# การแยกราเอนโดไฟต์ *Xylaria* spp. จากจังหวัดตราดและเมแทบอไลท์ที่มีแอกทิวิตีต้านมะเร็งปากมดลูก

นางสาวกุลณี ขจัดภัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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# ISOLATION OF ENDOPHYTIC *Xylaria* spp. FROM TRAD PROVINCE AND THEIR METABOLITES WITH ANTI-CERVICAL CANCER ACTIVITY

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A Thesis Submitted in Partial Fulfillment of the Requirements
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กุลณี ขจัดภัย : การแยกราเอนโดไฟต์ *Xylaria* spp. จากจังหวัดตราดและเมแทบอไลท์ที่มีแอกทิวิตีต้านมะเร็งปากมดลูก **(**ISOLATION OF ENDOPHYTIC *Xylaria* spp. FROM TRAD PROVINCE AND THEIR METABOLITES WITH ANTI-CERVICAL CANCER ACTIVITY**)** อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร. ธนาภัทร ปาลกะ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.ดร.ประกิตติ์สิน สีหนนทน์, 68 หน้า

สารเมแทบอไลต์ที่ได้จากจุลินทรีย์ถือว่าเป็นแหล่งสำคัญในการนำไปสู่ การพัฒนายาชนิดใหม่ ราเอนโดไฟต์ เป็นจุลินทรีย์ชนิดหนึ่งที่มีการนำมาศึกษาเพิ่มมากขึ้นในปัจจุบัน เพื่อเป็นแหล่งในการค้นหายาชนิดใหม่ ในการศึกษานี้ได้คัดแยกราเอนโดไฟต์ *Xylaria* spp. จากตัวอย่างใบไม้ที่เก็บได้จากจังหวัดตราด ซึ่งอยู่ทางภาคตะวันออกของประเทศไทยและทดสอบการออกฤทธิ์ทางชีวภาพของ เมแทบอไลท์จาก ไอโซเลตนั้นๆ พบว่าได้ราเอนโดไฟต์จำนวน 83 ไอโซเลต จำแนกราเอนโดไฟต์ได้ดั งนี้ *Xylaria* spp. (41 ไอโซเลต), *Daldinia* spp. (5 ไอโซเลต), *Fusarium* spp. (1 ไอโซเลต), *Collectotrichum* spp. (1 ไอโซเลต), *Pestalotiopsis* spp. (4 ไอโซเลต), *Phomopsis* spp. (2 ไอโซเลต), *Phyllosticta* spp. (2 ไอโซเลต), *Trichoderma* spp. (1 ไอโซเลต), *Nigrospora* spp. (4 ไอโซเลต) และ Mycelia sterilia (22 ไอโซเลต) เมื่อนำสารสกัดหยาบของราเอนไดไฟต์ *Xylaria* ทั้ง 41 ชนิดมาทดสอบการออกฤทธิ์ในการยับยั้งจุลินทรีย์ทดสอบ 5 ชนิด พบว่ามีสารสกัดหยาบ 9 ชนิดที่สามารถยับยั้งเชื้อจุลินทรีย์ทั้ง 5 ชนิดได้ดี จึงนำมาทดสอบความเป็นพิษต่อเซลล์มะเร็งปากมดลูก จากผลการทดลองพบว่าสารสกัดหยาบ X-17 มีความสามารถในการยับยั้ง การเจริญของ เซลล์ไลน์มะเร็งปากมดลูก CaSki ซึ่งมีไวรัส HPV โดยมีค่า $IC_{50}$ เท่ากับ 10.05±0.28 μg/ml และยังชักนำให้ วัฏจักรของเซลล์หยุด (cell cycle arrest) ทำให้เซลล์เกิดการตายแบบอะพอพโ ตซิส (apoptosis) ในเซลล์มะเร็ง เนื่องจากสารสกัดหยาบ X-17 มีฤทธิ์ต้านจุลินทรีย์และเซลล์มะเร็งสูง จึงเลือกเพื่อนำไปทดสอบฤทธิ์ต้านการอักเสบ แต่ไม่พบว่าสารสกัดหยาบ X-17 มีฤทธิ์กดการอักเสบ เมื่อนำสารสกัดหยาบ X-17 มาแยกด้วยเทคนิคลิควิด โครมาโทกราฟี ทำให้ได้สารบริ สุทธิ์เป็น cytochalasin D และ 2-*p*-tolylethanol เมื่อนำราเอนโดไฟต์ ไอโซเลต X-17 มาจำแนกสายพันธุ์ โดยตรวจสอบลำดับเบสของยีน ตำแหน่ง ITS (internal transcribe spacer) และศึกษาลักษณะทางสั ณฐานวิทยาและลักษณะการเจริญบนอาหารเลี้ยงเชื้อ พบว่า ราเอนโดไฟต์ ไอโซเลต X-17 คือ *Xylaria laevis.*

สาขาวิชา จุลชีววิทยาทางอุตสาหกรรม ลายมือชื่อนิสิต.....

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KUNLANEE KHAJADPAI: ISOLATION OF ENDOPHYTIC *Xylaria* spp. FROM TRAD PROVINCE AND THEIR METABOLITES WITH ANTI-CERVICAL CANCER ACTIVITY. ADVISOR: ASSOC.PROF. TANAPAT PALAGA, Ph.D., CO-ADVISOR : ASSOC. PROF. PRAKITSIN SIHANONTH, Ph. D., 68 pp.

Metabolites from microorganisms are considered to be a rich source of novel lead compounds for drug development. Endophytic fungi are increasingly used to screen for such compounds because they are relatively unexplored until now. In this study, we isolated endophytic *Xylaria* spp. from plant leaves sampling in Trad Province in eastern Thailand, and screened for biological activity of the isolated fungal metabolites. A total of 83 isolates included 41 *Xylaria* spp., 5 *Daldinia* spp., 1 *Fusarium* spp., 1 *Collectotrichum* spp., 4 *Pestalotiopsis* spp., 2 *Phomopsis* spp., 2 *Phyllosticta* spp., 1 *Trichoderma* spp., 4 *Nigrospora* spp. and 22 Mycelia sterilia. Crude extracts with ethyl acetate of all endophytic *Xylaria* spp. were screened for antimicrobial activity against 5 reference pathogenic bacteria and yeast. Moreover, nine crude extracts with strong antimicrobial activity were tested for cytotoxicity against human papiloma virus positive cervical cancer cell line, CaSki. Among these isolates, endophytic fungi isolate X-17 showed both strong antimicrobial and anticancer activity. Crude extract from X-17 showed cytotoxic activity against human cancer cell line, with the inhibitory concentration 50 ($IC_{50}$) of 10.05±0.28 µg/ml. Detailed study on the cytotoxicity of the X-17 against CaSki cell line demonstrated that this extract induced cell cycle arrest and apoptosis. Therefore, it was chosen for further test for anti-inflammatory activity. Nevertheless, crude extract from X-17 did not show anti-inflammatory activity. Purification of bioactive compounds from this extract was carried out by liquid chromatography and two pure compounds were obtained that were identified as cytochalasin D and 2-*p*-tolylethanol. In addition, morphology, cultural characteristics and ITS (internal transcribe spacer) sequencing analysis of endophytic isolate X-17 confirmed that it belongs to *Xylaria laevis*.

