vortexed and centrifuged at 15000 rpm for 8 min at room temperature. The 650 μl of supernatant was transferred to another 1.5-ml tube. The DNA was precipitated by adding an equal volume of isopropyl alcohol and keeping the tube at - 20ºC for 15 min. Centrifuged at 8000 rpm for 10 min at, the DNA pellet was washed with 500 μl of 70% ethanol and then centrifuged at 8000 rpm at 4 °C for 5 mins. The DNA pellet was dried and dissolved in 100 μl TE buffer (10 mM Tris-HCl (pH 8.0), 1 mMEDTA), and stored at -20ºC until used.

3.8.2.2 Polymerase Chain Reaction (PCR) of endophytic fungi DNA

The polymerase Chain Reaction (PCR) is the method used to produce large number copies of a gene. It is important to have enough templates for sequencing. Each PCR tube contained 100 µl of PCR reaction mix consisting of table 3-3. The PCR amplification was conducted using primers ITS1 (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATAT- GC).

Table 3-3: PCR reaction mixture for primer ITS1-4

Reagent	Volume (ul)
Distilled water	55
10x NH$_4$ buffer	10
25 mM dNTP	16
MgCl$_2$	5
20 uM ITS-1	1.5
20 uM ITS-4	1.5
Biotaq^{TM} DNA polymerase	1
DNA template	10

The tube was mixed gently and placed in the PCR machine (Takara Thermal Cycler Dice Standard TP-65). The PCR cycle was as follows; initial denaturation at 94°C for 2 mins, followed by 30 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds and extension at 72°C for 1 minute, and finished by a final extension at 72°C for 7 minutes. The PCR products were evaluated on a 1% agarose gel.

3.8.2.3 Sequencing

Each sequence was used as query sequence to search for similar sequences from GenBank using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

3.9 Determination of Bioactive Metabolites of Endophytic Fungal Isolate PT11 in Different Rice Malt

3.9.1 Effect of Rice Malt on Growth and Metabolites Production

Effect of different rice malts on bioactive metabolite production by endophytic fungus PT11 were performed by using MEB containing 8 rice malts such as rice R-258, rice RD6, rice Leuang Pratew, rice Khao Tah Haeng, rice Pathum Thani 1, rice

Jek Chuey, rice Muey Nawng, Japanese rice Sasanishiki and Akitakomachi and 2 malt extracts, Malt extract from Merck Ltd. and Malt extract from Lab-scan.

3.9.1.1 Media Preparation

Fifty Gram of each rice malt power was dissolved in 300 ml distilled water followed by shaking for 30 min to dissolve the malt, then filtered with filter paper to remove un-dissolved particles. The malt solution was mixed with 0.3 g peptone and 6 g glucose and adjusted to pH 5.4 and volume of 300 ml. The MEB media were autoclaved at 121ºC for 15 min.

3.9.1.2 Bioactive Compound Production

Fungal endophytic fungus isolate PT11 was grown on 2x150 ml of MEB medium containing 9 different malted rice powders compared with malt extract from Merck and Lab-scan incubated at room temperature for 14 days under static conditions.

The cultures were filtered through No. 4 filter paper. The filtered broths were extracted exhaustively with ethyl acetate. The solvent phase from culture broth and mycelia were pooled and evaporated to dryness under reduced pressure using a rotary evaporator and crude extracts obtained. The mycelia were dried at 80ºC until constant weight, biomass determined.

Ten milligram of crude extracts were dissolved in 1 ml of 10% of DMSO in sterile distilled water and kept at -20°C prior to testing for their antimicrobial properties by paper disc diffusion method in the same manner as described in section 3.6.2.

3.9.1.3 Bioactive Compound Profile

The bioactive compounds profiles of endophytic fungusPT11, which was grown on different rice malts were performed using Thin layer chromatography (TLC). The sovent systems were hexane:EtOAc;45:55, Hexane:EtOAc:MeOH; 50:49:1, hexane:EtOAc:MeOH;50:45:5, Chloroform:MeOH;95:5. Bioactive compound profiles were detected by visual appearance under ultraviolet light at wavelengths 254 and 365 nm and with exposure to iodine vapour.

3.9.2 Determination of Metabolite Profile of the Culture Broth Crude Extracts
        from Endophytic Fungal PT11

A few milligrams of the crude extracts of rice malts in section 3.9.1.2 were dissolved in ethyl acetate for metabolite profiles determination by using the Thin-layer chromatography (TLC) technique as described below.

Analytical Thin-layer chromatography (TLC)

Technique	One dimension ascending
Adsorbent	silica gel F_{254} coated on aluminium sheet (Merck)
Layer thickness	250 μM
Temperature	Room temperature (27-30ºC)
Distance	10 cm
Detection	1. Visual detection under UV light at 254 and 365 nm 2. Visual detection in iodine vapour

3.10 Isolation of Secondary Metabolites of Endophytic Fungus Isolate PT11

3.10.1 Cultivation of Selected Endophytic Fungus Isolate PT11

Fungal endophyte isolate PT11 was grown on MEA at room-temperature for 7 days. The agar culture were then cut into 6 mm diameter discs by a flamed cork hole borer. Eight discs were inoculated into 200 ml of MEB in 500 ml Erlenmeyer flak. The cultures were incubated at room temperature (25-30°C) for 30 days. A large scale of cultivation (20 L culture medium) was carried out using 100 ml of MEB in 500 ml Erlenmeyer flaks.

3.10.2 Extraction of Endophytic Fungus Isolate PT11

The fermentation broth of endophytic fungus isolate PT11 was filtrated through 4 layers of cotton gauze and exhaustively pressed. The filtrate was extracted with an equal volume of EtOAc 3 times. The solvent phase was evaporated to dryness

by using a rotary evaporator at 40°C. The crude extract of culture broth was obtained as a mixture white brown solid and brown viscous liquid (32 g).

The fungal mycelium (140 g dry weight) was grown and subsequently extracted with MeOH by stand overnight and then filtrated through filter paper. The solvent phase was evaporated to dryness by using a rotary evaporator at 40°C. The crude extract from fungal mycelium was obtained as a dark brown viscous liquid (5.6 g). The extraction scheme of the endophytic fungal isolate PT11 is described in Figure.

3.10.3 Isolation of Bioactive Compound of Endophytic Fungal Isolate PT11

The isolation and purification of secondary metabolites was performed using chromatography techniques.

The EtOAc crude extract of fungal PT11 was subjected to column chromatography (silica gel, 100 g), using a wet packing and dry loading method. Eluents of increasing polarity of hexane, hexane-EtOAc, EtOAc, EtOAc-MeOH and MeOH were used. Fractions were collected and examined. The similar fractions were pooled together based on an their TLC pattern and dried. The results from the isolation of crude extracts were presented in Table 3-4. Figure 3-2 and Figure 3-3 shows the details of the schematic isolation of secondary metabolites from PT11. The biological activity of each pool fraction was examined and described.

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┌─────────────────────────────────────────────────────────────────┐
│  ╭─────────────────────────────────────────────────────────────╮ │
│  │  Endophytic fungal isolate PT11 in 100x200 ml MEB in 500 ml  │ │
│  │                     Erlenmeyer flasks                         │ │
│  ╰─────────────────────────────────────────────────────────────╯ │
│                            │                                       │
│                         Filtration                                 │
│            ┌───────────────┴───────────────┐                       │
│            ▼                               ▼                        │
│  ┌──────────────────┐          ┌──────────────────┐               │
│  │     Mycelium     │          │   Culture broth  │               │
│  └──────────────────┘          └──────────────────┘               │
│            │                               │                        │
│            ▼                               ▼                        │
│  ┌──────────────────┐          ┌──────────────────┐               │
│  │  Extract with    │          │  Extract with    │               │
│  │      MeOH        │          │      EtOAc       │               │
│  └──────────────────┘          └──────────────────┘               │
│        Stand overnight                     │                        │
│            ▼                               ▼                        │
│  ┌──────────────────┐          ┌──────────────────┐               │
│  │   MeOH Phase     │          │   EtOAc Phase    │               │
│  └──────────────────┘          └──────────────────┘               │
│        Evaporation                   Evaporation                   │
│            ▼                               ▼                        │
│  ┌──────────────────┐          ┌──────────────────┐               │
│  │ 5.6 g MeOH crude │          │ 32 g EtOAc crude │               │
│  │     extract      │          │     extract      │               │
│  └──────────────────┘          └──────────────────┘               │
│                                            │                        │
│                                            ▼                        │
│                                 ┌──────────────────┐               │
│                                 │ Further separation│              │
│                                 │ and isolation of  │              │
│                                 │ secondary         │              │
│                                 │ metabolites using │              │
│                                 │ chromatography    │              │
│                                 │ techniques        │              │
│                                 └──────────────────┘               │
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Figure 3-2: Scheme of the endophytic fungal isolate PT11

Table: 3-4 The results from separation of EtOAc crude from mycelia extract

Fraction code	Fraction No.	Eluents	Appearance	Weight
Fr01	79-125	15 % EtOAc in Hexane	Yellow viscous liquid	45 mg
Fr02	126-169	30 EtOAc in Hexane	Yellow viscous liquid	57 mg
Fr03	211-220	30% EtOAc in Hexane	Yellow viscous liquid	114 mg
Fr04	225-236	30% EtOAc in Hexane	Yellow viscous liquid	56 mg
Fr05	250-260	30% EtOAc in Hexane	Yellow viscous liquid	134 mg
Fr06	261-294	35% EtOAc in Hexane	Yellow viscous liquid	120 mg
Fr07	336-360	40% EtOAc in Hexane	Yellow viscous liquid	5.89 g
Fr08	361-364	40% EtOAc in Hexane	Yellow viscous liquid	502 mg
Fr09	390-460	40% EtOAc in Hexane	Yellow viscous liquid	225 mg
Fr10	491-510	45% EtOAc in Hexane	Yellow viscous liquid	432 mg
Fr11	511-620	45% EtOAc in Hexane	Yellow viscous liquid	1.52 g
Fr12	621-670	50% EtOAc in Hexane	Yellow brown viscous liquid	321 mg
Fr 13	671-720	50% EtOAc in Hexane	Yellow brown viscous liquid	300 mg
Fr14	951-980	70% EtOAc in Hexane	Brown viscous liquid	2.1 g
Fr 15	990-1000	90 % EtOAc in Hexane	Brown viscous liquid	5.2 g
Fr 16	1001-1010	100% EtOAc	Brown viscous liquid	3.5 g
Fr 17	1011-1130	10% MeoH in EtOAc to 100% MeOH	Brown viscous liquid	4.5 g