Field of Study : Industrial Microbiology Student's Signature : .....
Academic Year : 2012 Advisor's Signature : .....
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## LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| 1 | ˚C | Degree Celsius |
| 2 | % | Percentage |
| 3 | / | Per |
| 4 | µg | Microgram |
| 5 | µl | Micro liter |
| 6 | µM | Micro molar |
| 7 | ATCC | American Type Culture Collection |
| 8 | $CDCl_3$ | Trichloromethane-d |
| 9 | $CH_2Cl_2$ | Dichloromethane |
| 10 | DMEM | Dulbecco's Modified Eagle Medium |
| 11 | DMSO | Dimethyl sulfoxide |
| 12 | EtOAC | Ethyl acetate |
| 13 | g | gram |
| 14 | HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| 15 | $^1$H NMR | Proton Nuclear Magnetic Resonance |
| 16 | IFNγ | interferon-gamma |
| 17 | ITS | Internal Transcribed Spacer |
| 18 | LPS | lipopolysaccharides |
| 19 | MEB | Malt extract broth |
| 20 | MeOH | methanol |
| 21 | MHB | Mueller-Hinton broth |
| 22 | mg | milligram |
| 23 | min | minute |
| 24 | ml | milliliter |
| 25 | NA | Nutrient agar |
| 26 | NB | Nutrient broth |
| 27 | nm | nanometer |
| 28 | PBS | Phosphate buffer saline |
| 29 | PCR | Polymerase chain reaction |

| | | |
|---|---|---|
| 30 | PDA | Potato dextrose agar |
| 31 | TLC | Thin layer chromatography |
| 32 | YMA | Yeast malt extract agar |
| 33 | YMB | Yeast malt extract broth |

# CHAPTER I

## BACKGROUND

In recent years, multidrug resistance in pathogens has become a serious medical problem. Standard treatments are often ineffective and infections persist and may spread to others. Moreover, tremendous increase in the incidence of cancer in the world's population is a serious health problem. Therefore, the need for novel bioactive compounds to assist and relief health condition of human are urgently required.

Endophytes are defined as all organisms that live inside plant tissue for at least part of their life cycle without causing any disease symptoms to the host (1). The relationship between endophytes and their host plants may range from symbiotic to near pathogenic (2). In other words, endophytic fungi are thought to interact mutualistically with their host plants. For example, they may contribute to their host plants by producing large numbers of substances that provide protection and ultimately survival to the plants (3).

One of the most interesting endophytic fungi is in the Family of Xylariaceae. The Xylariaceae represents one of the largest and most important fungal family in the phylum Ascomycota and the Fungi. The exact number of currently accepted taxa varies among researchers. For instance, sixty genera that potentially belong to the Xylariaceae were listed by Lumbsch & Huhndorf (2007) (4) and it has representatives in most countries of the world. Moreover, the Xylariaceae exhibits its greatest diversity in the tropics and is commonly found growing on dead wood or living in plant tissue for instance *Daldinia*, *Hypoxylon* and *Xylaria* (5).

*Xylaria* spp. are the rich source of bioactive compounds such as terpenoids, xyloketals, xanthones and cytochalasins ((6), (7), (8), (9)). Many of these compounds possess highly useful biological activities, such as anticancer (10), antifungal (11), antioxidant (12), antimicrobial (13), anti-inflammatory (14) and antiviral activities (15).

Thailand situates in a tropical region and is considered as one of the areas containing high percentages of unknown taxa of *Xylaria* spp. Therefore, it is

reasonable to expect the large numbers of interesting metabolites to be isolated from diversed *Xylaria* spp. The Trad Agroforestry Research Station is a research and training facility in eastern Thailand where has high biodiversity of plants and animals.

The aims of this study are to isolate endophytic *Xylaria* spp. from plants in Trad province, Thailand, and to screen for biological activities of the isolated fungal metabolites including antimicrobial, anti-inflammation and anticancer activities.

**The objectives of this study are:**

1. To isolate endophytic *Xylaria* spp. from Trad province
2. To screen for bioactive compounds derived from isolated endophytes with antimicrobial, anti-inflammation or anticancer activities

# CHAPTER II

# LITERATURE REVIEWS

## 2.1 Fungi and plants

Fungi are members of eukaryotic organisms. They differ from green plants in their lack of chlorophyll and they cannot photosynthesize their organic food from $CO_2$ and water. They have cell walls that contain chitin, unlike the cell walls of plants, which contain cellulose. The population of fungi was found in different hosts and environments, for example endophytic fungi, mychorrhiza, phylloplane fungi, wood-attacking fungi, plant parasitic fungi, litter decomposition and root-inhabiting fungi.

Symbiosis is a mode of life adopted by many fungi. De Bary, a nineteenth century mycologist and plant pathologist, described the word of symbiosis as some organisms live in close contact with organism of another species and obtain nutrients from them or benefit in other ways. Moreover, he noted that a symbiotic relationship might be parasitic which only one species benefits, or mutualistic with both partners benefiting. A parasite that causes damage to its host is a pathogen (16).

Some fungal inhabitants of plant form close contact with plant cells. Fungal hyphae are well suited for penetrating plant tissues. This ability is widely exploited for the invasion of living plants and the establishment of a symbiotic relationship. Some fungi are facultative symbionts, found both as free-living saprotrophs and within plants. Others are obligate symbionts, growing only in association with living plants (17). The hyphae of some symbiotic fungi penetrate plant cells, whereas those of other grow through tissue without entering cells. Some fungi are biotrophic, living within the plant and obtaining nutrients without causing host cell death. Some are necrotrophic, killing host cell and absorbing nutrients from the dead tissue. Other has both a biotrophic and later a necrotrophic phase (18). Many fungi are important plant pathogens causing severe crop losses whereas others, such as endophytic fungi, are mutualistic and can have an important role in plant nutrition.

## 2.2 Endophytic fungi

De Bary (1866) (19) gave first meaning of word “endophyte” as all microorganisms that colonize internal plant tissue, typically causing no apparent symptoms of disease. On the other hand, they were the cause of disease if they were stimulated or weaken. Later the term of endophyte is used with particular meaning by different researcher and for particular groups of hosts and microbes. Petrini (1991) considers the term endophyte to be purely topographical, “Endophytes colonize symptomlessly the living, internal tissue of their host, even though the endophyte may, after an incubation or latency period, cause disease”(1).

An endophyte can be found ubiquitously in all plant species in the world. Approximately 300,000 plant species growing in unexplored area on the earth are host to one or more endophytes (20). They may contribute to their host plant by producing a large numbers of substances that provide protection and ultimately survival to the plant.

Endophytes can be transmitted to host plant via either vertically (directly from parent to offspring) or horizontally (from individual to unrelated individual) (21). Vertically transmitted fungal endophytes are asexual and transmission of the systemically transmitted fungus passes from one generation to another through the seed, via fungal hyphae penetrating the host‟s seeds. The fungus enters the seeding from the seed and spread throughout the plant, enters new tissue 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 and/or insect vectors. 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.

In general, endophytic fungi are divided into two groups, the clavicipitaceous endophytes (C-endophytes) which infect some grasses and the nonclavicipitaceous endophytes (NC-endophytes) which can be recovered from asymptomatic tissues of nonvascular plants, ferns and allies, conifers, and angiosperms (22).

Keith Clay (1988) proposed that clavicipitaceous endophytes are defensive mutualists of host grasses (3). As this hypothesis became widely known, investigations into endophyte natural history, evolution, ecology, and physiology followed. As a result of the beneficial effects of C-endophytes, turf grass breeders now offer a variety of cultivars identified as „endophyte enhanced" (23).

## 2.3 Secondary metabolites from endophytic fungi

Practical applications of endophytes include plant protection and as research model systems for investigation of host-parasite interactions and evolution in natural system. Moreover, they produced potential biological control agents or bioactive compounds, sources of novel metabolites for medicine and industrial uses. Several in the group of bioactive compounds derived from endophytic fungi include compound, alkaloid, steroids, terpenoid, phenols, quinines and flavonoids, etc (6). They exhibited several biological activities such as anti-cancer (10), anti-fungal (11), anti-oxidant (12), anti-microbial (13), anti-inflammatory (14) and anti-viral activities (15). Many researchers report on bioactive compounds produced by endophytic fungi that are active against pathogenic microorganisms.

### 2.3.1 Principle for plant selection to isolate endophytic fungi

In order to get novel bioactive compounds from endophytic fungi, process of selecting of the plant is an important. Plants selection strategies should be as follows:

1. Plants that grow in area of high biodiversity in order to find housing endophytes with great biodiversity (24). Thus, tropical rainforest is interesting source for isolation and screening.
2. Plants that grow in special habitats especially in deteriorated ecological environment.
3. Plants surround by pathogens infected plants which show no symptom.
4. Plants that were used for traditional medicine.
5. Plants occupy a certain ancient land mass (25).