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

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┌─────────────────────────────────────────────────────────────────────────┐
│          ┌─────────────────────────────────────────────────┐             │
│          │  EtOAc crude extract of endophytic fungal PT11   │             │
│          │              (30 g dry weight)                   │             │
│          └─────────────────────────────────────────────────┘             │
│                              │                                            │
│                              │  Silica gel Column                         │
│                    ┌─────────┴─────────┐                                  │
│                    ▼                   ▼                                  │
│          ┌──────────────┐      ┌──────────────┐                           │
│          │     Fr07     │      │     Fr11     │                           │
│          └──────────────┘      └──────────────┘                           │
│                 │                     │                                   │
│                 ▼                     ▼                                   │
│        Silica gel Column     Silica gel Column                            │
│        EtoAc and hexane       EtoAc and hexane                            │
│            gradient               gradient                                │
│                 │                     │                                   │
│                 ▼                     ▼                                   │
│          ┌──────────────┐      ┌──────────────┐                           │
│          │   Fr07- SF1  │      │   Fr11-SF8   │                           │
│          └──────────────┘      └──────────────┘                           │
│                 │                     │                                   │
│                 ▼                     ▼                                   │
│        Silica gel Column     Silica gel Column                            │
│        EtoAc and hexane       EtoAc and hexane                            │
│            gradient               gradient                                │
│                 │                     │                                   │
│                 ▼                     ▼                                   │
│          ┌──────────────┐      ┌──────────────┐                           │
│          │  Compound 1  │      │  Compound 2  │                           │
│          └──────────────┘      └──────────────┘                           │
└─────────────────────────────────────────────────────────────────────────┘
```
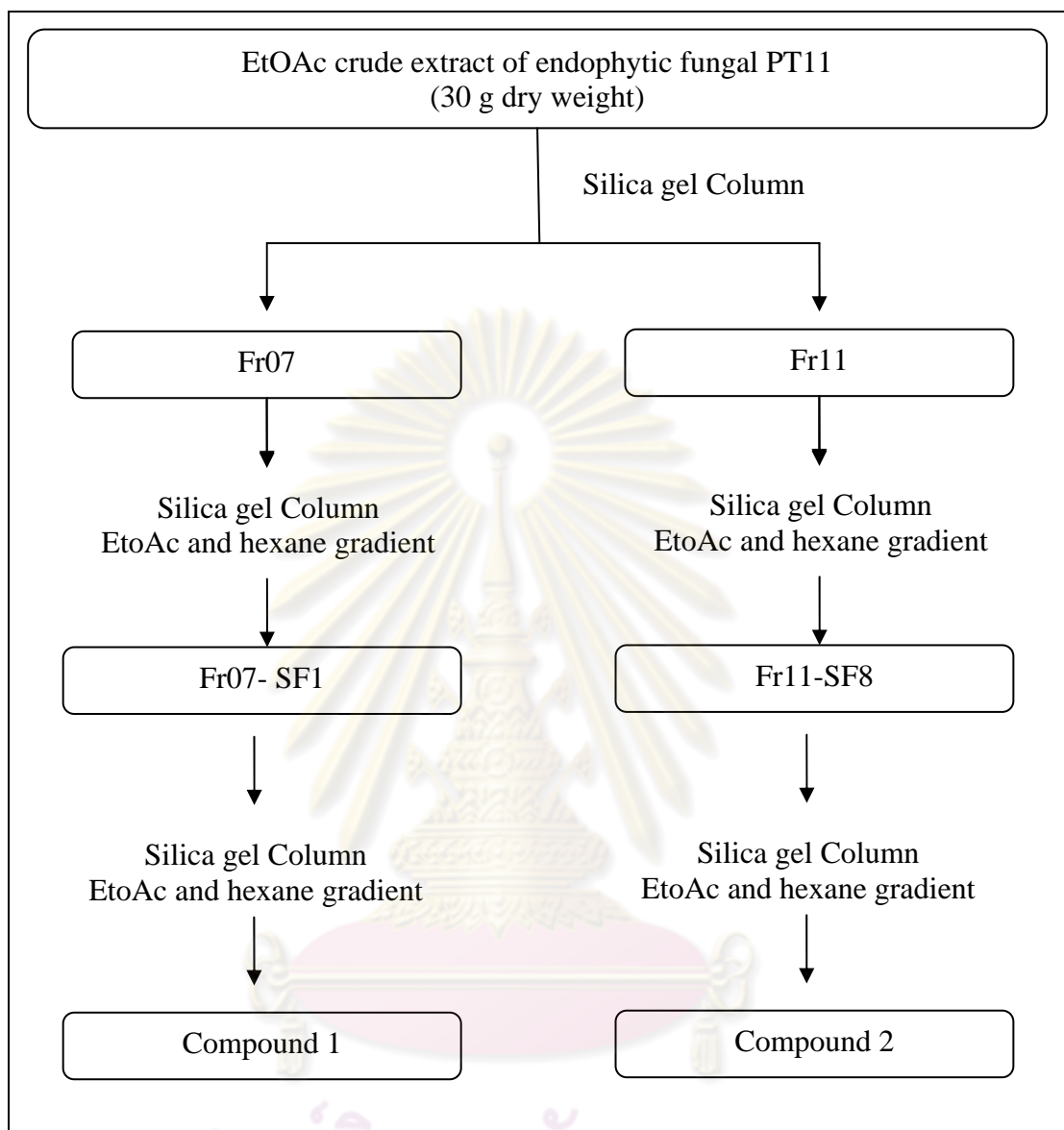
Figure: 3-3 Diagram of isolation of ethyl acetate crude extract of endophytic fungal PT11

3.11 Biological Activity Test

    3.11.1 Antimicrobial Activity Test

        3.11.1.1 Antimicrobial Activity of the Pool Fractions

Evaluation of the antimicrobial activity of the fractions was determined by the paper disc diffusion method in the same manner as described in section 3.6.2. Antimicrobial activity was performed against *B. subtilis* ATCC 6633, *S. aureus*

ATCC 25923, *P. auruginosa* ATCC 9023, *E. coli* ATCC 25922, *S. cerevisiae* TISTR 5169, *C. albicans* ATCC 1023.

- **Preparation of Pure Compounds**

Four mg of pure compounds were dissolved in 1 ml of 10% DMSO in sterile distilled water and kept at -20^{o}C before bioassay.

- **Preparation of Bacterial and Yeast inoculums**

Bacterial and yeast inoculums were prepared in the same manner as described in section 3.6.2.3 and 3.6.2.4

- **Assay Procedure**

Solutions of pure compounds and antibiotic drug standards were diluted with Mueller-Hinton Broth (MHB) and YMB for assays of antimicrobial activity. Fifty µl of the pure compound solution was dispended into each well into 96 well microculture plates, fifty µl of the final adjusted microbial suspension was inoculated into each well. One hundred µl of medium was the sterile control. A 100 µl of medium and microbial inoculums mixture acted as the growth control. The microculture plates were incubated at 37^{o}C for bacteria and *C. albicans* and 30^{o}C for *S. cerevisiae*, 24 h. The optical densities of the wells were measured with a microplate reader at 625 nm. The lowest concentration of pure compound showing complete inhibition of growth was recorded as minimal inhibition concentration (MIC).

3.11.2 Cytotoxicity Test

The Cytotoxicity activity of pure compound against human cancer cell culture in vitro was performed by the MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) colorimetric method (Palaga *et al*. 1996), described in section 3.6.3

3.11.3 Apoptosis Test

Induction of apoptosis cancer cell death of pure compound was examined as the same manner in section 3.7.

3.12 Biochemical Structure Analysis

3.12.1 Thin Layer Chromatography

Thin layer chromatography of fungal fermentation products was performed on Merck silica gel plated (0.25 mm thick) using various solvent systems.

3.12.2 Instruments and Equipments

3.12.2.1 Nuclear Magnetic Resonance spectrophotometer (NMR)

The ^{1}H NMR, ^{13}C NMR and 2D-NMR (COSY, HSQC, HMBC and NOESY) were obtained on a Varian, Mercury 400 using methanol-d4 (CD3OD).

3.12.2.3 Fourier Transform-Infrared spectrophotometer (FT-IR)

The IR spectra of all samples were recorded as KBR pellets using Nicolet Impect 410 Spectrophotometer.

3.12.2.4 Mass spectrometer (MS)

Low solution mass spectra were obtained with a Agilent 1100 HPLC-system connected to Agilent 1100 mass spectrometer with electrometer LCT, Micromass UK Limited.

3.12.2.5 Specific Optical Rotation

The optical rotation values were taken on a Perkin-Elmer 341 polarity at 20ºC.

3.12.2.6 Melting point Apparatus

Melting points were obtained on a Fisher-John Melting apparatus.

3.12.2.7 UV Spectrometer

UV-Vis spectra were record on Perkin Elmer Lamda 25 UV-Vis spectrophotometer in MeOH

3.12.2.8 Rotary Evaporator

The Eyela and Buchi rotary evaporator was used for the removal of volatile solvents.   CHAPTER   IV

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Isolation of endophytic fungi

A total of 106 isolates of endophytic fungi were obtained from healthy leave of *M. javanica* from Ayuthaya, Pathumthani and Amnatcharean provinces during April, 2006 – April, 2007. Forty-nine, twenty-six and thirty-one isolates of endophytic fungi were obtained from *M. javanica* leaves of Ayutthaya, Pathumthani and Amnatcharean province and designated as AY, PT and AM, respectively. The endophytic fungal isolates were characterized and identified according to culture characteristics, colony growth, and conidia morphology. The endophytic fungi were identified as *Colletotrichum* spp., *Fusarium* spp., *Phomopsis* spp., *Alternaria* spp., *Phyllostica* spp. *Xylaria* spp., *Penicillium* spp., a *Cladosporium* sp. *Aspergilus* spp. and the majority were mycelia sterilia. A list of isolated species is shown in Table B-1 (Appendix B). The most common endophytes assemblages in *M. javanica* leaves were Ascomycetes and their anamorphs.

The most common endophytic fungi presented in this study were *Phyllostica* spp., *Phomopsis* spp., *Alternaria* spp., *Colletotrichum* spp., *Xylaria* spp. and mycelia sterilia. Most of the taxa are common endophytic fungi, some of which were consistent with the fungi isolated from leaves of *M. speciosa* Korth (Kratom) studied by Singwongwatana (2005) and also similar to the fungal genera found in teak leaves (*Tectona grandis* L.) and rain tree (*Samanea saman* Merr.) growing in the campus of Chulalongkorn University. These were *Alternaria*, *Colletotrichum*, *Nigrospora*, *Phomopsis* and mycelia sterilia (Chareprasert et al., 2006). Gond et al. (2007) isolated endophytic fungi from healthy, living, and symptomless tissues of inner bark, leaf, and roots of the medicinal plant, *Aegle marmelos*. The results showed that maximum isolate recovery were hyphomycetes (78.5%) followed by ascomycetes (8.9%), coelomycetes (7.6%) and 5.1% isolates remained unidentified and were classified under mycelia Sterilia. Hata and Sone (2008) found that *Cytosphaera* sp., xylariaceous spp. and *Phomopsis* spp. were the most frequently isolated from

Neolitsea sericea leaves. Huang et al. (2007) reported that 42 endophytic fungal strains isolated from *Nerium oleander* were grouped into 14 different taxa, including Ascomycete sp., *Chaetomium* sp., *Cladosporium* sp., *Colletotrichum* sp., *Hyphomycete* sp., mycelia sterilia spp. (6 species), *Phoma* spp. (2 species), and *Torula* sp. Half of the fungal strains (50% relative frequency) lacking sporulating structures were grouped into mycelia sterilia spp. (Huang et al., 2007). Lin and co-workers (2007) isolated endophytic fungi from the pharmaceutical plant, *Camptotheca acuminate*. They reported that non-sporulating fungi (48.9%), *Alternaria* (12.6%), *Phomopsis* (6.9%), *Sporidesmium* (6.3%), *Paecilomyces* (4.6%) and *Fusarium* (4.6%) were dominant. This is consistent with previous reports (Petrini; 1991; Saikkonen; 1998). Most of the endophytic fungi did not produce conidia or spores when cultured on common mycological media (Wiyakruta et al., 2004).

4.2 Antimicrobial activities of endophytic fungi

All endophytic fungi isolates were grown in malt extract broth (MEB) for 30 days. Fermentation broth was separated from mycelium by filtration and extracted exhaustedly with ethyl acetate. The solvent phase was dried by evaporation, dissolved in 10% DMSO and ethyl acetate crude extracts were obtained.

The ethyl acetate crude extracts of fermentation broth of 106 endophytic fungi were tested for antimicrobial activities against 6 standard tested microorganisms as following *B. subtilis, S. aureus*, *E. coli*, *P. aeruginosa*, *S. cerevisiae* and *C. albicans* by paper disc diffusion assay. Figure 4-1, Figure 4-2 and Table B-2, B-2, B-2 (Appendix B) show the number and percentage of the ethyl acetate crude extracts, displaying antimicrobial activities with inhibition zones ranging from 7- 40 mm. Eighty isolates (75.47%) of endophytic fungi produced bioactive compounds that exhibited antimicrobial activity against at least one test microorganism with inhibition zones that ranging from 7-40 mm. There were 50 (47.17%), 57 (53.77%), 21 (19.81%), 26 (24.52%), 15 (14.15 %), 13 (12.26%) isolates   were inhibited *B. subtilis*, *S. aureus*, *E. coli*, *P. aeruginosa*, *S. cerevisiae*, and *C. albicans* respectively, and 6 (5.66%) isolates showed a broader antimicrobial spectrum. The broad spectrum isolates were AY 03, AY 10, PT08, PT11, AM04 and AM09. These six isolates could be good candidates for further studies of their antimicrobial activities.

The Gram-positive bacteria appeared to be more susceptible to the inhibitory effect of the ethyl acetate crude extracts than Gram-negative bacteria and yeast. The outstanding endophytic fungal isolate PT 11 had the strongest antimicrobial activity inhibiting against all bacteria and yeast, as shows in Figure 4-3 and Table 4-1. The results suggest that most effective endophytic fungal isolate PT 11 could produce effective bioactive compounds. Therefore, this fungus was selected for isolation and elucidation of the active metabolites.

Table 4-1: The antimicrobial activities of broad spectrum isolates

Isolate	Tested Microoorganisms and inhibition zone					
	B. subtilis	*S. aureus*	*P. aeruginosa*	*E. coli*	*S. cerevisiae*	*C. albicans*
AY 03	++	++	++	++	++	++
AY 10	++	++	++	++	++	++
PT 08	++	+++	+	++	+++	+++
PT 11	+++	+++	+++	+++	+++	+++
AM 04	++	++	++	++	+	+
AM 09	+	+++	++	++	+	++

+++ Inhibition zone above 20 mm

++ Inhibition zone between 10-20 mm

+ Inhibition zone less than 10 mm
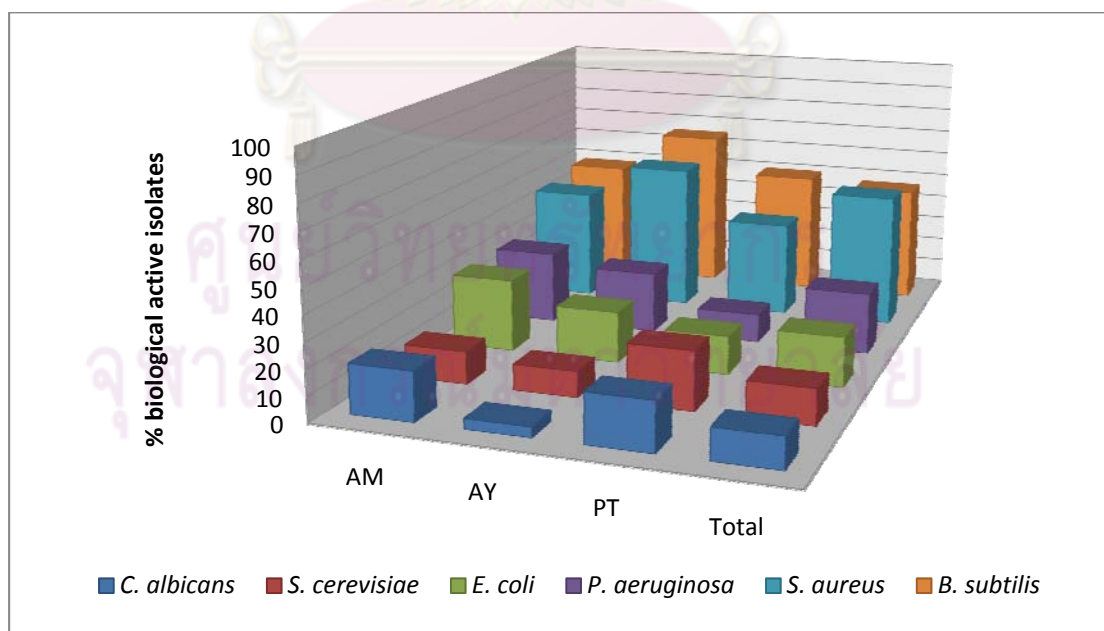
Figure 4-1: Percentage of active endophytic fungi exhibited antimicrobial activities against tested microorganisms in Ayuthaya, Pathumthani and Amnatcharean provinces

	B. subtilis	S. aerues	P. aeruginosa	E. coli	S. cerevisiae	C. albicans	Broad spectrum	No activity
Percentage	47.17	53.77	24.52	19.81	14.15	12.26	5.61	24.52
Isolate	50	57	26	21	15	3	6	26
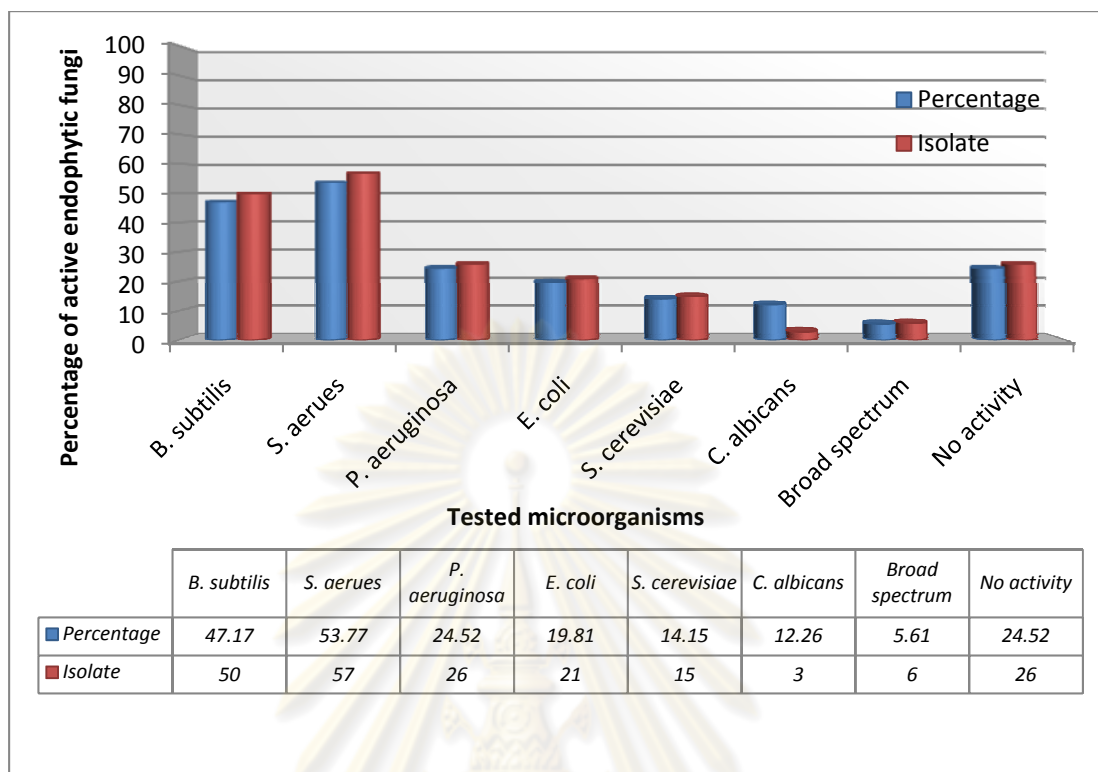
Figure 4-2: Percentage of active endophytic fungi exhibited antimicrobial activities against tested microorganisms; BS: *B. subtilis*, SA: *S. cerevisiae*, EC: *E. coli*, PA: *P. aeruginosa*, SC: *S. cerevisiae*, CA: *C. albicans*, and All: Broad spectrum
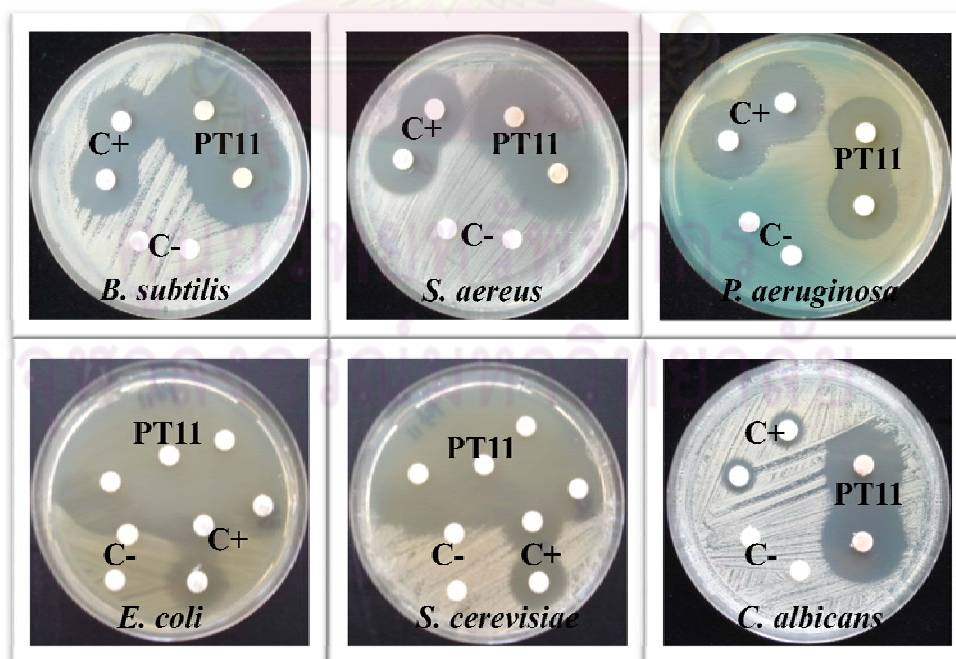
Figure 4-3:   Endophytic fungal PT11 crude extract exhibit broad spectrum

Several preliminary studies have been reported on screening of endophytic fungi from medicinal plants for production of antimicrobial activities found that more than 50% of isolates displayed antimicrobial activity against at least one pathogen and more than 30% had antibacterial and antifungal activities (Wang et al., 2006). For example, Gong and Guo (2009) reported that 56% of endophytic fungi from *Dracaena cambodiana* (Agavaceae) inhibited growth of at least one of the test organisms and 8 % showed broad spectrum inhibition. Wang et al. (2006) isolated endophytic fungi from *Quercus variabili*, 36 strains (53.7%) could produce substances that were inhibitory to all or some of the tested human pathogenic bacteria and fungi and 19.4% of strains showed a broader antimicrobial spectrum.

Some studies, however, have reported that less than 30% of endophytic fungi showed antimicrobial activities. For example, Phonpaichit et al. (2006) isolated endophytic fungi from surface sterilized leaves and branches of five *Garcinia* plants, *G. atroviridis*, *G. dulcis*, *G. mangostana*, *G. nigrolineata* and *G. scortechinii*, found in southern Thailand and these were screened for antimicrobial activity. The results revealed that 18.6% of the isolates displayed antimicrobial activity against at least one test microorganism with inhibition zones that ranged from 7-19 mm. Approximately half of the active isolates (43–53%, 30–37 isolates) displayed activity against strains *S. aureus* and *Cryptococcus neoformans* but only 4.3% inhibited *C. albicans*. Lin et al. (2007) also reported that 27.6% of the endophytic fungi isolated from *C. acuminata* displayed inhibition against more than one indicator microorganism. These bioactive endophytic fungal strains were found to be the largest and most widely distributed, non-sporulating fungi, and were also the major source of bioactive strains.

Most of the bioactive metabolite compounds from endophytic fungi reported more effective against Gram-positive bacteria than Gram-negative bacteria and pathogenic fungi. For example, most of the antimicrobial activities of endophytic fungi from teak and rain tree **were specific for Gram-positive** (Chareprasert et al., 2006). **None of the endophytes isolated from *Garcinia* sp inhibited Gram-negative bacteria (Pongpaichit et al., 2006).**

4.3 Anti cancer activities

The ethyl acetate crude extracts from the culture of 106 endophytic fungi were examined for anticancer activities against 6 cell lines (human skin cancer(A375), human breast cancer (BT 474), human gastric cancer (KatoIII), human intestine cancer (SW620), human liver cancer (HepG2) and human leukemia (Jurkat)) by MTT assay. The percentage of endophytic fungal crude extracts from Ayuthaya province displayed anticancer activities higher than crude extracts from Pathumthani and Amnatcharean provinces (Figure 4-4 and Table B-6, B-6 and B-7 in appendix B). Figure 4-5 shows number and percentage of endophytic fungi, having anticancer activities toward 6 cell lines below 40% of cell viability. The results showed that 62(58.49%), 48(54.28%), 64(60.38%), 62(58.49%), 65(61.32%) and 33(33.13%) endophytic fungi exhibited anticancer activities against A375, BT 474, KatoIII, SW620, HepG2 and Jurkat, respectively. Five endophytic fungi isolates showed cytotoxicity toward all cell lines.
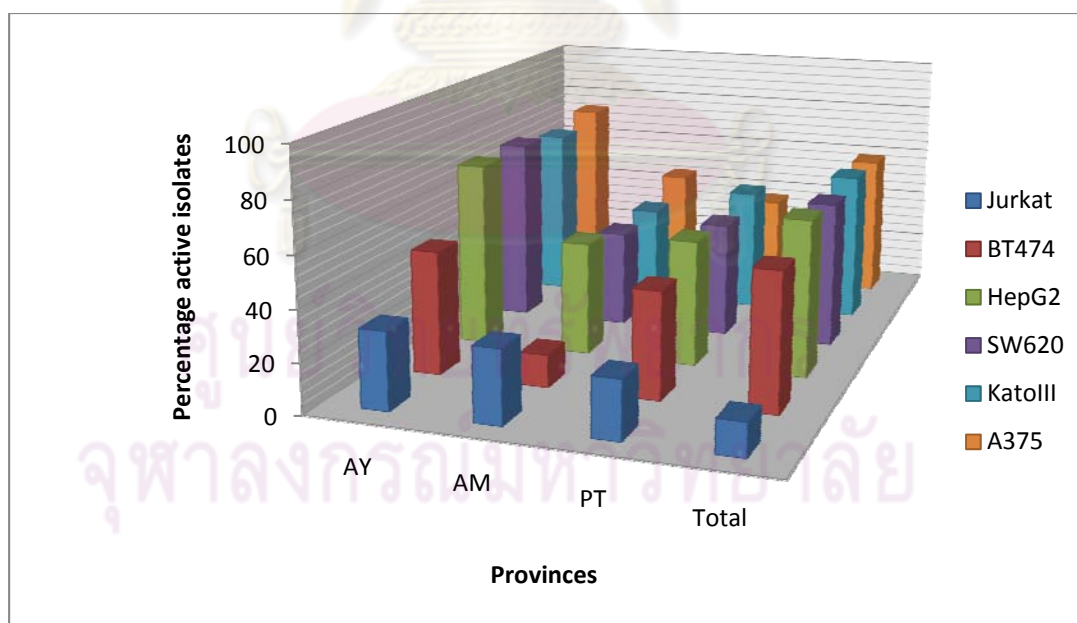
Figure 4-4: The percentage of anticancer active endophytic fungi isolates from different provinces

	A375	BT474	KatoIII	SW620	Jurkat	HepG2	All cell lines	No activity
▪ Percentage	58.49	45.28	60.38	58.49	31.13	61.32	4.71	15.09
▪ Isolate	62	48	64	62	33	65	5	16
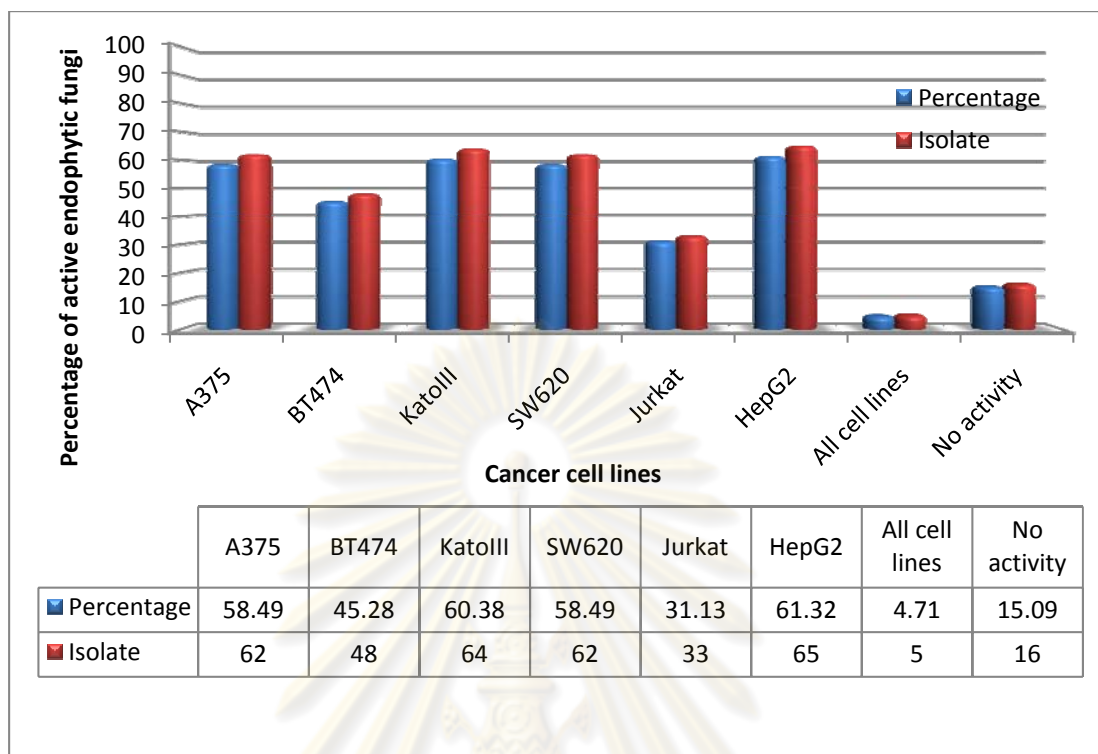
Figure 4-5: Percentage of active endophytic fungi displaying anticancer activities

Fourteen endophytic fungal isolates displayed strong specific cytotoxicity toward cancer cell lines as follows: the endophytic fungi AY 07, AY 16, AY39 and PT26 had specific activity to A375. Whereas, AY19, AY 25, AY41, AY 49, PT 01, AM28, and AM32 had strong specific cytotoxic activity against KatoIII cells. The endophytic fungal AY03, AY16, AY 25 and AY 30 showed strong actively towards SW620. The endophytic fungi AY07, AY 16, AY45, PT01 and AM17 exhibited HepG2. While, the endophytic fungi PT34, AM 28 also had specific cytotoxic activity against BT474 and PT01 demonstrated strong specific actively towards Jurkat (0% viability) (Table 4-2). These fungi were evaluated for their potential apoptotic-inducting cell death to specific target cancer cells.

Table 4-2: The endophytic fungal isolates having strong specific anticancer activities; shows as a percentage of viability

Isolate	A375	KatoIII	SW620	HepG2	BT474	Jurkat
AY 03	-	-	10.19±1.61	-	-	-
AY 07	21.03±4.60	-	-	7.11±2.25	-	-
AY16	-	-	7.87±0.53	15.01±0.75	-	-
AY 19	-	9.05±1.40	25.08±2.93	-	-	-
AY 25	-	15.53±4.33	-	-	-	0.0
AY 39	11.16±2.90	-	-	-	-	-
AY 41	-	11.13±4.22	13.55±3.11	-	-	-
AY 45	-	-	-	11.54±4.14	-	-
AY49	-	16.95±0.53	-	-	-	-
PT 01	-	28±1.65	-	-	-	0.0
PT 26	16.21±2.69	-	-	-	-	-
AM 17	-	-	-	14.32±0.34	-	-
AM 28	-	12.98±2.84	-	-	23.15±0.97	-
AM 32	-	13.28±5.22	-	-	-	-

Wiyakruta et al. (2004) reported that 17.0% of endophytic fungi from 81 Thai medicinal plant species exhibited anticancer activity against KB and BC-1 cancer cell lines, respectively. Li et al. (2005) also reported that 9.2 % of endophytic fungi from 12 Chinese traditional medicinal plants displayed antitumor activity against human gastric tumor cell line. Lin et al. (2007) reported that 4.0% of endophytic fungi isolated from the pharmaceutical plant, *Camptotheca acuminate* showed cytotoxic activity against tested tumor cell lines. These reports strongly support that for that endophytic fungi from medicinal plants are a promising sources of natural anticancer-active compounds.

4.4 Cytotoxicity of endophytic fungi by apoptosis

The fourteen isolates of the most specific active endophytic fungi (Table 4-2) were evaluated for their potential to induces apoptosis cell death (DNA fragmentation) in cancer cells when stained with Hoechst 33258 fluorescence stain. DNA fragmentation (apoptotic body) in particular has been used as an indication of apoptosis when observed under fluorescence microscopy. After treatment with each