### 2.3.2 Antimicrobial compounds

Antibiotic defined as low-molecular-weight organic natural substances produced by microorganisms that are active at low concentrations against other microorganisms (26). Many researchers reported a large number of antimicrobial compounds isolated from endophytes with considerable antimicrobial activity against pathogenic microorganisms including bacteria, fungi, virus and protozoans. Moreover, they can be used not only as drugs but also as food preservatives in the control of food spoilage and food-borne disease. Some examples of antibiotic compounds produced from endophytic fungi are shown in Table 2.1

**Table 2.1** Antibiotic produced by endophytic fungi

| Fungal endophyte | Host | Antibiotic | Target pathogen | Reference |
|---|---|---|---|---|
| *Acremonium zeae* | Maize | Pyrrocidines A, B | *Aspergillus flavus*, *Fusarium verticillioides* | Wicklow *et al.* (2005)(27) |
| *Verticillium* sp. | *Rehmannia glutinosa* | Massariphenone, Ergosterol peroxide | *Pyricularia oryzae* P-2b | You *et al.* (2009)(28) |
| *Phomopis cassiae* | *Cassia spectabilis* | Cadinane sesquiterpenes | *Cladosporium sphaerospermum*, *Cladosporium cladosporioides* | Silva *et al.* (2006)(29) |
| *Muscodor albus* | Tropical tree | Tetrohydofuran, 2-methyl furan, 2-butanone, Aciphyllene | *Stachybotrys chartarum* | Atmosukarto *et al.* (2005)(30) |
| *Periconia* sp. | *Taxus cuspidata* | Fusicoccane diterpenes | *Bacillus subtilis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Salmonella* Typhimurium | Kim *et al.* (2004)(31) |

| Fungal endophyte | Host | Antibiotic | Target pathogen | Reference |
|---|---|---|---|---|
| *Ampelomyces* sp. | *Urospermum picroides* | 3-O-Methylalaternin, Altersolanol A | *Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis* | Aly *et al.* (2008)(32) |

### 2.3.3 Anticancer compounds

Cancer is a major cause of death worldwide and present most anti-cancer drugs show nonspecific toxicity to proliferating normal cells. This effect leads to undesired side effects. In addition, it is not effective against many forms of cancer. Thus, searching for the novel drugs is important and urgently. Bioactive compounds produced by endophytic fungi could be alternative approaches for new discovery. Some general example of anti-cancer agent produced by endophytic include taxol, camptothecin and gliocladicillin A and B (33).

Taxol is a compound interfering with the multiplication of cancer cells. It is used for treatment of breast cancer, lung cancer and refractory ovarian cancer (34). Taxol was isolated from the bark of trees in the Taxus family (*Taxus brevifolia*), which was the first most common source (35). Nevertheless, these trees are rare, slow growing and produce small amount of product, resulting in high price of the drug. Endophyte *Taxomyces andreanae* is an alternative approach to obtained taxol cheaper than taxol from the trees. Furthermore, many researchers reported that taxol was produced from different genera of endophyte such as *Taxodium diatichum* (36), *Wollemia nobilis* (37), *Phyllosticta spinarum* (38), *Bartalinia robillardoides* (39), *Pestalotiopsis terminaliae* (40) and *Botryodiplodia theobromae* (41).

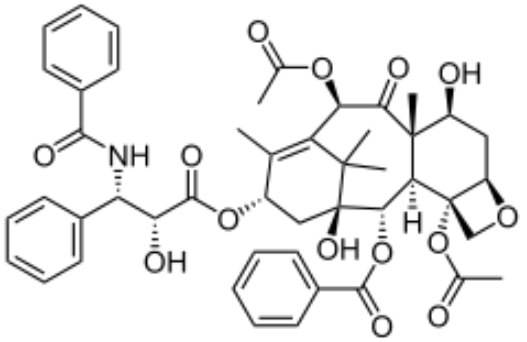



**Figure 2.1** Chemical structure of Taxol isolated from Endophyte *Taxomyces andreanae*.

Camptothecin is a pentacyclin quinoline alkaloid that was isolated from *Entrophospora infrequens* (42) and *Neurospora* sp. (43) endophytic fungi of *Nothapodytes foetida*. It shows excellent anticancer activity with a unique mechanism of action involving interference with DNA replication and gene transcription.

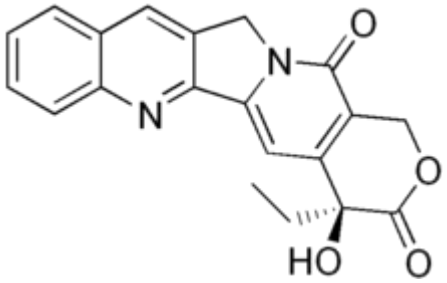



**Figure 2.2** Chemical structure of Camptothecin isolated from Endophytic fungi.

Gliocladicillin A and B were reported as effect in anti-tumor agents *in vitro* and *in vivo*. Chen *et al.* (2009) reported that Gliocladicillin A and B inhibited growth of tumor cell lines HeLa, HepG2 and MCF-7. Moreover, his further study demonstrated that both preparations arrested the cell cycle at G2/M phase in a dose-dependent manner and induced apoptosis through up-regulation of expression of p53, p21, and cyclin B, and activation of caspases-8, -9 and -3. In addition, *in vivo* studies showed that Gliocladicillin A and B exhibits significant inhibitory effects against cell growth of melanoma B16 cells implanted into immunodeficient mice (44).

### 2.3.4 Anti-oxidant compounds

Normally, anti-oxidants are commonly found in medicinal plants, vegetables and fruits. However, metabolites from endophytes can be potential sources of natural anti-oxidant for example pestacin and isopestacin were obtained from endophytic fungi *Pestalotiopsis microspora* isolated from *Terminalia morobensis* (45, 46). Another compound is Graphislactone A that was isolated from endophytic fungi *Cephalosporium* sp. associated with *Trachelospermum jasminoides* which showed free radical-scavenging and antioxidant activity (47). Moreover, it was has a reported by Liu *et al.* (2007) that phenolic and flavonoid extracted from endophytic *Xylaria* in medicine plant *Ginkgo biloba* showed strong antioxidant (48).

## 2.4 Xylariaceae

Xylariaceae is a Family of Ascomycetous fungi and generally accepted to consist of at least 60 genera (4). They were characterized by stromata which are usually well developed and generally consist of fungi only. They commonly found growing on death wood or living in plant tissue and have a worldwide distribution especially in the tropics where in many cases the Xylariaceae emerge as the dominant residents (49). In Thailand the species of Xylariaceae belonging to *Hypoxylon*, *Daldinia* and *Xylaria* were usually found as both saprophyte and endophyte (50).

Endophytic *Xylaria* spp. are interesting source of this family which was found many bioactive compounds. There are many reports on bioactive compounds produced by endophytic *Xylaria* spp. that are active against pathogenic microorganisms as listed below.

Liu *et al.* (2007) reported on phenolic and flavonoid derivertives (48) were isolated from endophytic *Xylaria* sp. (strain number YX-28), from *Ginkgo biloba*. It showed antioxidant activity. Total phenolic and flavonoid contents extracted by different solvents (methanol and hexane) were determined using Folin–Ciocalteu procedure and the flavonoid–aluminium method. The results indicated that the methanol extract exhibited strong antioxidant capacity in both 2, 2-diphenyl-1-picrylhydrazyl (DPPH) analysis and beta-carotene–linoleic acid model system.

Gu and Ding (2008) reported that two new tetralone derivatives named Xylariol A and B (8) were isolated from ethyl acetate extract of the culture broth of *Xylaria hypoxylon* AT-028. It showed moderate cytotoxic activities against hepatocellular cell line (Hep G2 cells) in the *in vitro* cytotoxic assay with $IC_{50}$ values of 22.3 and 21.2µg/ml, respectively.