crude extract at a 5% (v/v) final concentration, the cancer cells morphology were determined. Six isolates produced apoptotic bodies when observed by fluorescence microscopy, indicating that these crude extracts had induced apoptosis.

After treatment of jurkat-human leukemia cells with PT01 crude extract for 24 h, the result showed that PT01 induced DNA fragmentation with morphologically features of apoptosis of about 90.04%. Whereas, AY16 induced 88 % apoptotic cell death in human colon cancer (SW620) cells after treated for 36 h. AY 41 had apoptosis-inducing activities against the human breast cancer and gastric cancer (Kato III) at 84.80% after incubate for 24 h, while AY03 and AY 07 were showed weak induction of apoptosis in SW620 at 20.44% and liver cancer (HepG2) cells at 19.26 %, respectively. On the other hand, AY 19, AY 25, AY 39, AY 45, AY 49, PT26, AM17, AM28 and AM 32 did not show apoptosis inducing activity. Therefore, the non-inducing apoptotic isolates may have the ability to cause anti-proliferation of cancer cells by necrosis.

The results from fluorescence microscope of Jurkat, Kato III, SW620 and HepG2 cells after treated with ethyl acetate crude extracts, cell morphological changes such as cell shrinkage, DNA fragmentation and formation of apoptotic bodies when compared to the cancer cells treated with etoposide as positive control. On the contrary, the DNA of treated with 0.1 % DMSO (v/v) and untreated cells (negative control) were display evenly across the nucleus and visible as faint blue circles and did not show these apoptotic characteristics (Figure 4-6).
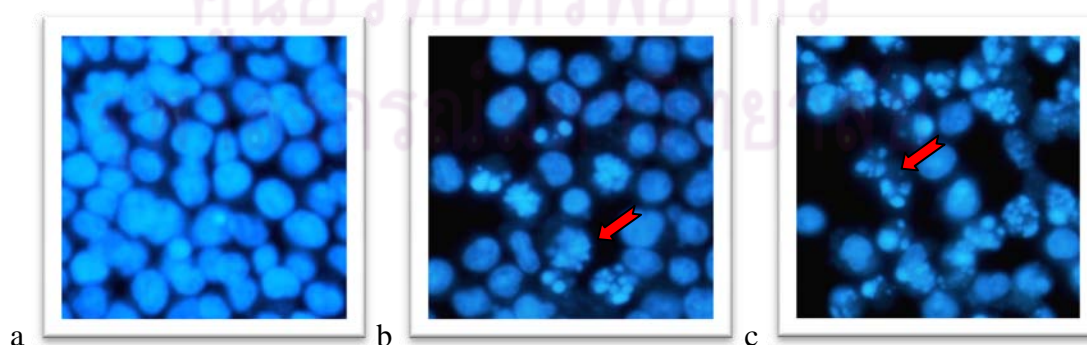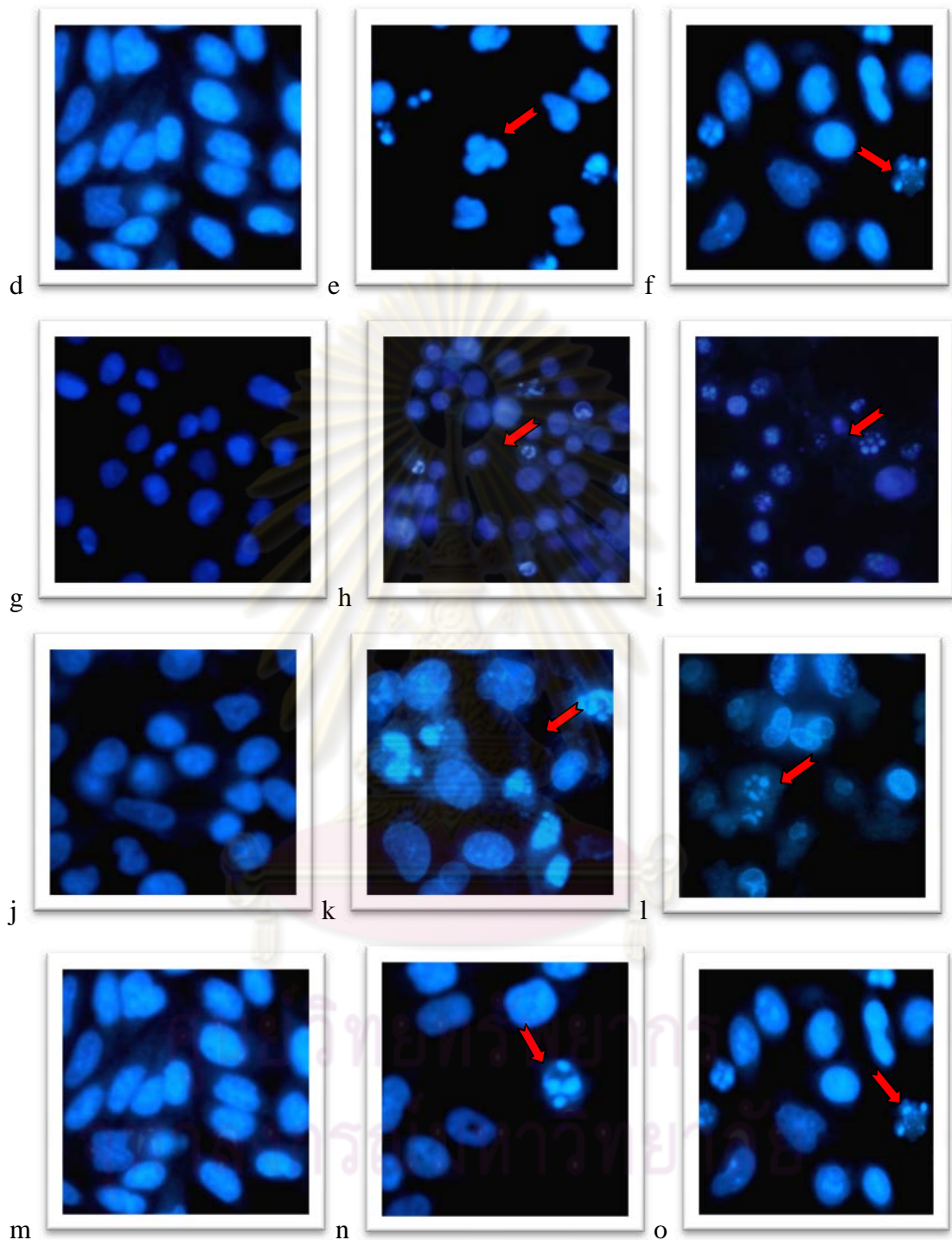
Figure 4-6: Endophytic fungal crude extracts induced apoptosis in human cancer cell lines; Jurkat (a, b, c), SW620 (d, e, f), Kato III (g, h, i) and HepG2 (j, K, l) Image a, d, g and k show untreated cells; b, e, h, k and n treated with endophytic fungal isolate PT01, AY16, AY 41, AY 07 and AY03 crude extract respectively; c, f, I, l and o

treated with etoposide. Treated cells display nuclear fragmentation and apoptotic bodies (arrows). Magnification x400
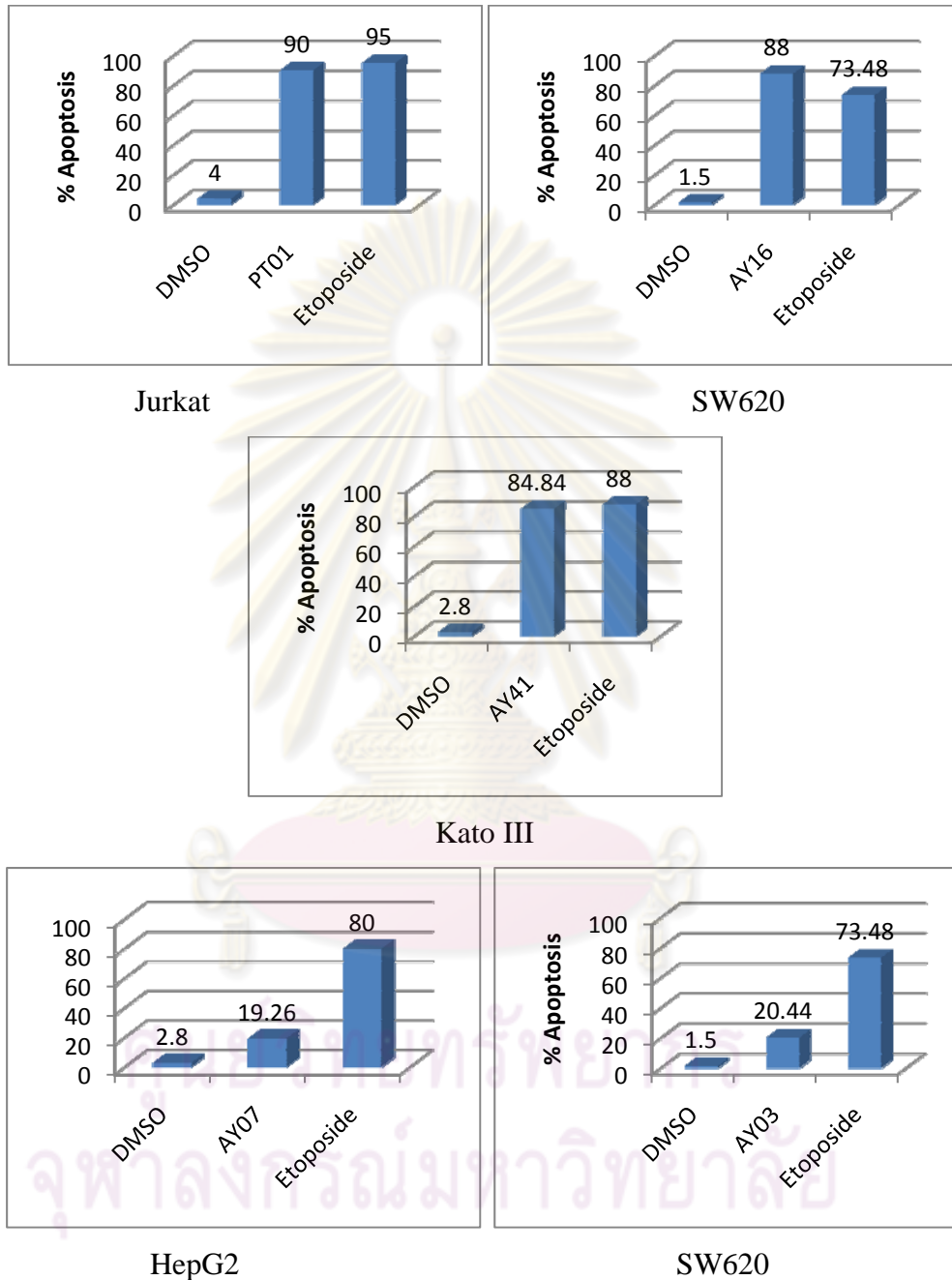
Figure 4-7: Percentage apoptosis of endophytic crude extract isolate PT01, AY16, AY 41, AY07 and AY 03 in Jurkat, Sw620, KatoIII, HepG2 and SW620, respectively

Cancer is a disease caused by an abnormal growth of cells, which tend to proliferate in an uncontrolled way because of genetic defects in programmed cell death.

Apoptosis is just one form of programmed cell death which the cell plays as an active role in its own demise; cells may be eliminated by a number of alternative mechanisms including necrosis. Necrosis is typically described as a "non-specific" from of cell death, characterized by rupture of plasma membrane and damage to surrounding cells and tissue. Apoptosis is a genetically controlled process that plays important roles in embryogenesis, metamorphosis, cellular homeostasis, and as a defensive mechanism to remove infected, damaged, or mutated cells. Molecules involved in cell death pathways are potential therapeutic targets in immunologic, neurologic, cancer, infectious, and inflammatory diseases. Apoptosis is a complex sequential process of genetically determined self-destruction that ultimately leads to the activation of proteases with certain substrate specificities, the caspases, and nucleases that produce membrane blebs, degrade DNA into nucleosomesized fragments and condensate cellular compartments (Reimann and Schmitt, 2007). The remainders, so called apoptotic bodies, are subject to phagocytosis devoid of a harmful inflammatory response. Molecules involved in cell death pathways are potential therapeutic targets in immunologic, neurologic, cancer, infectious, and inflammatory diseases (Khan and Pelengaris, 2007) Apoptosis occurs in several pathological situations and is characterized by morphological changes that include progressive cell shrinkage, condensation and fragmentation of nuclear chromatin, and membrane blebbing (Park et al, 2008). Apoptosis is an energy-dependent mode cell death requiring active participation of the target cell (Gangadevi and Muthumary, 2007). Addition, Taxol is an important anticancer drug used wildly in the treatment of human malignancies, particularly ovarion and breast cancer and its mode of action was revealed by induced apoptotic cell death. Therefore, this is potential for selected endophytic fungi to produce novel-anti-cancer approaches that specifically target and induce cancer cells to apoptosis. The results, indicated that endophytic fungal PT01, AY16 and AY 34 should be investigated further and their isolated and characterized bioactive compounds.

Endophytic fungi are one of the most unexplored and diverse group of organisms that make symbiotic associations with higher life forms and may produce

beneficial substances for host (Khan et al., 2007). Fungi have been widely investigated as a source of bioactive compounds.

The results of biologically activities of fungal crude extracts of *M. javanica*, suggest that the bioactive metabolites of endophytes producers may vary with the biotopes, different environments as well as a organism level. Schulze and others (2002) reported that in optimizing the search for new bioactive secondary metabolites, it is relevant to be consider that: (1) the secondary metabolites a fungus synthesizes may correspond with its respective ecological niche and (2) that metabolic interactions may enhance the synthesis of secondary metabolites. Thus, the fungi screened should originate from biotopes from which fungi have not been previously isolated for biochemical purposes and they should have metabolic interactions with their environment. This is an example of intelligent screening and is a strategy for exploiting the untapped potential for secondary metabolites that fungi offer. Similar evaluation for their potential to induce apoptosis cell death (DNA fragmentation) in cancer cells as reported by Tan & Zou (2001) who reviewed the diversity of metabolites that have been isolated from endophytic fungi emphasizing their potential ecological role. Many reports revealed that in a microbe-plant relationship, endophytes contribute substances which posses various types of bioactivity (a potent antitumor agents), for example, amptothecin (Puri *et al*., 2005), podophyllotoxin (Eyberger *et al*., 2006) and vincristine (Yang *et al*., 2004).

Thus, the endophytes from *M. javanica* from different habitats can increase the chance of finding novel compounds to possessing biological activity.

4.5 Identification selected endophytic fungi

The active endophytic fungi were identified on basis of microscopic morphology and nucleotide sequence of ITS region.

4.5.1 Identification of active antimicrobial activities fungal endophyte PT11

4.5.1.1 Morphological identification

The fungal isolate PT11 did not produce spore or conidia on PDA, MEA and SA. Therefore, the isolate PT11 was classified as Mycelium sterilia. The colony characteristic shows white colour on MEA and mycelia characteristics of this fungus is shown in Figure 4-8 and Figure 4-9

Figure 4-8: Endophytic fungal isolate PT11colony grew on MEA at room temperature for 10 days
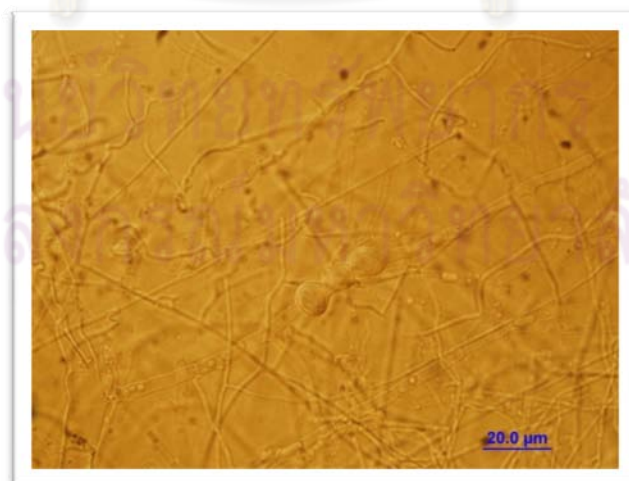
Figure 4-9: Light micrograph of endophytic fungal mycelium isolate PT11

4.4.1.2 Molecular identification

The rDNA ITS region of endophytic fungus isolate PT11 was amplified with the conserved fungal primer ITS1f and ITS4. The length of corresponding fragment was 511 bases. The nucleotide rDNA sequence of ITS was automatically aligned to compared with the available ITS sequences in the GenBank DNA database (http://www.ncbi.nlm.nih.gov/). The ITS sequence of endophytic fungal isolate PT11 was closely related to 99% identity of *Nodulisporium*.

4.5.2 Identification of active anticancer fungal endophyte PT01

Endophytic fungal isolate PT01 was chosen for cytotoxicity compounds. This was because crude extract of fungal PT01 showed 100% inhibition Jurkat cells and inhibited Kato III (28% viability). PT01 induced apoptosis towards Jerkat-leukemia cells. Therefore, this fungus was specific to Jurkat cells.

Colony characteristics of PT01 grown on MEA, mycelium is gray and produced red pigment. This fungal also produced pycnidia and conidia (Figure 4-10, 4-11 and 4-12).
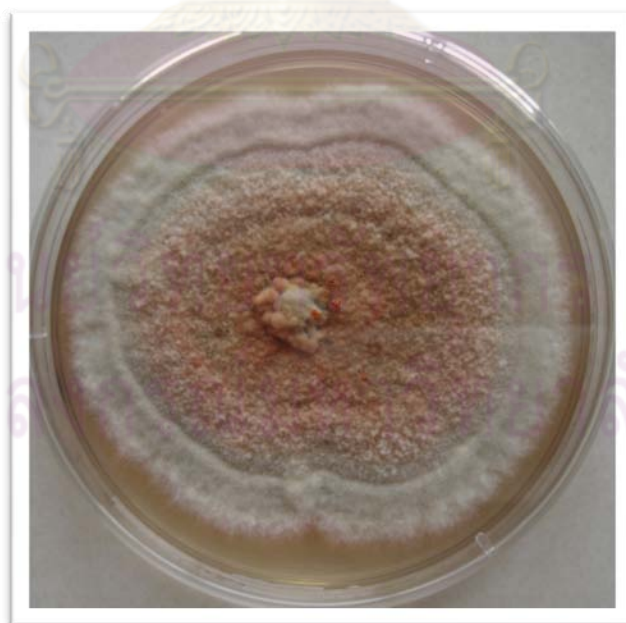
Figure 4-10: Endophytic fungal isolate PT 01colony grew on MEA at room temperature for 10 days

Figure 4-11: Light micrograph of endophytic fungal mycelium isolate PT 01
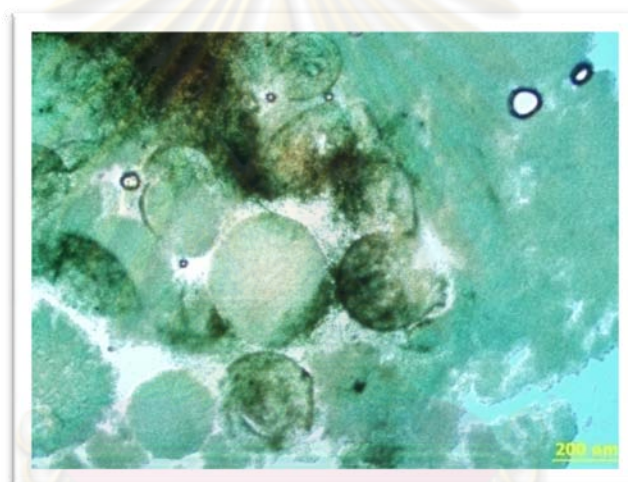
Figure 4-12: Mycelial characteristic of endophytic fungal isolate PT 01 on MEA

4.5.2.1 Molecular identification

The rDNA ITS region of endophytic fungus isolate PT01 was amplified with the conserved fungal primer ITS1f and ITS4. The length of corresponding fragment was 511 bases. The nucleotide rDNA sequence of ITS was automatically aligned to compare with the available ITS sequence of known species in the GenBank DNA database (http://www.ncbi.nlm.nih.gov/) The ITS sequence of endophytic fungal isolate PT01 was closely related to 99% identity of *Phoma herbarum*.

4.6 Bioactive metabolites of endophytic fungal isolate PT11 in different rice malts

4.6.1 Bioactive compound production and extraction

The fungal isolate PT11was cultured in 150 ml of MEB supplement with different 2% rice malts, RD6, R-258, Muey Nawng, Leuang Pratew, Jek Chueng, . Khao Tah Haeng, Pathum Thani 1, Sasanishiki, Akitakomachi compared with 2 malt extracts, (Merck and Lab-Scan) in static condition at room temperature (27-35ºC) for 14 days. The culture broth was filtrated and extracted with 3x100 ml of ethyl acetate. The solvent phase was dried under rotary vacuum and the crude extract obtained for future studies. Mycelial determined biomass as cell dry weight. The results shown in Table 4-3.

Table 4-3: Biomass, crude extract concentration and appearance of crude extract from endophytic fungi PT11

Rice malt	Cell dry weight (g/l)	Crude extract (mg/l)	Appearances of crude extract
1. RD6	9.13	566.95	Yellow brown viscous liquid
2. R-258	3.98	466.90	Yellow brown viscous liquid
3. Muey Nawng	8.47	1100.55	Yellow brown viscous liquid
4. Leuang Pratew	6.27	600.30	Yellow brown viscous liquid
5. Jek Chueng	6.58	900.45	Yellow brown viscous liquid
6. Khao Tah Haeng	6.58	1700.85	Yellow brown viscous liquid
7. Pathum Thani 1	4.91	667.00	Yellow brown viscous liquid
8. Sasanishiki	3.68	1300.65	Yellow brown viscous liquid
9. Akitakomachi	6.77	566.95	Yellow brown viscous liquid
10. Malt extract (Merck Ltd.)	2.63	566.95	Yellow brown viscous liquid
11. Malt extract (Lab-Scan)	3.15	125.00	Yellow brown viscous liquid

The highest amount of crude extract yield was obtained from MEB containing Khao Tah Haeng malt at concentration 1.7 mg/ml with a final mycelial biomass of 6.58 g/l. Sasanishiki malt, Muey Nawng malt and Jek Chueng malt were showed high crude extract yield concentration of 1.3 mg/ml, 1.1mg/ml and 0.9 mg/ml, respectively. Whereas RD6, Leuang Pratew malt, Pathum Thani 1 malt, Akitakomachi malt, malt extract from Merck Ltd. and R-258 revealed moderate bioactive compound crude extract, but malt extract from Lab-scan showed minimum crude extract concentration at 0.12 mg/ml. The maximum biomass concentration (8.47 g/l) was found in MEB containing RD6 malt. The fungal grew very well in MEB containing Muey Nawng malt, in contrast with Malt extract from Merck and Sasanishiki the fungus grew very slowly but they exhibited high product yield (gram product per gram biomass) 0.35 and 0.22, respectively. It is possible that malted rice effected on growth as well as secondary metabolite compound production.

Vahidi et al. (2006) reported that a Basal medium combination with malt extract and combination of glucose plus malt extract as carbon source increased biomass and activity of the antifungal agent produced by *Gymnopilus spectabilis*. Medium containing glucose and malt extract was showed inhibited 100% spore germination of *Aspergilus niger*.

Gogoi et al. (2208) studied impact of submerged culture condition on growth and bioactive metabolite produced by the endophyte *Hypocrea* sp., the result showed that glutamic acid in combination with sucrose enhanced growth as well as metabolite synthesis of *Hypocrea* sp.

Malting is a process involving germination and drying of cereal seeds, the major objective being to promote the development of hydrolytic enzymes that are not active in ungerminated seeds (Dewar et al., 1997). During malting, the seed changes by α and β amylases present in the grain and partial degradation (hydrolysis, catalysed by enzymes) of substances (cell wall, gums, protein, starch) in the endosperm. (Ayernor and Ocloo, 2007).

Germinating rice or Malted rice is the one of malt source, during the process of being germinated, nutrients in the grain change drastically. Nutrient stored in grain rice is decomposed by biochemical reaction during germination. Starch breakdown during germination is caused by the synergistic action of hydrolytic enzyme such as α

amylase, β amylases, debrancing enzyme and α-glucosidase. Protein was hydrolyzed to amino acid and peptide (Sutinium et al., 2008).

Rice malt or germinated rice with a greater amount of the naturally-occurring amino acid gamma-aminobutyric acid (GABA), carbohydrate (oligosaccharide, reducing sugar; maltose, glucose and dextrin), small molecule of peptide (glutamic acid, prolin, leucine, aspartic acid) , various vitamin (B, E, folic acid) and minerals auch as Ca, Cu, Fe, Mg, Mn,P, K, zn. The hydrolysis enzyme in rice seed breakdown the nutrients in rice grain to fermentable sugar, amino acid, vitamins and minerals, thus malts from rice are preferred material for most users. Rice is a major crop grown in Thailand. It is less expensive compared with malt extract.   This is suggest that rice malt can be one of the possible ways to improve the production of bioactive compounds from PT1.

4.6.2 Determination of metabolite profile

Metabolite profile of culture broth crude extracts of 8 rice malts and 2 malt extracts were determined by TLC and detected under UV light at 254 and 365 nm and with iodine vapor. The resulting metabolite profiles are shown in Figure 4-13 -4-15.

The metabolite profile of 11crude extracts were detected under UV light and in iodine vapour, the pattern of metabolites profiles showed similarity in all detection methods. This confirms that all rice malts could induce endophytic fungal PT11 produce the same metabolites. Most spot in the metabolite profiles were detected under UV light at 356 nm and with iodine vapour. While some compounds were visible spots under UV light at 254 nm.   The same pattern of spots when detected with   UV light at 254 nm showed R_f value 0.3, 0.5.0, 0.53, 6.0 and 0.86 in hexane:ethyl acetate:methanol, 50: 45:05 solvent system,   hexane:ethyl acetate: methanol, 50: 49 :01 solvent system, Chloroform: methanol, 95:05.

Figure: 4-13: The metabolite profiles of culture broth crude extracts of different rice malts from endophytic fungus PT11 on TLC sheets; 1: RD6, 2: R-258, 3: Muey Nawng, 4: Leuang Pratew, 5: Jek Chueng, 6: Khao Tah Haeng, 7: Pathum Thani 1, 8: Sasanishiki, 9: Akitakomachi, 10: Malt extract (Merck), and 11: Malt extract (Lab-

Scan), The solvent system used was hexane:ethyl acetate: methanol, 50: 45:05, (A) Detected under UV light at wavelength 254 nm, (B) Detected under UV light at wavelength 365 nm and (C) Detected with iodine vapour
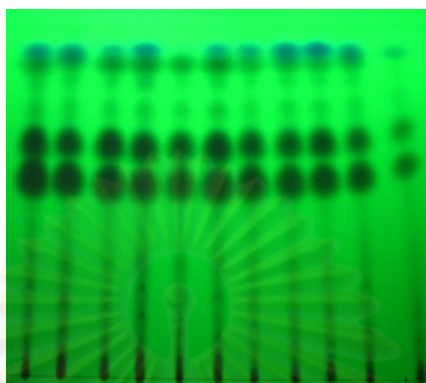
1 2 3 4 5 6 7 8 9 10 11
(A)
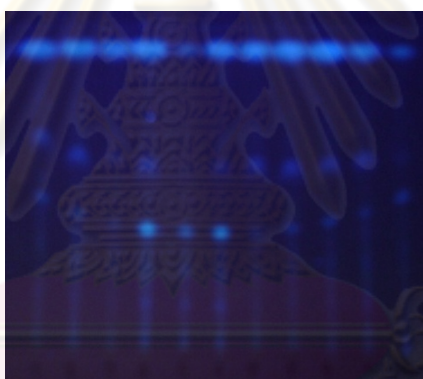
1 2 3 4 5 6 7 8 9 10 11
(B)
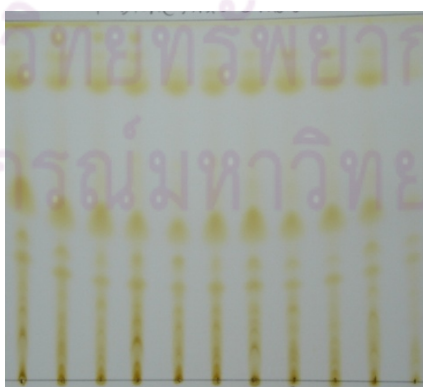
1 2 3 4 5 6 7 8 9 10 11
(C)

Figure: 4-14: The metabolite profiles of culture broth crude extracts of different rice malts from endophytic fungus PT11 on TLC sheets; 1: RD6, 2: R-258, 3: Muey Nawng, 4: Leuang Pratew, 5: Jek Chueng, 6: Khao Tah Haeng, 7: Pathum Thani 1, 8:

Sasanishiki, 9: Akitakomachi, 10: Malt extract (Merck), and 11: Malt extract (Lab-Scan), The solvent system used was hexane:ethyl acetate: methanol, 50: 49 :01, (A) Detected under UV light at wavelength 254 nm, (B) Detected under UV light at wavelength 365 nm and (C) Detected with iodine vapour

(A)

(B)

(C)

Figure: 4-15: The metabolite profiles of culture broth crude extracts of different rice malts from endophytic fungus PT11 on TLC sheets; 1: RD6, 2: R-258, 3: Muey

Nawng, 4: Leuang Pratew, 5: Jek Chueng, 6: Khao Tah Haeng, 7: Pathum Thani 1, 8: Sasanishiki, 9: Akitakomachi, 10: Malt extract (Merck), and 11: Malt extract (Lab-Scan), The solvent system was used as Chloroform: methanol, 95:05, (A) Detected under UV light at wavelength 254 nm, (B) Detected under UV light at wavelength 365 nm and (C) Detected with iodine vapour

Various factors such as temperature, pH, incubation period, salinity, carbon and nitrogen sources and amino acid play a major role in the production of antimicrobial agents. The biosynthesis of secondary metabolite is directly related to culture conditions.

Maximum antimicrobial agent production by the fungus was recorded on the 8th day of incubation. The trophophase (growth phase) of the endophytic fungus lasted for six days, but the idiophase (production phase) reached its top at the 8th day of incubation and remain almost constant after nine days (Stinson et al. 2003)

Amino acid supplement may have some role by sharing their carbon ring or both carbon and nitrogen skeleton in the primary or secondary metabolism processes of microorganisms (Noaman et al., 2004).

Malted rice is rich source of sugar, amino acid, vitamins and minerals. It is good carbon source for fermentation to produce valuable metabolites.

4.6.3 Determination antimicrobial activity

All crude extracts were evaluated for antimicrobial activities against 6 standard tested microorganisms by the paper diffusion method. The 7 mm of paper disc was impregnated with 30 µg crude extract and placed on inoculated plates. After incubate for 24h at 37ºC for bacteria and 30ºC for yeast inhibition zone were measured. The result showed that all crude extracts presented significant antimicrobial activities against all tested microorganisms (Table 4.5). The effective of all crude extracts to each tested microorganism did not differ because all rice malts and malt extracts produced the same metabolite profile (Figure 4-14). The crude extract showed strong effective to yeast than bacteria. Figure 4.16 shows the antimicrobial activity of endophytic fugal PT 11 when grown on MEB containing rice RD6 malt and rice R-256 malts.

Table 4-4: The antimicrobial activities of broad spectrum isolates

Rice malt	Tested microoorganisms and diameter inhibition zone (cm)					