Recently, Oliveira *et al*. (2011) reported that the dihydroisocoumarin (3R,4R)-3,4-dihydro-4,6-dihydroxy-3-methyl-1-oxo-1H-isochromene-5-carboxylic acid was isolated from *Xylaria* sp., a fungus associated with *Piper aduncum* (Piperaceae). It showed antifungal and acetylcholinesterase (AChE) inhibitory activities *in vitro*. Therefore, dihydrocoumarins should be considered as potential lead compounds in the development of new compounds for use in the drug design for Alzheimer"s disease treatment (51).

**2.5 Inflammation**

Inflammation is a complex process of an immune response to tissue damage from stimuli such as foreign substances, irritants and pathogens. The classic signs of inflammation are redness, swelling, heat, pain and loss of function that result from dilation of the blood vessels leading to an increased blood supply and from increased intercellular spaces resulting in the movement of leukocytes, protein and fluids into the area of inflammation (52). Inflammation is divided into two parts consisting of acute and chronic inflammation. The immediate response to an injury and usually only sustains in a short duration for minutes/hours or days is refer to as acute inflammation. On the other hand, chronic inflammation is often carried out as long as many weeks or months where inflammatory process continues during tissue damage. Chronic inflammation is a major cause of many diseases such as artherosclerosis, rheumatoid arthritis and cancer.

Macrophage is the key play during inflammation. Macrophages play a crucial role in the inflammatory response through the release of a variety of factors, such as nitric oxide (NO), prostaglandin mediators and proinflammatory cytokines (TNF-α, IL-1β, IL-6), in response to an activating stimulus (53), e.g. lipopolysaccharide (LPS). Production of these mediators can be found in several inflammed tissues.

### 2.5.1 Nitric oxide

Nitric oxide (NO) is a gaseous signaling molecule that regulates various physiological and pathophysiological responses in the body. These include circulation and blood pressure, platelet function, host defense, neurotransmission in central nervous system and in peripheral nerves (54). NO is synthesized from L-arginine in catalysis reaction by nitric oxide synthase (NOS) enzyme. NO is a mediator of inflammation. Moreover, nitric oxide has both proapoptotic and anti-apoptotic properties, depending on the concentration and flux of NO and the source which NO is derived (55).

## 2.6 Cancer

The term “Cancer” which mean crab in Latin, was defined by Hippocrates, father of medicine, in the fifth century BC to describe a disease which abnormal cells proliferate in an uncontrolled and spread through the body. Such cells can arise in a variety of tissue and organ and each of these sites contain different cell types that may be affected. It had considered one of the major causes of deaths worldwide (56).

Tumor can be divided in two different categories based on the growth patterns. One group consists of benign tumors which grow in a confined local area or cells do not spread to other parts of the body and they are not cancerous. In contrast, malignant tumors can invade surrounding tissues, enter the bloodstream or lymphatic system and spread to distant parts of the body by a process called metastasis (Figure 2.3) (57). Differences in the properties of the two tumor types are shown in Table 2.2. The term of cancer is referring to any malignant tumor.

**Table 2.2** Some properties of benign and malignant tumors

| Properties | Benign | Malignant |
|---|---|---|
| Growth pattern | Local growth only | Spread by invasion and metastasis |
| Life threatening | Rarely | Often |
| Growth rate | Usually slow | May be rapid |
| State of differentiation | Well differentiated | Variable |

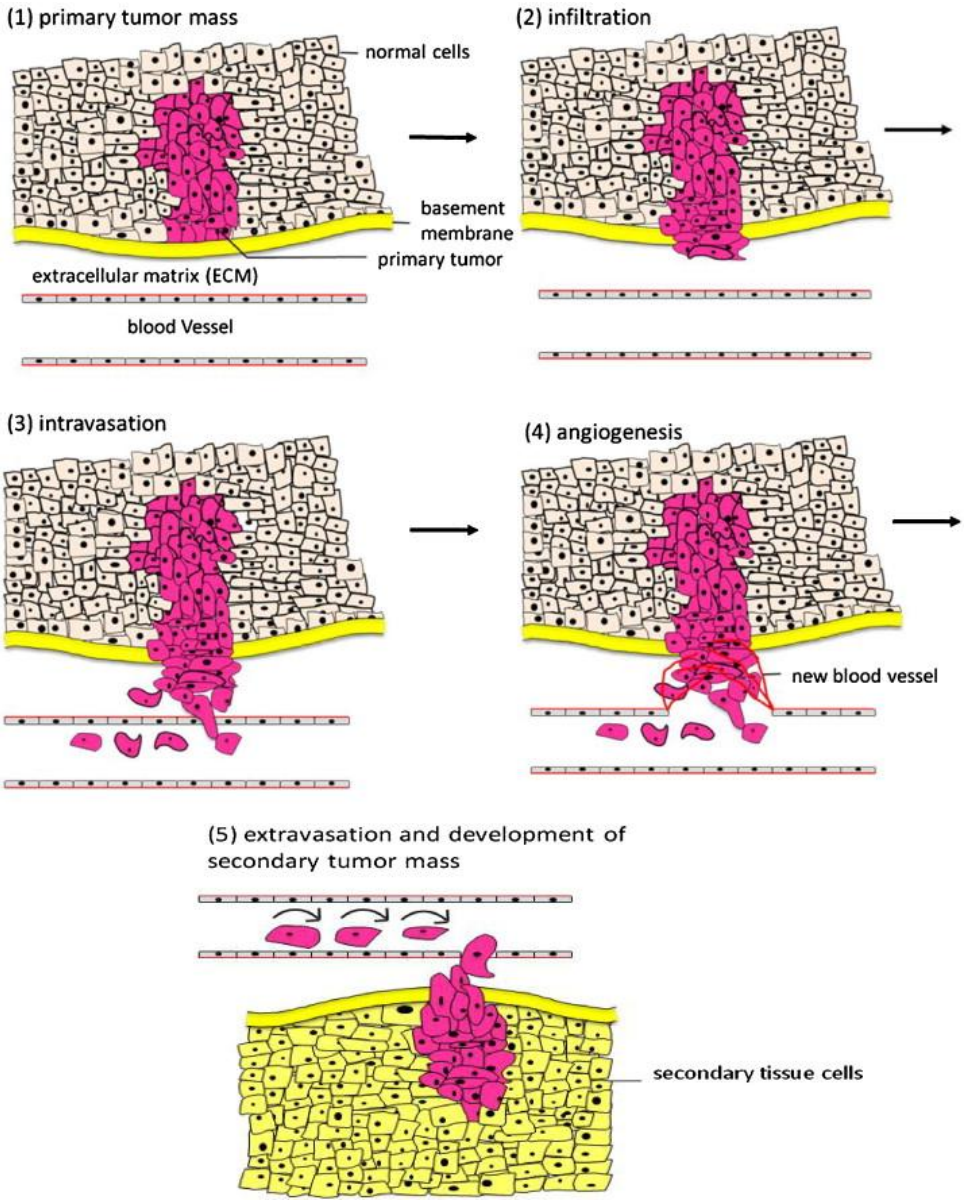



**Figure 2.3** The main steps in the formation of a metastasis.

### 2.6.1 Cervical cancer

Cervical cancer threatens the lives of women all over the world and it is the principal cancer of women in most developing countries, where 80 percent of tumor cases were found (58). In Thailand, cervical cancer is the big killer disease of Thai women about 20.9 per population of hundred thousand with 6,300 new cases each year (59). Cervical cancer affects mostly women between the ages of 45 and 50. Most cervical cancers begin in the cells lining the cervix from squamous cells. These cells do not suddenly change into cancer but the normal cells of the cervix first gradually develop pre-cancerous changes that turn into cancer.

Almost all cervical cancers were caused by infection with HPV (human papilloma virus). HPV is a common virus that is spread through sexual intercourse. There are more than 80 types of HPV which have been identified and about 40 types of them infect genital tract (60). A high-risk HPV is the underlying cause of cervical cancer which includes type 16, 18 and 31. On the other hand, low-risk HPV type 6 and 11 are not associated with cancer, but cause genital warts.