	B. subtilis	*S. aureus*	*P. aeruginosa*	*E. coli*	*S. cerevisiae*	*C. albicans*
RD6	3.0		3.0	2.0	>4.5	>4.5
R-258	3.0	2.3	3.0	1.9	>4.5	>4.5
Muey Nawng	3.0	2.3	3.0	2.0	>4.5	>4.5
Leuang Pratew	3.0	2.3	3.0	2.0	>4.5	>4.5
Jek Chueng	2.8	2.4	3.0	2.0	>4.5	>4.5
Khao Tah Haeng	2.9	2.5	3.0	2.0	>4.5	>4.5
Pathum Thani 1		2.5	3.0	2.0	>4.5	>4.5
Sasanishiki	3.0	2.5	3.0	2.0	>4.5	>4.5
Akitakomachi	3.0	2.5	3.0	2.0	>4.5	>4.5
Malt extract (Merck)	3.0	2.4	3.0	2.0	>4.5	>4.5
Malt extract (Lab-Scan)	2.8	2.3	3.0	2.0	>4.5	>4.5
Positive control	2.0	1.6	2.5	2.2	2.0	2.0

Positive control for bacteria is 30 µg of Amicacin. Positive control for yeast is 50 µg of Nystatin.

B. subtilis   *S. aureus*   *P. aeruginosa*

E. coli   *S. cerevisiae*   *C. albicans*
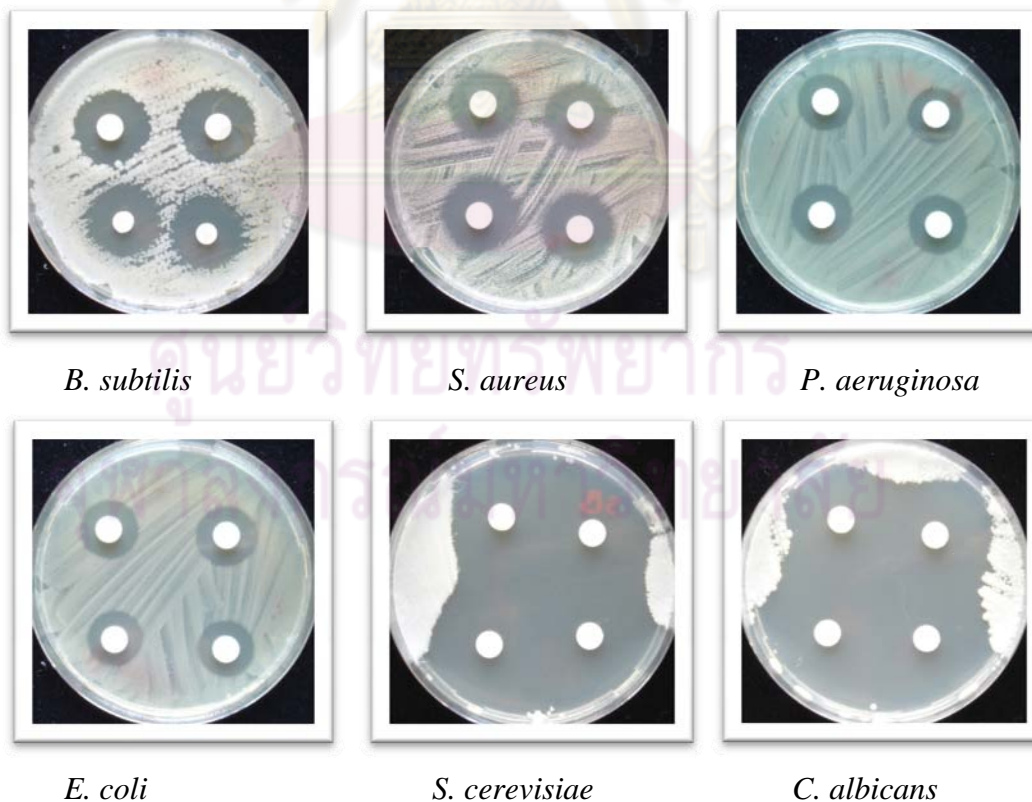
Figure 4-16: Antimicrobial activity of endophytic fungus PT11, grown on MEB containing rice RD6 malt and rice R-256 malts.

4.6.4 Autobiography test

The crude extract from culture broth of Malt extract (Merck) were examined to visual metabolites spot which having antimicrobial activities by autobiography test using standards tested microorganisms. The results showed that metabolites spot R_f value 0.50 and 0.53 in hexane: ethyl acetate: methanol, 50: 49:01 solvent system when detected with UV light at 254 nm displayed activities against all tested microorganisms (Figure 4-16).
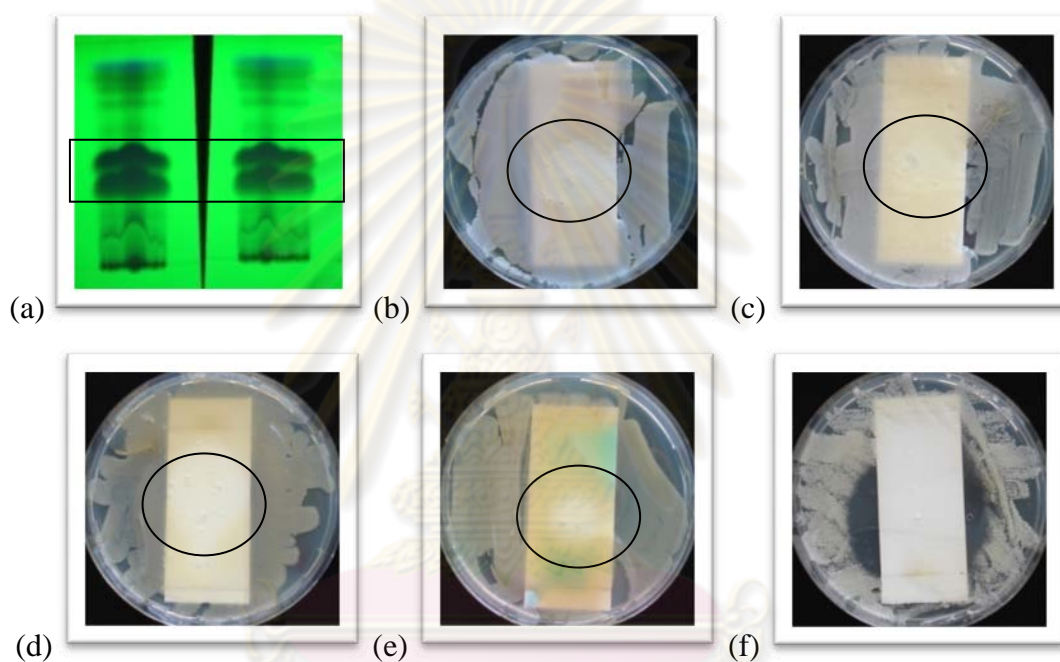
Figure 4-17: Autobiography test image; (a) TLC plate loading PT11 crude extract, (b) *B. subtilis*, (c) *S. aureus*, (d) *E. coli*, (e) *P. aeruginasa* and (f) *C. albicans*

4.7 Chemical constituents of endophytic fungal isolate PT11

Endophytic fungal PT11 was cultured in 20 L of MEB for 4 weeks. The culture broth and mycelium were separated and extracted with EtOAc and obtained 32 g of EtOAc crude extract from culture broth and 5.6 g MeOH crude extract from mycelium.

4.7.1. Isolation and purification of bioactive compounds in EtOAc crude extract from culture broth of PT11.

Preliminary tested antimicrobial activity of crude extract from culture broth and mycelium crude extract were found that the crude extract of mycelium showed

very low antimicrobial activities than crude extract from culture broth. The metabolites profile of culture broth crude extract and mycelium crude extract visually on TLC did not show different. Therefore this study could isolate bioactive compounds from culture broth crude extract.

Secondary metabolites from the culture broth extract of fungal PT11 were purified by silica gel column chromatography using various solvent mixtures of hexane, hexane-ethyl acetate, ethyl acetate, ethyl acetate-methanol and methanol, to yield 17 fractions, divided based on the similarity of the TLC patterns. All fractions were tested for antimicrobial activities found that 3 fractions (Fr05 (134 mg), Fr07 (5.89 g) and Fr11 (1.52 g)) showed antimicrobial activities. The active fractions were re-subjected on silica gel column chromatography.

The R_f value of Fr 05 showed between 0.50 - 0.53 in hexane: ethyl acetate: methanol, 50: 49:01 system the same manner of previous autobiography studies in section 4.5.4 and Figure 4-15. The TLC pattern of Fr05 showed mixed at least 4 compounds. This fraction was re-subjected on silica gel column and eluted with 30% EtOAc in hexane to 100% EtOAc. Only 15 mg of mixed compounds were obtained. Therefore the mixture compounds did not enough for further elucidation.

Two compounds were isolated from the culture broth of endophytic fungus PT11. Compound 1 (158.10 mg) was obtained from Fr07 as an amorphous white solid. Compound 2 (35 mg) was obtained from Fr11 as an amorphous white solid. These compounds were tested for biological activities

4.7.2 Structure elucidation of compound 1

Compound 1 was obtained from the combined fraction Fr07-SF1 of culture broth ethyl acetate crude extract, eluted with EtOAC:hexane (40:60→60:40). Compound 1 was a white amorphous solid, m.p. 78-80°C. The structure of compound 1 was elucidated using spectroscopic technique. The IR spectrum of compound 1 is shown in Figure D1 (in appendix D).

The ^{1}H NMR spectrum of compound 1 indicated that it possesses five signals at δ 1.5, 2.7, 3.3, 5.0 and 6.5 ppm (Figure D2 in appendix D).

The ^{13}C-NMR spectrum of compound 1 showed carboxyl group at δ171 and 161 ppm (Figure D3 in appendix D).

The LC-MS spectrum of compound 1 showed the $[M+H]^{+}$peak at m/z 156.04(100%), 157.05 (7.8%) and 158.05 (1.1%) (Figure D-7 in appendix D). The mass spectrum indicated a molecular weight 156.14.

Chemical formula of compound 1 may be $C_7H_8O_4$ as a new compound which supported by ^1H and ^{13}C NMR, 2D NMR techniques (Figure 4.18).
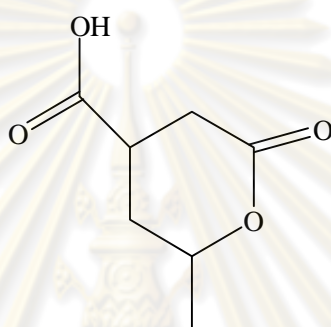
Figure 4-18: The Chemical structure of compound 1

4.7.2 Biological activities

4.7.2.1 Antimicrobial activity of pure compounds

The antimicrobial activity of pure compounds was evaluated by the antimicrobial susceptibility test, broth microbial method. The pure compounds were examined at a concentration of 0.0.01-400 µg/ml (two-fold dilution). Antimicrobial activity tests were performed against 6 standard tested microorganisms. The lowest concentration of pure compound showing complete inhibition of growth is recorded as the minimal inhibitory concentration (MIC).

The results showed that compound 1 and compound 2 did not show inhibited tested antimicrobial at concentration 400 µg/ml.

4.7.2.2. Anticancer activity of pure compounds

The in vitro activity of pure compounds from endophytic PT 11 was examined for anticancer activities against 6 cell lines as follows: human skin cancer (A375), human breast cancer (BT 474), human gastric cancer (KatoIII), human

intestine cancer (SW620), human liver cancer (HepG2) and human leukemia (Jurkat) usinf MTT assay.

The result showed that compound 1 exhibited only Jurkat cells with 31.04±1.17 cells viability. This compound was then examined for potential to induce apoptosis cell death in jurkat. After treatment of the Jurkat with compound 1 at concentration 50 µg/ml for 24 h, the treated cells were stained with Hoechst 33258 flourescence dye and observed apoptosis under fluorescence microscopy and phase contrast microscopy. The compound 1 had apoptosis-inducing activity against the Jurkat at 66.35±1.46%. The features under fluorescence microcopy and 3D phase contrast microcopy of Jurkat showed obvious morphological changes and apoptotic bodies (arrow in Figure 4-19) after 24 h, the cells became rounded up, shrunken in size.

a

20.0 µm
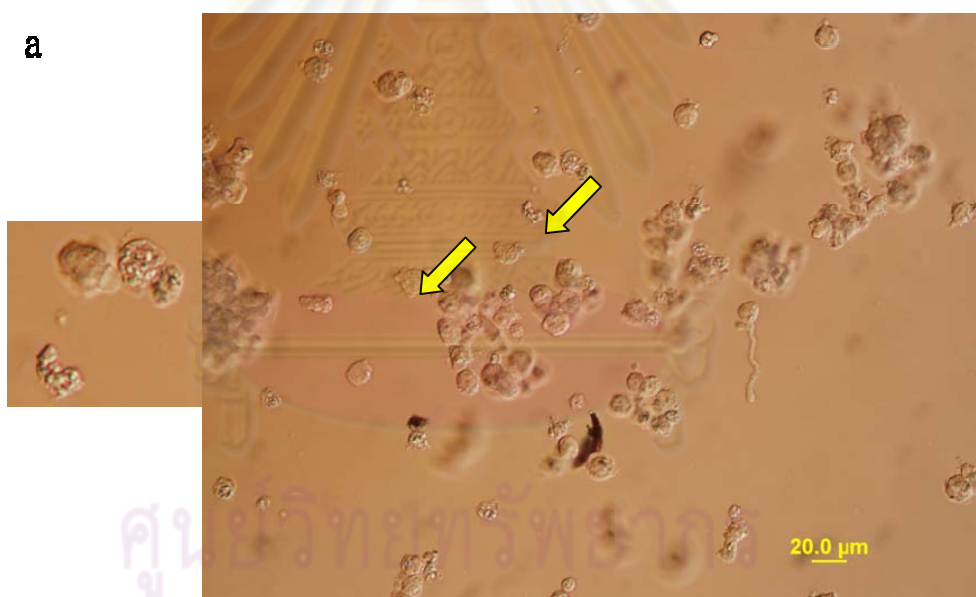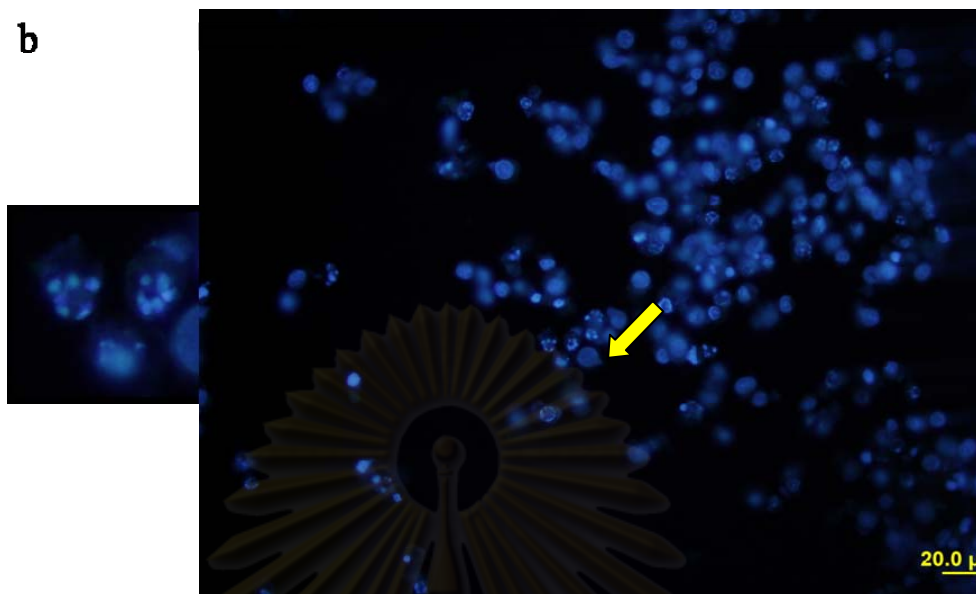
Figure 4-19: Photomicrographs observed under (a) 3D Phase contrast microscopy and (b) Fluorescence microscopy of Jurkat cells treated with 50 µg/ml compound 1 after 24 h. Treated cells display apoptotic bodies (arrows) (Magnification x400)

The cytotoxicity effect of the Cpmpoun1 on Jurkat cells was determined by measuring the cytotoxic dose that killed 50% (IC_{50}) of the cell population compared untreated control. The IC_{50} was determined by MTT method.

The compound Compound 1 showed cytotoxicity against Jurkat cells with IC_{50} 37.5±0.76 µg/ml (Figure 4- 19).
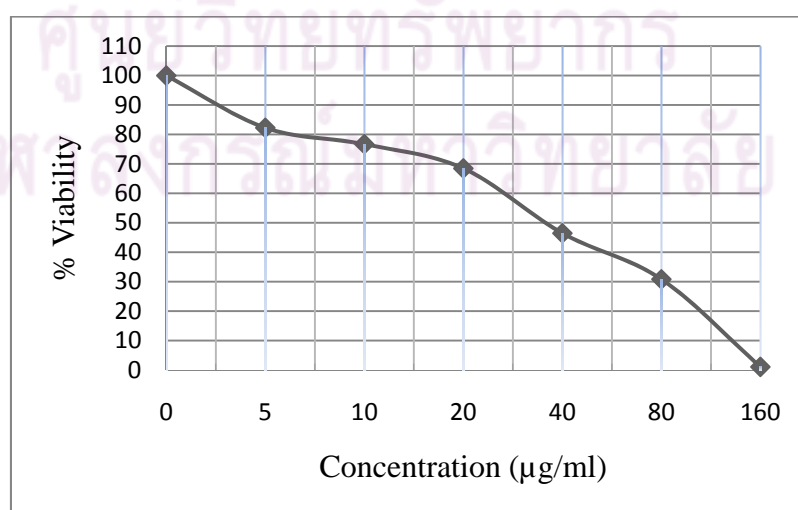
Figure 4-20: The cytotoxicity effect of compound 1 against Jurkat cells.

As the results the mixture from Fr05 exhibit antimicrobial activities but yield of bioactive compounds very low. Optimization the culture conditions and process parameters of isolate bioactive compounds from endophytic isolate PTT11 will be investigated to improve production of the biologically active compounds. The results of section 4.5 showed that PT11 grown in MEB containing rice malt higher yield than the MEB supplemented with malt extract.

PT11-SF6 showed specific cytotoxicity toward Jurkat cells. This bioactive compound need to be further characterized. Searching for bioactive compounds with activity at low concentrations and with reasonably low toxicity to humans is necessary. The compound 1 displayed cytotoxic activity specific to the target jurkat cells *in vitro*. From results indicated that, the PT11-SF6 could be a good candidate for therapeutic anticancer drug.

An endophytic isolate PT11 is closely related to undescribed specie of *Nodulisporium* (anamorph Hypoxylon, Xylariaceae). Bioactive compounds obtained from this fungus include Nodulisporic acid A, B (Ondeyka et al.1997, Ondeyka et al.2002, Kasettrathat et al. 2008), Nodulisporic C, 3-hydroxy-1-(2,6-dihydroxyphenel)-butan-1-one (Dai et al. 2006), tetronic acid (Kasettrathat et al. 2008), and volatile compounds (terpenes) (Mann et al. 2008). Nodulisporic acid A is a potent, long-lasting nontoxic systemic orally-active agent that kills fleas on dogs or cats (Ondeyka et al. 2002). Dai et al. 2006 have been isolated 7 metabolites from endophytic fungus *Nodulisporium* sp. from *Juniperus cedre*, only one compound exhibit herbicidal, antifungal and antibacterial activity.

CHAPTER V
CONCLUSION

Endophytic fungi were isolated in these studies from Krathum na (*M. javanica*) leaves of 3 Ayutthaya, Pathumthani and Amnatcharean provinces by using surface sterilization method and tested for bioactive metabolites. A total of 106 endophytic fungi were isolated from Ayutthaya (49 isolates), Pathumthani (26 isolates) and Amnatcharean (31 isolates) provinces. The endophytic fungi were identified as *Colletotrichum* spp., *Fusarium* spp., *Phomopsis* spp., *Alternaria* spp., *Phyllostica* spp. *Xylaria* spp., *Penicillium* spp., *Phoma* sp., *Cladosporium* sp. *Aspergilus* spp. and the majority were mycelia sterilia. *Phomopsis* sp., *Colletotrichum* sp., *Phyllostica* sp., *Xylaria* sp. were common endophytes assemblages in *M. javanica* leaves from 3 provinces.

The results from antimicrobial activities tested found that 80 (75.47%) isolates of endophytic fungi could produce bioactive compounds that exhibited antimicrobial activity against at least one test microorganism with inhibition zones that ranging from 7-40 mm. Out of these 50 (47.17%), 57 (53.77%), 21 (19.81%), 26 (24.52%), 15 (14.15 %), 13 (12.26%) isolates inhibited *B. subtilis*, *S. aureus*, *E. coli*, *P. aeruginosa*, *S. cerevisiae*, and *C. albicans* respectively. Six (5.66%) isolates such as AY03, AY10, PT08, PT11, AM04 and AM09 showed a broad antimicrobial spectrum. The outstanding endophytic fungal isolate PT 11 showed the strongest antimicrobial activity inhibition against all bacteria and yeast.

Seventy five percent of endophytic fungi displayed anticancer activities toward 6 cell lines below 40% of cell viability. Sixty two (58.49%), 48(45.28%), 64(60.38%), 62(58.49%), 65(61.32%) and 33(31.13%) of endophytic fungi exhibited anticancer activities against A375, BT 474, KatoIII, SW620, HepG2 and Jurkat, respectively. Five endophytic fungus isolates showed cytotoxicity toward all cell lines.

Fourteen endophytic fungal isolates displayed strong specific cytotoxicity toward cancer cell lines as following the endophytic fungi AY 07, AY 16, AY39 and AM28 and AM32 had strong specific cytotoxic activity against KatoIII cells.

The endophytic fungal AY03, AY16, AY 25 and AY 30 showed strong active toward SW620. The endophytic fungal AY07, AY 16, AY45, PT01 and AM17 exhibited HepG2. While, the endophytic fungal BT34, AM 28 also had specific cytotoxic activity against BT474 and PT01 and AM 25 demonstrated strong specific activity toward Jurkat (0% viability).

The strong specific cytotoxicity endophytic fungi were evaluated for their potential apoptotic-inducting cell death in specific target cancer cells. Five isolates were potential induced apoptosis such as PT01 induced DNA fragmentation and morphologically features of apoptosis about 90.04%. Whereas, AY16 induced 88 % apoptotic cell death in human colon cancer (SW620) cells after treated for 36 h. AY 41 had apoptosis-inducing activities against the human breast cancer gastric cancer (Kato III) at 84.80% after incubate for 24 h, while AY03 and AY 07 were showed weakly induction apoptotic in SW620 at 20.44% and liver cancer (HepG2) cells at 19.26 %, respectively. The endophytic fungal PT01 was chosen for further study for bioactive compound. Because of it was showed strong specific induced apoptosis to Jerkat cells up to 90%.

Fungal isolate PT11 was chosen for further study for bioactive compound because the fungus showed the strongest antimicrobial activity inhibited against all bacteria and yeast. Moreover, it showed cytotoxicity to all cancer cell lines. Based on morphological of the endophytic PT11 was classified as Mycelium sterilia. Based on the nucleotide rDNA sequencing of ITS, it was closely related to 99% identity of *Nodulisporium* sp.

Comparison of metabolites from culture broth of fungal isolate PT11 cultivated in 8 difference malted rice, RD6, R-258, Muey Nawng, Leuang Pratew, Jek Chueng, . Khao Tah Haeng, Pathum Thani 1, Sasanishiki, Akitakomachi. There were no different in metabolite profile and antimicrobial activities but mycelia biomass and metabolites concentrations were different. The highest amount of crude extract yield was obtained from MEB containing Khao Tah Haeng malt at concentration 1.7 mg/ml with a final mycelia biomass concentration 6.58 g/l. The maximum biomass concentration (8.47 g/l) was found in MEB containing RD6 malt. Malt extract from Lab-scan showed minimum crude extract concentration at 0.12 mg/ml.

Compound 1 was isolated from culture of endophytic isolate PT11. The compound 1 was a white amorphous solid which chemical formula $C_7H_8O_4$. The compound 1 displayed specific cytotoxic activity induces 66.35±1.46% apoptosis in Jurkat with IC_{50} 37.5±0.76 µg/ml.

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APPENDICES

Appendix A
Media and Reagents

1. Medium for Endophytic fungi

1.1 Malt Extract Agar (MEA) Medium

Malt extract	20.0 g
Glucose	20.0 g
Peptone	1.0 g
Agar	15.0 g
Distilled water	to make 1000 ml
Final pH 5.0-5.5	

1.2 Potato-Dextrose Agar (PDA) Medium

Potatoes, peeled and diced	200.0 g
Glucose	20.0 g
Agar	15.0 g
Distilled water	to make 1000 ml

Boil 200.0 g of peeled, diced potatoes for one hour in a 1000 ml of distilled water. Filter, and make up the filtrate to one liter. Add the glucose and agar and dissolve by steaming and sterile by autoclaving at 121°C, 15 lb/in^2 for 15 minutes.

1. 3 Souboroud Dextrose Agar (SDA) Medium

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

2. Medium for antimicrobial assay

2.1 Nutrient Agar (NA) Medium

Beef extract	3.00 g
Peptone	5.00 g
Agar	15.00 g
Distilled water	to make 1000 ml

2.2 Yeast- Malt extract Agar (YMA) Medium

Yeast extracts	3.00 g
Malt extracts	3.00 g
Peptone	5.00 g
Glucose	10.00 g
Agar	15.00 g
Distilled water	to make 1000 ml

3. Medium and reagents for anticancer assay

3.1 RPMI 1640 medium (Stock reagent)

RPMI 1640 dry powered medium	10.40 g
Sodium hydrogencarbonate	2.00 g

Dissolve RPMI 1640 power and Sodium hydrogencarbonate in 800 ml of deionized distilled water, and then adjust pH to 6.9-7.4 with 1N HCl solution. Make up the stock reagent to one liter and filtered through 0.22 μm filter paper into sterilized bottom volume 90 ml and keep at 4°C.

3.2 Completed RPMI 1640 medium (working reagent)

The RPMI medium (stock reagent)	90 ml
Activated Fetal Bovine Serum	10 ml
Sodium pyruvate	1 ml
0.25 mM HEPES	1 ml
Penicillin G (10 U/ml)	10 μl
Streptomycin sulfate (50 U/ml)	50 μl
Mixed	

3.3 RPMI 1640 freezing medium for freezing cancer cells

Completed RPMI 1640 medium	9 ml
10% DMSO	1 ml

4. Phosphate buffered saline (PBS, Ca^{2+}, Mg^{2+}, Free)

NaCl	8.00 g
KCl	0.20 g
Na_2HPO_4	1.44 g

KH_2PO_4	0.24 g
Deionized distilled water	1,000 ml

Mix to dissolve and adjust pH to 7.2 and sterile by autoclaving at 121°C, 15 lb/in^2 for 15 minutes

5. MTT (3-4, 5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide) solution

Dissolve 50 mg of MTT in 50 ml of phosphate buffer saline (pH 7.4) and filter through 0.22 μm filter paper into sterilized bottom and keep at 4°C and protected from the light by wrapped the bottom with aluminum foil.

6. 0.04 N of HCl in isopropanol

Add 0.331 ml of HCl into 80 ml of isopropanol and then adjust volume to 100 ml with isopropanol and storage at room temperature.

7. 1% glutaraldehyde in PBS

Dissolve 1 g of glutaraldehyde in 100 ml of PBS pH 7.4 and storage at 4°C.

8. Stains

8.1 Lactophenal-cotton Blue

Lactophenol

Lactic acid	100 ml
Phenol	100.0 g
Glyceral	200 ml
Distilled water	100 ml

Dissolve phenol in distilled water without heat, and then add lactic acid and glycerol

Cotton blue solution

Saturated solution of cotton blue

(Soluble aniline blue)	10 ml
Glyceral	10 ml
Distilled water	80 ml

Mixed equal part of lactophenol and cotton blue solution

8.2 Hoecst stain solution

The 5.62 mg of solid dye was dissolved in 1 ml of distilled water to make concentrated stock solutions up to 1 mM/ml. Stock solutions stored refrigerated or frozen, protected from light. Note: The Hoechst stains should not be resolubilized in phosphate-buffered saline (PBS), but dilute solutions of the dye may be used with PBS or other phosphate-containing buffers. Solutions of Hoeschst dye should be stored at 2–6°C, protected from light. Stock solutions in water are stable for at least 6 months when refrigerated. For long-term storage the stock solution can be aliquoted and stored at ≤–20°C.

Appendix B

Table B-1: Endophytic fungi from *M. javanica*

Ayutthaya Province		Pathumthani Province		Amnatcharean Province	
Isolate	Species	Isolate	Species	Isolate	Species
AY 01	*Aspergillus* sp.	PT 01	*Phoma* sp.	AM 01	*Penicillium* sp.
AY 02	*Penicillium* sp.	PT 02	*Collectotrichum* sp.	AM 02	Asprgilus sp.
AY 03	**Cladosporium sp.**	PT 03	Mycelia serilia	AM 03	*Xylaria* sp.
AY 04	Mycelia serilia	PT 04	*Alernaria alternata*	AM 04	Mycelia serilia
AY 05	*Fusarium* sp.	PT 05	**Cladosporium sp.**	AM 05	*Dardinia* sp.
AY 06	Mycelia serilia	PT 06	Mycelia serilia	AM 06	*Aspergilus* sp.
AY 07	Mycelia serilia	PT 07	Mycelia serilia	AM 07	*Cladosporium* sp.
AY 08	*Phomopsis* sp.	PT 08	Mycelia serilia	AM 08	*Colletotrichum* sp.
AY 09	*Dardinia* sp.	PT 09	Mycelia serilia	AM 09	Mycelia serilia
AY 10	Mycelia serilia	PT 10	Mycelia serilia	AM 10	Mycelia serilia
AY 11	Mycelia serilia	PT 11	**Noludosporium sp.**	AM 11	Mycelia serilia
AY 12	*Phyllostica* sp.	PT 12	*Phyllostica* sp.	AM 12	Mycelia serilia
AY 13	*Phyllostica* sp.	PT 13	*Phyllostica* sp	AM 13	Mycelia serilia
AY 14	Mycelia serilia	PT 14	Mycelia serilia	AM 14	*Phyllostica* sp.
AY 15	Mycelia serilia	PT15	Mycelia serilia	AM 15	*Fusarium* sp.
AY 16	*Penicilium* sp.	PT 16	Mycelia serilia	AM 16	Mycelia serilia
AY 17	*Fusaruim* sp	PT 17	Mycelia serilia	AM 17	Mycelia serilia
AY 18	*Phomopsis* sp.	PT 18	Mycelia serilia	AM 18	Mycelia serilia