Although the causes of cervical cancer are not well understood, certain risk factors have been identified. They include women with multiple sexual partners, those who started having sexual relation before the age of 18, women with HPV infection, smokers, those with a high number of births and finally women with a history of cervical cancer in the family (61).

The treatments methods chosen for treating each patient with cervical cancer depend on many reasons such as the stage of disease, the size of the tumor, the patient's desire to have children and the patient‟s age (62). There are commonly 3 treatments methods used to treat cervical cancer including surgery, chemotherapy, and radiation therapy (63). Sometimes they are used alone, and there are times when they are used in conjunction with one another. However, the prevention is an important. Gardasil is a vaccine which prevents infection against HPV responsible for most cervical cancer case (64). Moreover, risk avoidance from the risk factor is a way to do.

## 2.7 Apoptosis

Apoptosis (programmed cell death) is a process that function thorough out life, eliminating cells after they are no longer needed, such as during differentiation, or when they aged, such as for white blood cells following a few months of function. Another function of apoptosis is to rid the body of defective cells. For example, cells infected with virus invoke apoptosis to trigger their own destruction thereby limiting reproduction and the spread of virus. Cell with damage DNA also trigger apoptosis especially if the damage cannot be repaired. This ability to destroy genetically damaged cell is especially useful in helping prevent the development of cancer.

There are two pathways, the intrinsic and extrinsic apoptotic pathways (Figure 2.4) (65). The intrinsic pathway also known as mitochondrial pathway triggers apoptosis in response to DNA damage, defective cell cycle, detachment from the extracellular matrix, hypoxia, loss of survival factor and other type of severe cell stresses. This pathway involves the activation of pro-apoptosis cascade which turn activates the mitochondria. This event leads to release of a group of mitochondrial proteins including cytochrome C that activates the caspase cascade. Another is an extrinsic pathway which responds to engagement of death receptors by their ligands. This pathway stimulates the apoptotic caspase cascade (66).

The first observed morphology changes of cell going apoptosis is cell shrinkage. After that, small bubble-like protrusions of cytoplasm (blebs) starts forming at the cell surface as the nucleus and other cellular structures begin to disintegrate. Genomic DNA is degraded into small pieces and the entire cell breaks apart forming small fragments called apoptotic bodies. Finally, apoptotic bodies are swallowed up by nearby phagocytes. These genetically regulated programmed cell deaths are distinct from necrosis. Apoptosis cleanly eliminate specific cells with minimal effect on the microenvironment and nearby cells. In contrast, necrosis is causes inflammation of surrounding tissue due to released components of dead cell (67).

The main strategy to treat cancer includes surgery, radiotherapy and chemotherapy (68). These approaches work primarily by damaging proliferation of the cell at the level of DNA replication or cell division, and induce apoptotic cell

suicide as a secondary response to the damage. As mentioned above, apoptosis represents a universal and exquisitely efficient cellular suicide pathway that is a therapeutic goal for cancer therapy. Understanding of cellular damage responses and the physiological cell death mechanism has improved, leading in turn to new insights into drug-induced cell death. Drugs of different structure and specificity induce the characteristic morphological changes associated with apoptosis and it is now believed that apoptotic pathways contribute to the cytotoxic action of most chemotherapeutic drugs (69).

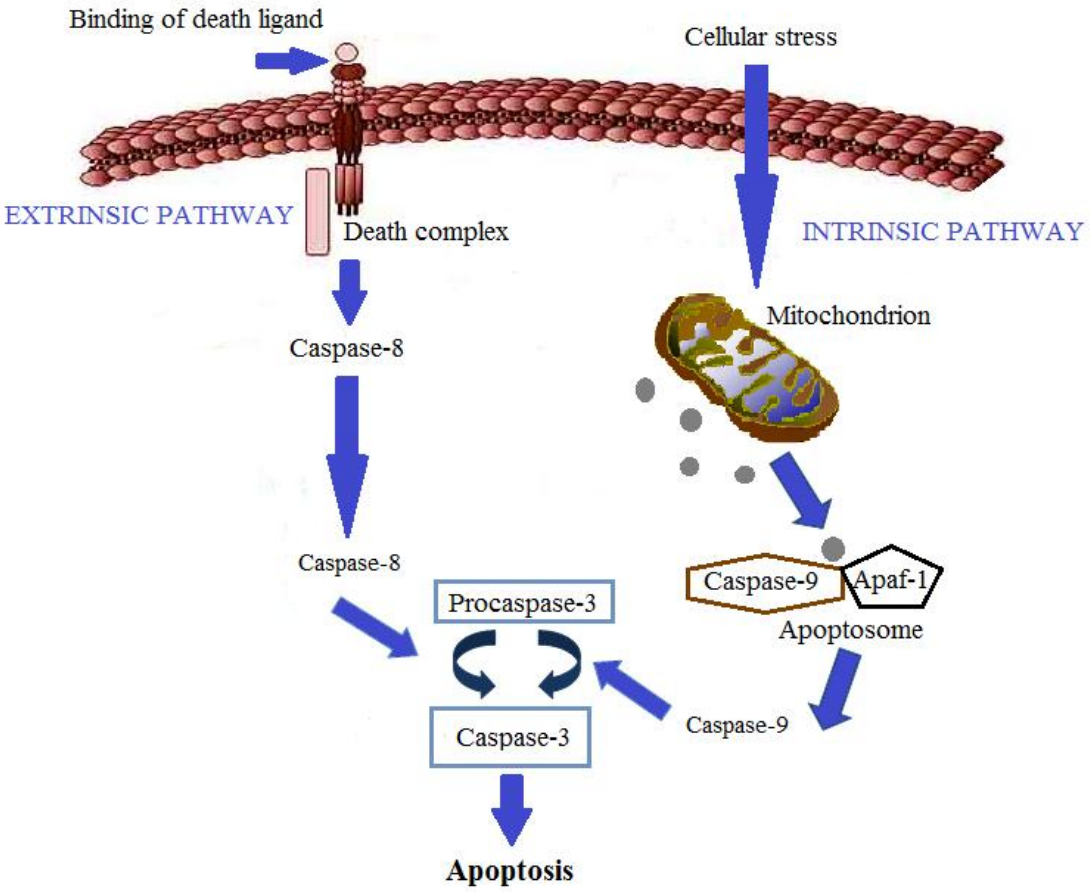



**Figure 2.4** Apoptosis pathways: Intrinsic and extrinsic pathway

# CHAPTER III

# MATERIALS AND METHODS

## 3.1 Collection of plant samples

Healthy leaves were collected from Trad Agroforestry Research and Training Station, Trad province, Thailand, in July 2010. The leaf samples consist of *Dipterocarpus alatus*, *Cinnamomum inners*, *Artocarpus heterophyllus*, *Mangifera indica*, *Peltophorum dasyrachis*, *Anthocephalus chinensis*, *Cinnamomum porrectum*, *Denarocalamus latifloma*, *Dracaena sanderiana* and *Baccaurea ramiflora*. All samples were stored in sterile plastic bags in an icebox and were used to isolate endophytic fungi within 48 h. of collection.

## 3.2 Isolation of endophytic fungi

Healthy leaves were carefully washed with running tap water and cut into small pieces of approximately 5×5 $mm^2$. The samples were surface sterilized using the method described by Blodett *et al*. (2000) (70). Briefly, the samples were immersed in 95% ethanol for 1 min, 10% sodium hypochlorite solution for 5 min, 95% ethanol for 30 seconds and then rinsed in sterile water and allowed for surface to dry on filter paper under sterile condition. The surface sterile samples were placed on potato dextrose agar (PDA) and incubated at 30ºC for 2-3 weeks. The hyphal tips of the endophytic fungus growing out from the plant tissue were subsequently transferred to fresh PDA plate and incubated at room temperature for 7-14 days. The purity of culture was determined by colony morphology. The endophytic fungal isolates were maintained on PDA for future study.