AY 19	Mycelia serilia	PT 19	*Phomopsis* sp	AM 19	*Xylaria* sp.
AY 20	Mycelia serilia	PT 20	*Phomopsis* sp	AM 20	Phomopsis sp.
AY 21	Mycelia serilia	PT 21	*Phomopsis* sp	AM 21	Mycelia serilia
AY 22	Mycelia serilia	PT 22	Mycelia serilia	AM 22	Mycelia serilia
AY 23	Mycelia serilia	PT 23	Mycelia serilia	AM 23	Mycelia serilia
AY 24	*Alternaria* sp.	PT 24	*Xylaria* sp.	AM 24	Mycelia serilia
AY 25	*Xylaria* sp.	PT25	*Phomopsis* sp.	AM 25	*Phomopsis* sp.
AY 26	*Phyllostica* sp.	PT26	*Phyllostica* sp.	AM 26	*Xylaria* sp.
AY 27	Mycelia serilia			AM 27	*Xylaria* sp.
AY 28	*Aspergilus* sp.			AM 28	Mycelia serilia
AY 29	Mycelia serilia			AM 29	*Phomopsis* sp
AY30	*Aspergilus* sp.			AM 30	Mycelia serilia
AY 31	Mycelia serilia			AM 31	Mycelia serilia
AY 32	*Phomopsis* sp.				
AY 33	Mycelia serilia				
AY 34	*Xylaria* sp.				
AY 35	*Phomopsis* sp.				
AY 36	Mycelia serilia				
AY 37	Mycelia serilia				
AY 38	Mycelia serilia				
AY 39	*Colletotrichum* sp.				
AY 40	*Colletotrichum* sp.				
AY 41	*Phomopsis* sp.				
AY 42	Mycelia serilia				
AY 43	Mycelia serilia				
AY 44	*Alternaria alternata*				
AY 45	*Xylaria* sp.				
AY 46	*Phomopsis* sp.				
AY 47	*Collettotrichum* sp.				
AY 48	Mycelia sterilia				
AY 49	Mycelia sterilia				

Table B-2: The antimicrobial activities of endophytic fungi from Ayutthaya Province

Isolate	Test organisms and Inhibition zone (mm)					
	B. subtilis	*S. aureus*	*P. aeruginosa*	*E. coli*	*S. cerevisiae*	*C. albicans*
AY 01	-	-	-	-	-	-
AY 02	+	+	+	+	-	-
AY 03	++	++	++	++	++	++
AY 04	-	-	-	-	-	-
AY 05	+	++	-	+	-	-
AY 06	++	++	-	-	-	-
AY 07	++	++	++	++	-	-
AY 08	+++	++	+++	+	+	-
AY 09	+	++	+	+	-	-
AY 10	++	++	++	++	++	++
AY 11	++	++	+	-	-	-
AY 12	-	-	-	-	-	-
AY 13	-	-	-	-	-	-
AY 14	++	++	-	-	-	-
AY 15	+	++	-	-	-	-
AY 16	+	+	-	-	-	-
AY 17	++	++	-	-	-	-
AY 18	+	++	-	++	-	-
AY 19	-	+	-	-	-	-
AY 20	++	++	++	-	++	-
AY 21	-	+	-	-	-	-
AY 22	+	-	-	-	-	-
AY 23	-	++	-	-	-	-
AY 24	++	++	-		+	-
AY 25	+	-	-	-	-	-
AY 26	++	+++	-	-	-	-
AY 27	-	-	-	-	-	-
AY 28	-	-	-	-	-	-
AY 29	-	-	-	-	-	-
AY30	-	+	+	-	-	-
AY 31	++	-	-	-	-	-
AY 32	++	++	-	-	-	-
AY 33	-	+	-	-	-	-
AY 34	-	+	-	-	-	-
AY 35	-	+	-	-	-	-
AY 36	+	+	++	-	-	-
AY 37	+	+	+	+	-	-
AY 38	+	+	++	-	-	-

Table B-2: Continuous

Test organisms and Inhibition zone (mm)						
Isoalte	*B. subtilis*	*S. aureus*	*P. aeruginosa*	*E. coli*	*S. cerevisiae*	*C. albicans*
AY 39	+++	+++	-	-	-	-
AY 40	+	-	-	-	-	-
AY 41	+	+	-	-	-	-
AY 42	-	-	-	-	-	-
AY 43	+	-	+	+	-	-
AY 44	+	-	-	-	-	-
AY 45	+	-	-	-	-	-
AY 46	-	-	-	-	-	-
AY 47	+	-	-	-	-	-
AY 48	+	-	-	-	-	-
AY 49	-	+	-	-	-	-

+++   Inhibition zone above 20 mm

++   Inhibition zone between 10-20 mm

+   Inhibition zone less than 10 mm

-     No activity

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Table B-3: The antimicrobial activities of endophytic fungi from Pathumthani Province

Test organisms and Inhibition zone (mm)						
Isolate	*B. subtilis*	*S. aureus*	*P. aeruginosa*	*E. coli*	*S. cerevisiae*	*C. albicans*
PT 01	++	+	-	-	++	+
PT 02	-	-	-	-	-	-
PT 03	-	+	-	-	-	-
PT 04	-	-	-	-	-	-
PT 05	+	++	-	-	-	-
PT 06	++	++	-	-	+	++
PT 07	++	-	++	++	-	-
PT 08	++	+++	+	++	+++	+++
PT 09	+++	+++	-	-	-	-
PT 10						
PT 11	+++	+++	+++	+++	+++	+++
PT 12						
PT 13	-	-	-	-	-	-
PT 14	-	-	-	-	-	-
PT15	++	-	-	-	-	-
PT 16	++	-	-	-	-	-
PT 17	++	+++	-	-	-	-
PT 18	-	-	-	-	-	-
PT 19	-	-	-	-	-	+
PT 20	-	-	-	-	-	-
PT 21	+	+	-	-	-	-
PT 22	-	-	-	-	-	-
PT 23	+	-	-	-	++	-
PT 24	+	++	-	+	+	-

+++   Inhibition zone above 20 mm
++   Inhibition zone between 10-20 mm
+   Inhibition zone less than 10 mm
-    No activity

Table B-4: The antimicrobial activities of endophytic fungi from Amnatcharean Province

Test organisms and Inhibition zone (mm)						
Isolate	*B. subtilis*	*S. aureus*	*P. aeruginosa*	*E. coli*	*S. cerevisiae*	*C. albicans*
AM 01	++	-	-	-	-	++
AM 02	-	++	+	++	-	-
AM 03	++	-	+	++	-	-
AM 04	++	++	++	++	+	+
AM 05						
AM 06						
AM 07	-	++	-	+	++	++
AM 08	-	-	-	-	-	-
AM 09	+	+++	++	++	+	++
AM 10	-	-	-	-	-	-
AM 11	++	++	+	-	-	-
AM 12	-	-	-	-	-	-
AM 13	-	-	-	-	-	-
AM 14	++	++	++	-	-	-
AM 15	++	+++	++	++	-	-
AM 16	+	-	-	-	-	++
AM 17	+	-	-	-	-	-
AM 18	-	-	-	-	-	-
AM 19	+++	++	-	-	++	-
AM 20						
AM 21	++	+	++	-	-	-
AM 22	-	-	-	-	-	-
AM 23	++	++	-	-	-	+
AM 24	-	-	-	-	-	-
AM 25	-	-	-	-	-	-
AM 26	+	+	-	-	-	-
AM 27	+	+	-	-	-	-
AM 28	++	++	++	++	-	-
AM 29	+	++	-	-	-	-
AM 30	-	-	-	-	-	-
AM 31						

+++   Inhibition zone above 20 mm

++   Inhibition zone between 10-20 mm

+   Inhibition zone less than 10 mm

-      No activity

Table B-5: The anticancer activities of endophytic fungi from Ayutthaya Province

Percentage of cell viability						
Isolate	A375	KatoIII	SW620	HepG2	BT474	Jurkat
AY 01	-	-	-	-	-	-
AY 02	-	-	++	-	-	-
AY 03	-	-	++	-	-	-
AY 04	-	++	++	++	-	-
AY 05	++	++	-	-	+++	-
AY 06	+++	++	+++	+	++	+
AY 07	+	-	-	+++	-	-
AY 08	+	+++	+	-	++	-
AY 09	+	++	-	++	++	+++
AY 10	+	+	++	-	-	-
AY 11	++	++	+	+	-	-
AY 12	+++	++	+	-	++	-
AY 13	++	++	++	++	+	-
AY 14	+	++	-	-	+	+++
AY 15	+	++	+	+	++	-
AY 16	++	-	+++	+	-	-
AY 17	-	++	++	+	-	-
AY 18	+	+++	++	+	++	-
AY 19	-	+++	+	-	-	-
AY 20	++	+++	+++	++	++	++
AY 21	+	+++	++	++	-	-
AY 22	+++	+++	++	++	-	-
AY 23	-	+++	-	++	-	-
AY 24	-	+++	++	++	-	-
AY 25	-	+++	-	-	-	+++
AY 26	+	+++	++	++	-	-
AY 27	-	+	++	+++	+++	+
AY 28	-	-	+	-	-	-
AY 29	++	-	-	+	++	-
AY30	+	+	-	-	-	-
AY 31	+	+++	+++	++	++	-
AY 32	+	++	+	+	++	-
AY 33	++	++	++	++	++	-
AY 34	-	-	+	-	+++	-
AY 35	-	++	+	++	-	+
AY 36	++	++	-	++	+	+
AY 37	++	+++	++	+	++	++
AY 38	+	++	-	++	-	-
AY 39	++	-	-	-	-	-
AY 40	++	+++	+	++	-	-

Table B-5: Continuous

Percentage of cell viability						
Isolate	A375	KatoIII	SW620	HepG2	BT474	Jurkat
AY 41	-	++	++	-	-	-
AY 42	+	-	+	-	-	+
AY 43	-	-	-	-	-	-
AY 44	-	-	-	-	-	-
AY 45	-	-	-	++	-	-
AY 46	++	-	-	++	-	-
AY 47	-	-	-	+	-	-
AY 48	-	-	-	-	-	-
AY 49	-	++	-	-	-	-

+++   % Viability rate is between 0-10 %
++   % Viability rate is between 11-20%
+   % Viability rate is between 21-30%
-       % Viability rate is higher than 31%

Table B-6: The anticancer activities of endophytic fungi from Pathumthani Province

Isolate	Percentage of cell viability					
	A375	**KatoIII**	**SW620**	**HepG2**	**BT474**	**Jurkat**
PT 01	+	+	-	++	-	+++
PT 02	-	-	-	-	-	-
PT 03	+	+	++	++	-	-
PT 04	-	-	-	-	-	-
PT 05	+	++	++	++	+	++
PT 06	-	-	-	-	-	-
PT 07	-	+	+	-	-	-
PT 08	+	++	++	+	+	-
PT 09	-	++	+	++	+	-
PT 10	++	+++	++	++	+	-
PT 11	-	++	++	+++	+	+
PT 12	+	-	++	-	+	-
PT 13	-	-	-	-	+	-
PT 14	-	-	-	-	-	+
PT15	-	-	-	-	-	-
PT 16	-	-	+	+	-	-
PT 17	++	++	-	++	++	-
PT 18	-	-	-	-	-	-
PT 19	-	-	-	-	-	-
PT 20	++	+	+	++	-	-
PT 21	-	-	-	-	-	-
PT 22	-	-	-	-	-	+
PT 23	++	++	-	+	-	-
PT 24	++	-	-	-	+	-

+++   % Viability rate is between 0-10 %

++   % Viability rate is between 11-20%

+   % Viability rate is between 21-30%

-      % Viability rate is higher than 31%

Table B-7: The anticancer activities of endophytic fungi from Amnatcharean Province

Percentage of cell viability						
Isolate	A375	KatoIII	SW260	HepG2	BT474	Jurkat
AM 01	-	-	-	-	-	-
AM 02	+	-	-	+	++	-
AM 03	-	-	-	-	++	-
AM 04	-	-	-	-	-	-
AM 05	-	-	-	-	++	-
AM 06	-	-	-	-	-	-
AM 07	-	+	-	+	++	-
AM 08	-	-	-	-	-	-
AM 09	-	-	-	-	-	-
AM 10	-	-	-	-	-	-
AM 11	-	++	+	+	+	+
AM 12	+	-	-	-	-	+
AM 13	-	-	-	-	-	-
AM 14	++	+++	+	++	++	++
AM 15	+	++	++	++	+++	-
AM 16	-	-	-	+	-	-
AM 17	-	-	-	++	-	-
AM 18	+++	++	++	++	-	+
AM 19	++	++	++	++	+++	-
AM 20	-	-	-	-	-	-
AM 21	+++	++	++	-	+++	+
AM 22	+	-	-	+	-	-
AM 23	+++	+++	+++	-	++	++
AM 24	+	++	++	+	+	-
AM 25	+	-	++	-	+	+
AM 26	-	-	-	-	-	-
AM 27	++	++	++	+	++	-
AM 28	-	++	-	-	-	+
AM 29	-	-	-	-	-	-
AM 30	+++	-	++	++	+	
AM 31	+	+	++	++	-	+

+++   % Viability rate is between 0-10 %

++   % Viability rate is between 11-20%

+   % Viability rate is between 21-30%

-      % Viability rate is higher than 31%

Appendix C

TGCAAACGCGCGCCCGAGCTGTACAAGCGGAGGAATCATTAACGA
GTTACAAAACTCCCAACCCTATGTGAACATACTATTGTTGCCTCGGCGGCG
CCGCGATAGCGGCCCGCCGGTGGACCTAAACGCTAATTGTAACCACTGTA
TCTCTGAATGTGTAACTGTAATACGTTAAAACTTTCAACAACGGATCTCTT
GGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT
TGCAGAATTCCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAG
TATTCTAGTGGGCATGCCTATTCGAGCGTCATTTCGACCCTTACGCCCTGT
TGCGTAGTGTTGGGACTCTGCGTGTTACAGCGCAGTTCCTGAAAGCAATT
GGCGGAGCTAGAGCCCACTCTAGGCGTAGTAAATACCATTCTCGCTTCTG
TAGTGGCTTTGGCGGCTAGCCAGAAAACCCCTATATTTCTAGTGGTTGACC
TCGGATTAGGTAGGAATACCCGCTGAACTTAAGCATATCAAAAGG

Figure C-1: Nucleotide sequences of partial 18s region, complete ITS region of enophytic isolate PT11

Appendix D
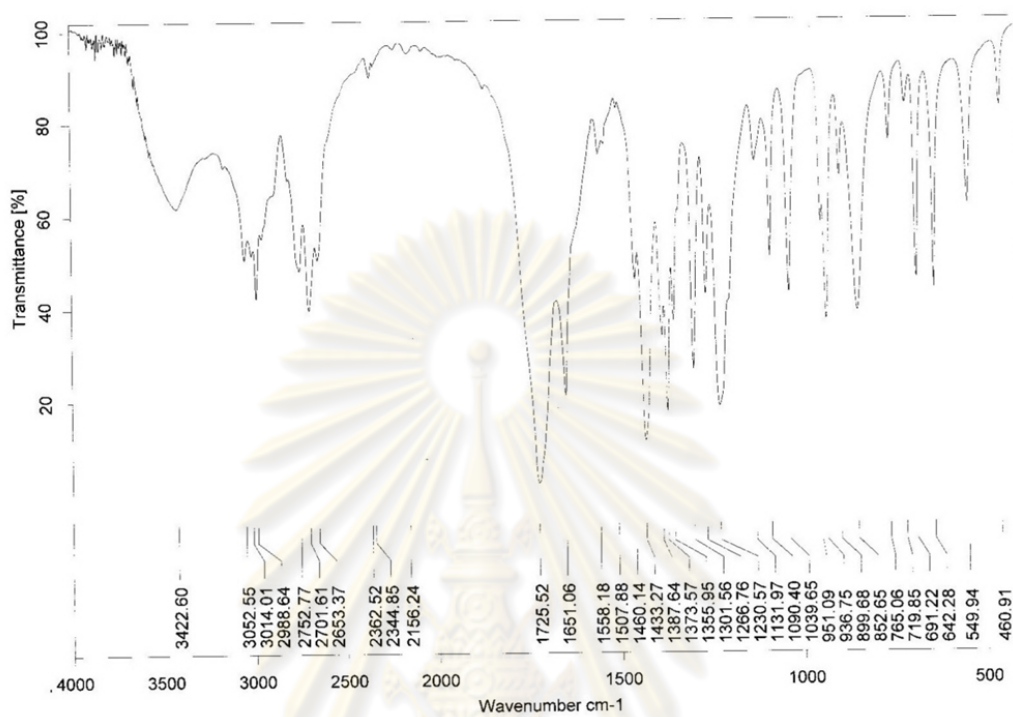
Figure D-1: IR spectrum of compound 1
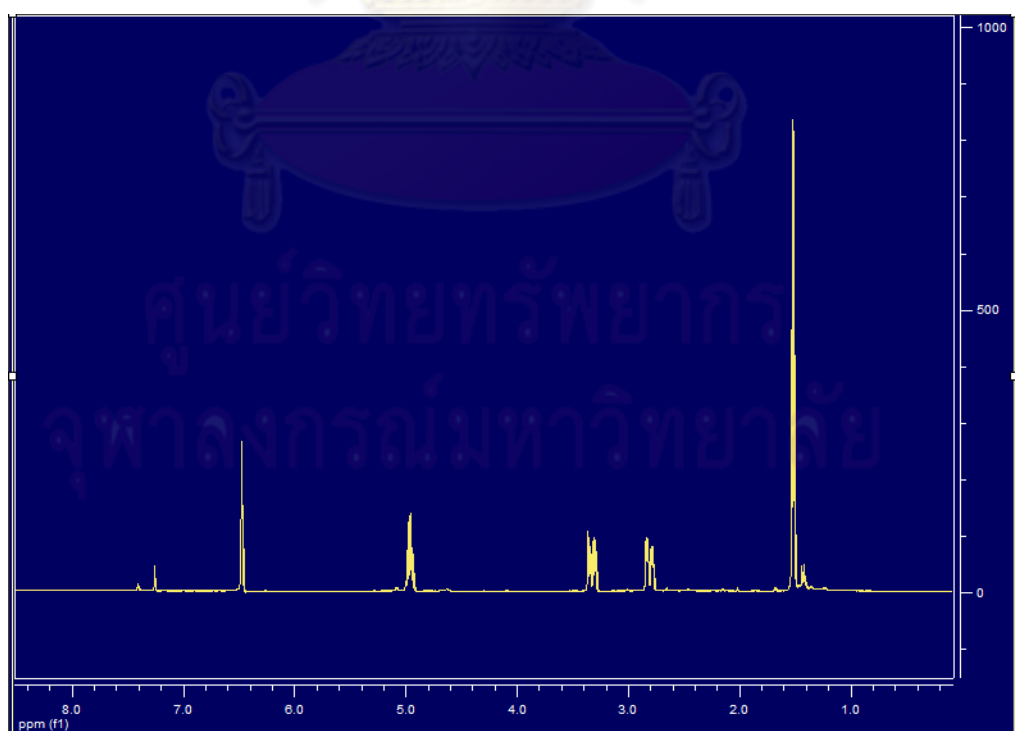
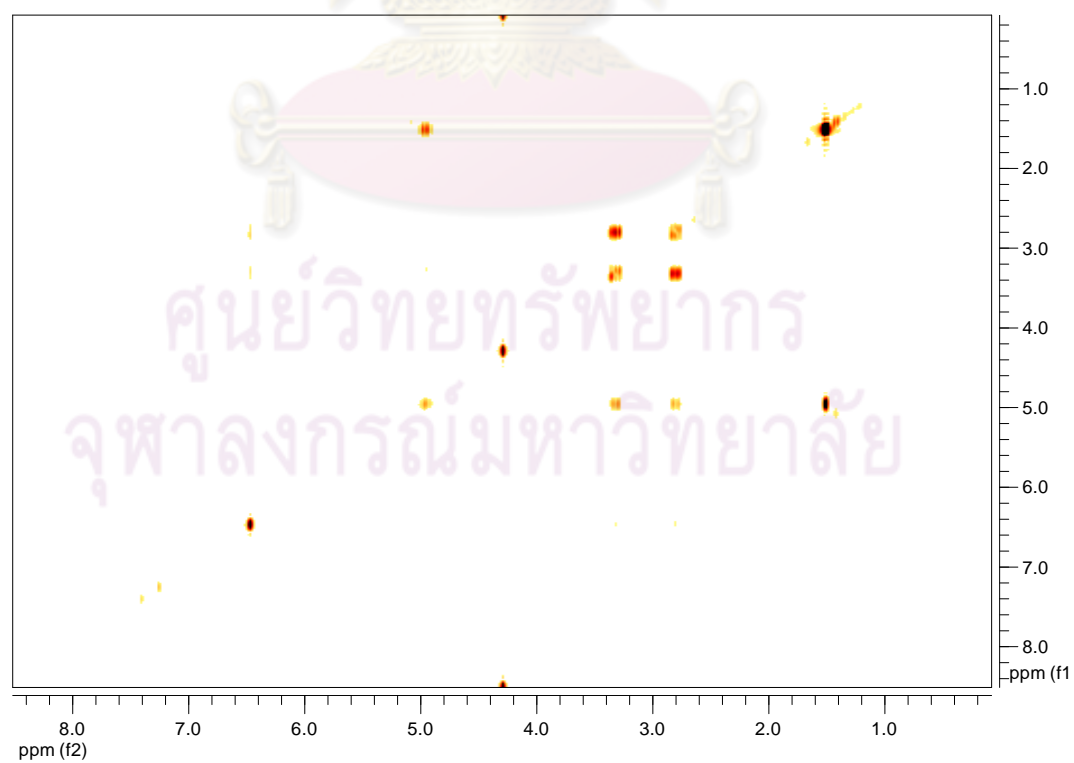Figure D-2: ^{1}H-NMR spectrum of compound1
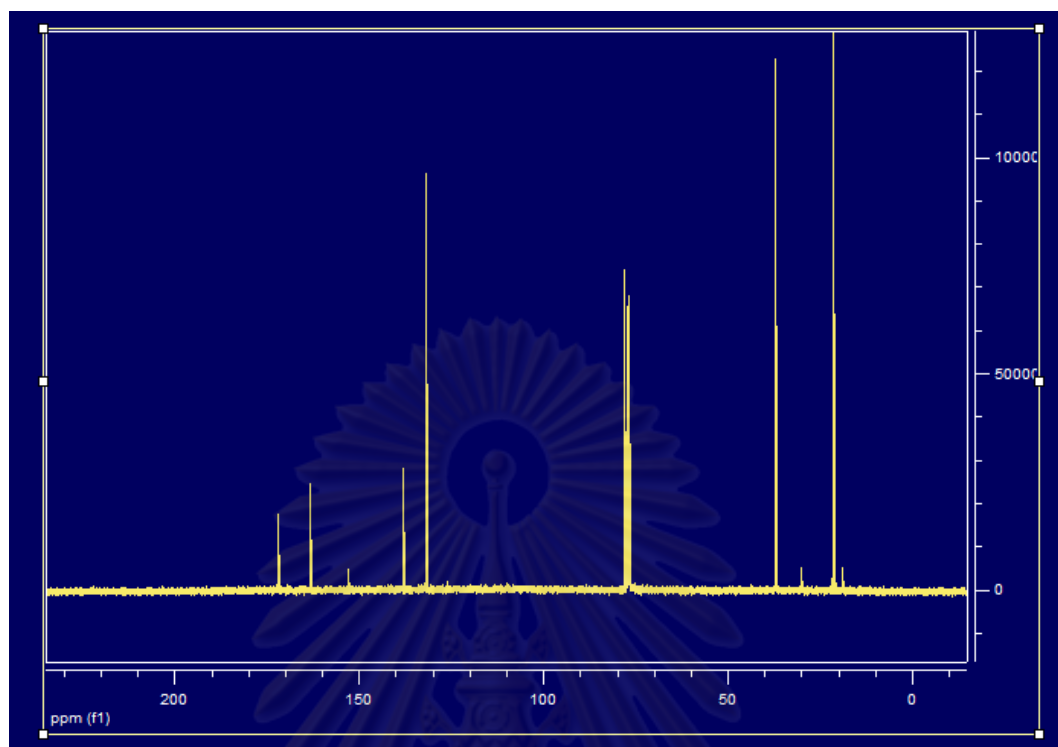
Figure D-3: ^{13}C-NMR spectrum of compound1

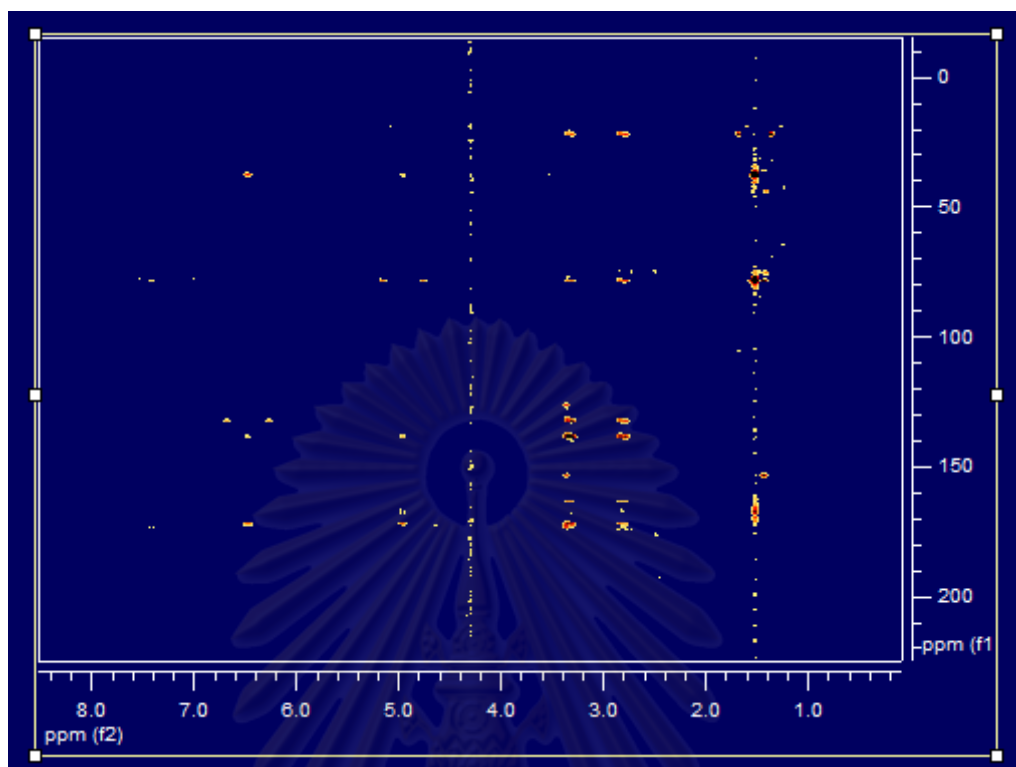Figure D-4: NOESY spectrum of compound1

Figure D-5: HMBC spectrum of compound1

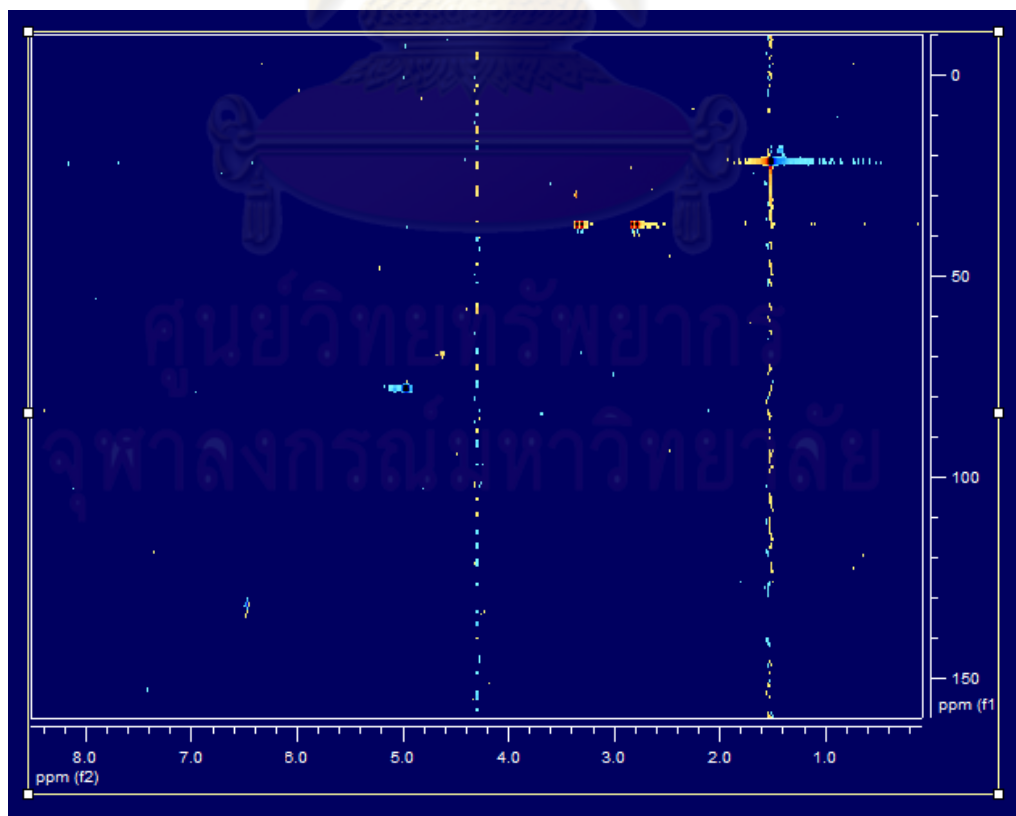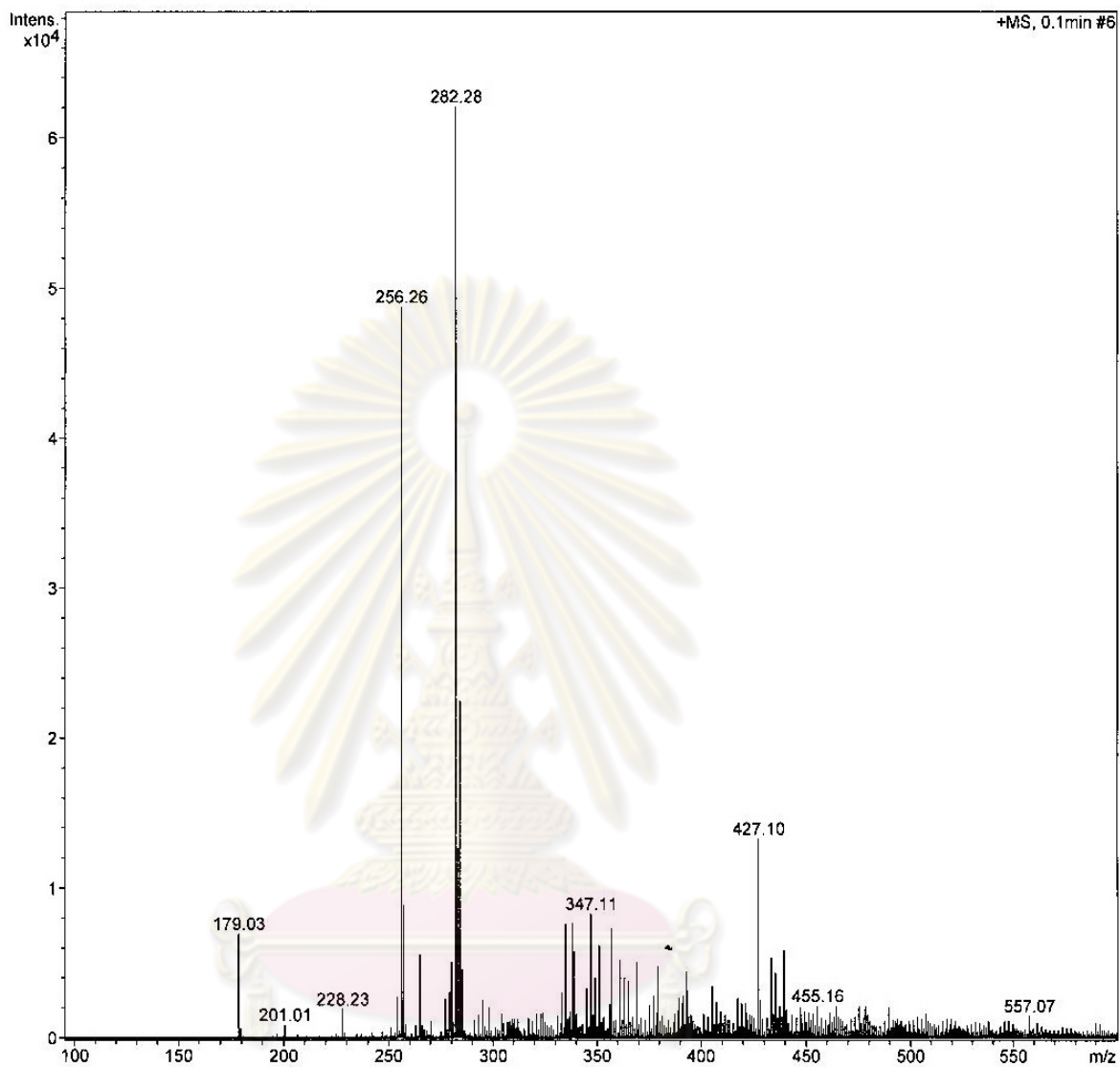Figure D-6: COSY spectrum of compound1

Figure D-7: LC-MS spectrum of compound1

BIOGRAPHY

Miss Thirawatna Pharamat was born on April 16, 1966 in Amnatcharoen province, Thailand. She graduated with Bachelor Degree of Science in Biology from the faculty of science, Ramkhamhaeng University, Thailand in 1988. She graduated with Master Degree of Science in Biotechnology, Chulalongkorn University, Thailand in 1998. She has been studying for a Degree of Doctor of Science, Program in Biotechnology, Faculty of Science, Chulalongkorn University, Thailand since 2004.