## 3.3 Maintenance of endophytic fungi

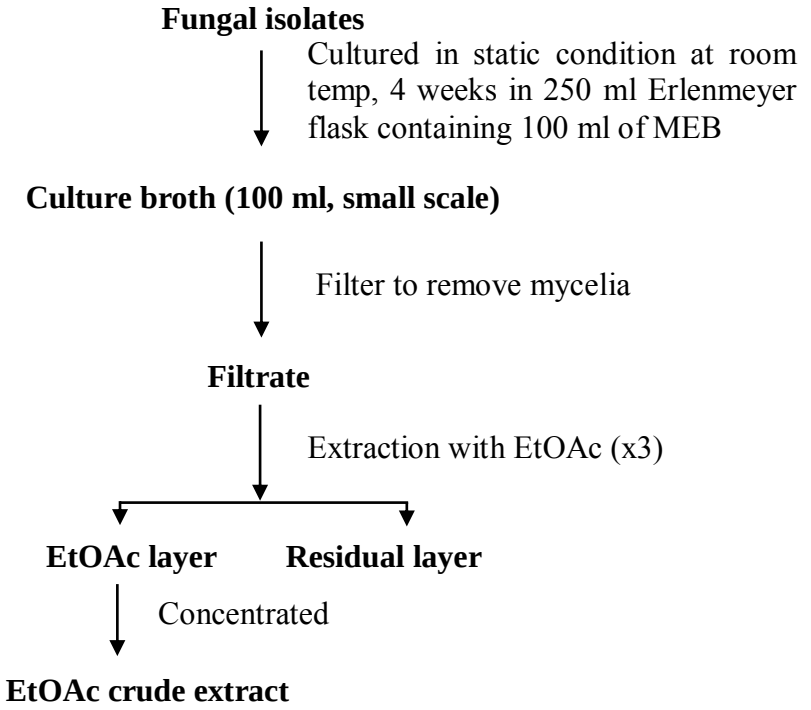
The pure cultures of endophytic fungi were grown on PDA plates at room temperature for 7-10 days and the grown culture was cut and placed on PDA slant and

incubated at room temperature. After 7-14 days, the culture was kept at 4˚C. This method was used to preserve the culture for 2-3 months. On the other hand, the pure culture was cut by cork borer and kept in sterile water at room temperature for longer period of storage around 6 months to 1 year.

### 3.4 Fermentation and extraction

The cultures of endophytic fungi were grown on PDA plates at room temperature for 7-14 days, depending on the growth rate of each isolate. Five pieces (6x6 $mm^2$) of the grown culture were cut by cork borer and inoculated into 250 ml Erlenmeyer flasks containing 100 ml of malt extract broth (MEB), followed by static culture condition at room temperature for 30 days.

The fermentation broth was filtered and the filtrate was extracted three times with ethyl acetate (EtOAc). EtOAc was removed by rotary evaporator. The mycelia were frozen at -20˚C for DNA extraction. The general extraction procedure of fungal culture broth is shown in **Scheme 3.1**




**Scheme 3.1** General procedure for extraction of fungal culture broth

The crude extracts were dissolved in 10% dimethylsulphoxide (DMSO) (Sigma Aldrich, USA) and used for testing for their biological activities.

### 3.5 Selection of endophytic fungal isolate

Thirty eight endophytic *Xylaria* spp. were isolated from 10 leaves of different trees and they were selected for the further study based on their antimicrobial activity by paper disk diffusion method.

#### 3.5.1 Test Microorganisms

An *in vitro* antimicrobial susceptibility test was performed by using reference pathogenic microorganisms including two Gram positive bacteria, *Staphylococcus aureus* (ATCC 6538-P), *Bacillus subtilis* (ATCC 6633) and two Gram negative bacteria, *Pseudomonas aerogenosa* (ATCC 9027), *Escherichia coli* (ATCC 8739) and yeast, *Candida albicans* (ATCC 90028).

#### 3.5.2 Preparation of bacteria inoculums

Test bacteria were grown on Nutrient agar (NA) for 24 h. at 37˚C. Selected four or six single colonies were inoculated into 5 ml of Nutrient broth (NB) and incubated in shaking incubator 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).

#### 3.5.3 Preparation of yeast inoculums

Yeasts were grown on Yeast malt extract agar (YMA) for 24 h. at room temperature. Selected four or five single 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 (OD 0.1 at 625 nm).

#### 3.5.4 Paper disk diffusion method

After adjusting the turbidity of the inoculum suspension, a cotton stick was dipped into the inoculum suspension. The dried surface of NA plate for bacteria and YMA plate for yeast were inoculated by streaking the plate in one direction, then streaking at right angles to the first streaking and subsequently finished by streaking

diagonally. The procedure was ended by using the cotton stick to streak the outside diameter of the agar. The surface of the medium was allowed to dry for 3-5 min.

Afterward the disks (6 mm in diameter) impregnated with crude extract solution were placed on inoculated plates. All of the plates were incubated at 37˚C for bacteria and at room temperature for yeast for 24 h.

The diameter of inhibition zones were used as an indicator of antimicrobial activity. Streptomycin and nystatin (Bio BASIC INC., Canada) were used as positive control for bacteria and yeast, respectively. DMSO was used as a negative control.

**3.5.5 Determination of minimum inhibitory concentration (MIC)**

Solution of test crude extracts in DMSO (25.6 mg/ml) was diluted with Mueller-Hinton broth (MHB). The test crude extracts were tested at the concentrations between 0.5 and 256 $\mu$g/ml.

Fifty microliters of MHB containing the test crude extracts were dispensed into each well of microtiter plates (96-well plates) for the evaluation of antibacterial activities. Sterile crude extract-free medium containing the corresponding amount of DMSO was dispensed in the control wells. The finally adjusted bacterial suspensions were inoculated into each well with the volume of 50 $\mu$l. Crude extract-free MHB (100 µl) were used as the sterility control. The experiments were carried out in duplicate. After incubation at 37˚C for 24 h., *p*-iodonitrotetrazolium (20 µl) (INT) solution (1mg/ml) (TCI, USA) was added into each well. The antibacterial assay plates were further incubated for 1 h. Growth in each well was indicated by a color change from colorless to violet. Crude extract that inhibit microbial growth would prevent the development of a violet color. The well that shows no change in color indicates antimicrobial activity of the crude extract.

## 3.6 Screening of endophytic fungi for their biological activity

### 3.6.1 Cell line and media

RAW 264.7 macrophage-like cell line (ATCC TIB-71) was maintained in Dulbecco's Modified Eagle Medium (DMEM) (Hyclone, Logan, UT, USA) and Human papilloma virus positive cervical cancer cell lines, CaSki was cultured in RPMI-1640 (Hyclone, Logan, UT, USA) medium. All of them containing 10% fetal

bovine serum (v/v) (FBS) (Hyclone, England), 100 U/ml penicillin (M&H Manufacturing Co.Ltd, Bangkok, Thailand), 0.5 mg/ml streptomycin (M&H Manufacturing Co.Ltd, Bangkok, Thailand), 1 % sodium pyruvate (Hyclone, England) and 1% HEPES (Hyclone, England) and incubated at 37˚C in 5% $CO_2$ incubator.

**3.6.1.1 Cell preparation**

RAW264.7 cell line was harvested from non-treated culture plates by cold PBS. Harvested cells were pelleted by centrifugation at 1000 rpm for 5 min. The culture supernatant was discarded and cell pellets were resuspended in complete DMEM. For adhesive cervical cancer cell lines, CaSki, cells were detached from a tissue culture treated flasks by treating with 0.25% (w/v) Trypsin-0.53 mM EDTA (Hyclone, England) for 3-5 min. Complete RPMI 1640 media were added and cells were pelleted by centrifugation at 1000 rpm for 5 min. The culture supernatant was discarded and cell pellets were resuspended in complete DMEM/ RPMI 1640 media. Trypan blue dye (Hyclone, England) exclusion method was used to determine the number of viable cells present in a cell suspension for all experiments.

**3.6.1.2 Cell preservation for storage**

After harvesting cells by centrifugation, cells were resuspended in 1 ml cold DMEM/ RPMI 1640 freeze media containing 10% DMSO (v/v) and stored in cryogenic vials. The vials were stored at -80˚ C for short term storage or overnight at -80˚C and moved to the liquid nitrogen tank for long term storage.

**3.6.1.3 Thawing cell from stock**

The frozen cells in cryogenic vials were quickly thawed in the water bath. Nine milliliter serum-free DMEM/ RPMI 1640 media were added to cell suspensions and centrifuged at 1000 rpm for 5 min. The supernatant was discarded and DMEM/ RPMI1640 complete media were added. Thawing cells in complete media were placed in a tissue culture treated plate/flask and maintained for further experiments.

**3.6.2 MTT assay**

The cytotoxicity and anti-cancer activity was measured by an MTT assay. RAW 264.7 and CaSki cell line were maintained in complete DMEM and RPMI1640

medium, respectively. They were seeded in 96-well plates ($1x10^5$ cell/ml, 100µl perwell) and incubated at 37° C in a humidified atmosphere with 5% $CO_2$.

Cells were treated with crude extracts at various concentrations for 24 hrs. Four hours before the end of the treatment, MTT solution (5mg/ml;10 µl) (Alfa Aesar, UK) was added and incubated until the end of treatment. At the end of the treatment, 0.4 N HCl in isopropanol (100µl) was added to dissolve formazan and the absorbance was measured using a microculture plate reader at 540 nm. Percent of cell viability (%) was calculated using the following formula.

$$\text{Percent of cell viability (\%)} = \frac{\text{(OD test-OD blank)x100}}{\text{OD control -OD blank}}$$

The cytotoxic activity of the crude extracts was determined by inhibition concentration ($IC_{50}$).

Three crude extracts (X-12, X-17 and X-21) were analyzed by $^1H$ NMR. The crude extracts of the fungi that show interesting $^1H$ NMR signals were selected for large scale cultivation and extraction.

### 3.7 Anti-inflammatory activity assay

The effect of metabolites on the production of nitric oxide was investigated by Griess reaction to measure the amount of nitrite produced by activated macrophages. RAW 264.7 cell line was seeded into microtiter plates ($1x10^4$ cell/well; 100 µl) and the plates were incubated overnight at 37°C in a humidified atmosphere of 5% $CO_2$. The next day, cells were pretreated with crude extract or vehicle control for 1 h. and stimulated with 100 ng/ml LPS from *Salmonella* sp. (Sigma Aldrich, USA) and 10 ng/ml recombinant mouse IFN γ (R&D Systems, Canada) for 24 h. under the same condition. After that the culture supernates were collected for measuring nitric oxide.

To measure the amount of nitric oxide produced, culture supernates were added in microtiter plates (50 µl/well). Sulfanilamide (50 µl/well) was added to the wall and incubated at room temperature for 10 min in dark. After this step, *N*-(1-

napthyl) ethylenediamine (NED) (50 µl/well) was added and the mixture was incubated under the same condition. After 10 min of incubation, bubbles were removed with needle and the absorbance was measured at a 540 nm by microplate reader. The result was compared with the standard nitrite. Percent of inhibition against nitric oxide production (%) was calculated using the following formula.

$$\% \text{ Inhibitoion of nitric oxide production} = \frac{(\text{NO test-NO min}) \times 100}{\text{OD max -OD min}}$$

### 3.8 Apoptotic assay by Hoechst staining

CaSki cell line ($1x10^5$ cells/ml) was cultured on coverslips in 24 well plates and incubated at 37° C in a humidified atmosphere with 5% $CO_2$. After 24 h. of incubation, the cells were treated with crude extracts. Negative control cells were treated with DMSO. Positive control cells were treated with etoposide (20µM) (Sigma Aldrich, USA). After the incubation period, cells were washed with phosphate buffer saline (PBS) at pH 7.4 and fixed with 1% glutaraldehyde for 2 h. at room temperature in dark condition. Finally cells were washed with PBS, stained with Hoechst 33342 (1mM) (Sigma, USA) and covered with a glass slid. Apoptotic cells were observed under fluorescence microscopy (Olympus Optical, Japan).

### 3.9 Cell cycle analysis

Cell cycle was analyzed by propidium iodide staining (PI staining). After cells were treated and harvested, cells were washed by PBS once before resuspending in 200 µl cold 70% ethanol for fixation. After that incubated on ice at 4 h. or kept at -20˚C until further analysis. Ethanol was removed by centrifugation at 2000 g for 5 min and washed with 500 µl PBS. Cell pellet was resuspended in 250 µl PBS which contained 0.1 mg/ml RNaseA and incubated at 37˚C for 30 min. Finally, twelve point five microliters of 1 mg/ml propidium iodide was added into samples and incubated at room temperature in dark at least 30 min before cell cycle was analyzed by Flow

cytometer FC500 (Beckman Coulter, USA). Acquired data were analyzed by CXP analysis (Beckman Coulter, USA).

### 3.9.1 Statistical analysis

The data were analyzed by an independent Student's t-test using SPSS software. $p$ value < 0.05 was considered to be statistically significant.

## 3.10 Purification of bioactive compounds of endophytic *Xylaria* sp.

In a large scale fermentation, the endophytic *Xylaria* sp. X17 was cultivated in 500 ml Erlenmeyer flask (x100) containing 200 ml of MEB under static conditions at room temperature for 30 days. After that, the fermentation broth (20litres) was filtered and extracted with an equal volume of EtOAc three times. The solvent phase was evaporated using a rotary evaporator.

The EtOAc crude extract (6.82 g) was loaded onto a Sephadex LH-20 column (Pharmacia Code No. 17-0090-01) and eluted with MeOH. Similar fractions were pooled together based on their TLC pattern resulting in 9 fractions (P1 to P9). Afterwards, all fractions were tested for their cytotoxic activity against a CaSki cell line.

Fraction P5 was further subjected to $SiO_2$ column chromatography (CC) and eluted with MeOH: $CH_2Cl_2$ in gradient condition (2%, 4%, 6%, 8% and 10%) and yielded 5 fractions (P5S1 to P5S5). Fraction P5S2 was reloaded on silica gel and eluted with 10% MeOH: $CH_2Cl_2$ to yield Compound 1. Fraction P5S3 was purified by chromatography on a $SiO_2$ column and eluted with increasing polarity of 60 and 70% acetone: hexane to yield 5 fractions (P5S3S1 to P5S3S5) and then fraction P5S3S3 was reloaded on silica gel and eluted with 10% MeOH: $CH_2Cl_2$ to yield 4 fractions (P5S3S3S1 to P5S3S3S4). Fraction P5S3S3S2 was further purified by $SiO_2$ column using 5% MeOH in $CH_2Cl_2$ as eluent to yield compound 2.

### 3.10.1 Thin-layer chromatography (TLC)

Thin-layer chromatography (TLC) was carried out on a silica gel F254 coated on aluminium sheet (Merck). Detection was visualized under ultraviolet light at wavelengths of 254 and 356 nm and dipped with $(NH_4)_6Mo_7O_{24}$ solution in 5% $H_2SO_4$ /EtOH.

### 3.10.2 Column chromatography

Column chromatography was performed using Sephadex LH-20 and Silica gel 60H (Merck code No. 7734 and No. 9385) as packing materials.

### 3.10.3 Nuclear magnetic resonance spectroscopy (NMR)

The NMR spectra were recorded in $CDCl_3$ using a Bruker AV400 spectrometer at 400 MHz for $^1H$ NMR as an internal standard.

## 3.11 Identification of selected endophytic fungi

### 3.11.1 DNA extraction

Ten to thirty milligram of fresh mycelia was grinded into small pieces. The DNA was extracted using GF-1 Plant DNA Extraction Kit (Vivantis, USA) according to the manufacturer's instructions. DNA was eluted from the column into a clean microcentrifuge tube by preheated Elution Buffer (50 - 100µl) and stood at room temperature for 10 min. The column was then centrifuged at 10,000 rpm for 1 min to elute DNA. DNA solution was stored at -20˚ C until use.

### 3.11.2 Polymerase chain reaction (PCR)

The selected endophytic fungi was identified based on the nucleotide sequences of rDNA of the internal transcribe spacer (ITS) region. This region was amplified using universal primers ITS1 (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (71). The PCR reaction was performed in a total volume 25 µl under condition as shown in Table 3.1

**Table 3.1** Composition of PCR reaction mixture

| Reagent | Volume (µl) |
|---|---|
| 10 µM primer ITS-1 | 1 |
| 10 µM primer ITS-4 | 1 |
| Distilled water | 9.5 |
| KAPA DNA polymerase (2X) | 12.5 |
| DNA template (0.5-1 ng/µl) | 1 |

The PCR cycles were as follows:

| | | | |
|---|---|---|---|
| Initial denaturation | 95˚C | 5 min | |
| Denaturation | 95˚C | 1 min | 35 cycles |
| Annealing | 50˚C | 1 min | |
| Extension | 72˚C | 1.30 min | |
| Final extension | 72˚C | 10 min | |
| Hold | 4˚C | | |

The PCR products were evaluated on 1% agarose gel electrophoresis in 0.5X TBE buffer.

### 3.11.3 Purification of the PCR products

The PCR product was purified by FavorPrep GEL/PCR Purification kit (Favorgen, Taiwan) according to the manufacturer's instruction. For eluting of DNA fragment, 40µl of preheated elution buffer was added to the center of FADF column. The FADF column was let to stand for 10 min and centrifuged to elute DNA. The purified DNA was visualized by 1% agarose gel electrophoresis under UV light and stored at -20 ℃.

### 3.11.4 DNA sequence data analysis

The purified DNA fragment obtained was sent to commercial nucleotide sequencing service (1$^{st}$ BASE, Singapore) for DNA sequencing using primer ITS1. The obtained nucleotide sequences were analyzed by blast search for similar sequences from GenBank database using BLAST program.

# CHAPTER IV

# RESULTS AND DISCUSSION

## 4.1 Isolation of endophytic fungi

Healthy leaves of 10 plants collected from Trad province in July 2010 were collected for isolation of endophytic fungi by sterile technique. A total of 83 isolates were isolated, including 41 *Xylaria* spp., 5 *Daldinia* spp., 1 *Fusarium* spp., 1 *Collectotrichum* spp., 4 *Pestalotiopsis* spp., 2 *Phomopsis* spp., 2 *Phyllosticta* spp., 1 *Trichoderma* spp., 4 *Nigrospora* spp. and 22 mycelia sterilia (Figure 4.1). A list of isolates is shown in Table 4.1. All isolates were maintained on potato dextrose agar (PDA).

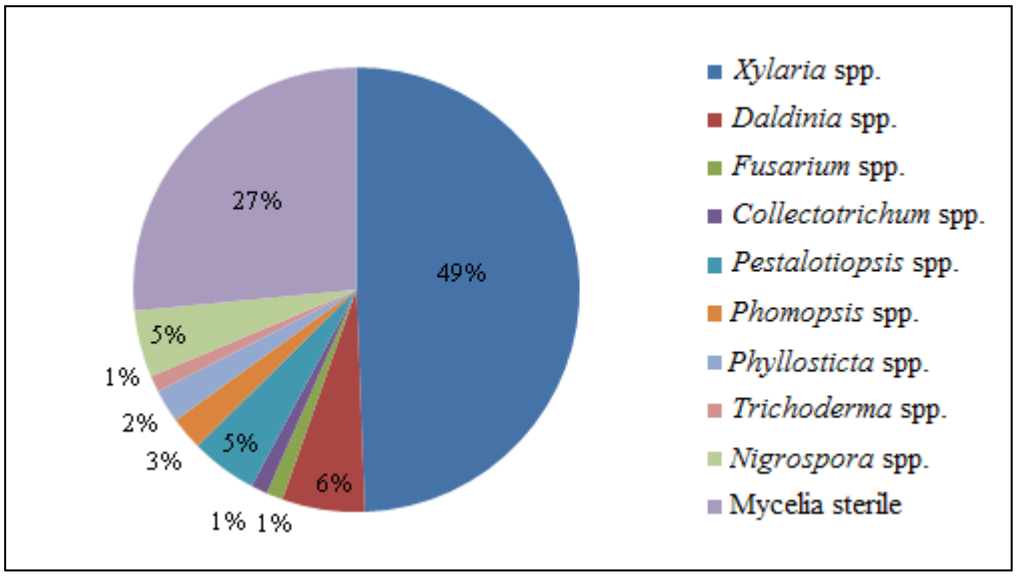



**Figure 4.1** A pie graph showing distribution of endophytic fungi isolated in this study from Trad Agroforesty Research and Training Station in Trad province, Thailand.

In Thailand, several studies on endophytic fungi have been reported, namely, from leaf of *Tectona grandis* (Verbenaceae) at Khao Yai National Park in Nakhonratchasima province (72) , leaf of *Dalbergia oliveri Gamble* (Leguminosae) at

Viengsa district (73), Nan province and leaf of *Terminalia chebula* in Tak Province (74) but the isolation of endophytic fungi from Trad province has not been reported.

Xylariaceae is a large family of fungi that exhibits its greatest diversity in the tropics. Eight genera of the Xylariaceae have been recorded as endophytes including *Anthostomella*, *Biscogniauxia*, *Daldinia*, *Hypoxylon*, *Kretzschmaria*, *Nemania*, *Rosellinia*, and *Xylaria* (49). However, the common endophytic genus is *Xylaria.* Due to *Xylaria* species are the major fungal isolates found in this study, they were therefore chosen for further bioactive compound analysis.

Endophytic *Xylaria* species are also found in other place of Thailand. Pongcharoen *et al*. (2008) reported the isolation of endophytic *Xylaria* sp. PSU-D14 from the leaves of *Garcinia dulcis* collected in Songkhla Province. The bioactive compounds from this isolate showed antifungal activity against *Candida albicans* (75). Moreover, *Xylaria* species were found in northern of Thailand such as Queen Sirikit Botanic Garden and Doi Suthep National Park in Chiang Mai, and Naresuan University, Phayao Campus, in Phayao (76).

**Table 4.1** Details of host plants and endophytic fungi isolated in this study

| Isolate | Host | Taxa | Code |
|---|---|---|---|
| TRDA1 | *Dipterocarpus alatus* | *Xylaria* sp.1 | X1 |
| TRDA3 | | *Xylaria* sp.2 | X2 |
| TRDA5 | | *Xylaria* sp.3 | X3 |
| TRDA7 | | *Daldinia* sp. | Dal, Pae |
| TRDA11 | | Mycelia sterilia | M1 |
| TRDA21 | | Mycelia sterilia | M2 |
| TRDA23 | | Mycelia sterilia | M3 |
| TRCI1 | *Cinnamomum inners* | *Xylaria* sp.4 | X5 |
| TRCI3 | | *Daldinia* sp. | |
| TRCI20 | | Mycelia sterilia | |
| TRAH1 | *Artocarpus heterophyllus* | *Xylaria* sp.5 | X6 |
| TRAH3 | | *Xylaria* sp.6 | KN2 |
| TRAH5 | | *Fusarium* sp. | orange |
| TRAH8 | | *Collectotrichum* sp. | KN5 |
| TRAH16 | | Mycelia sterilia | |
| TRAH17 | | Mycelia sterilia | |
| TRAH31 | | Mycelia sterilia | |
| TRAH32 | | Mycelia sterilia | |

**Table 4.1** Details of host plants and endophytic fungi isolated in this study (continued)

| Isolate | Host | Taxa | Code |
|---|---|---|---|
| TRMI1 | *Mangifera indica* | *Xylaria* sp.7 | X7 |
| TRMI3 | | *Xylaria* sp.8 | X8 |
| TRMI4 | | *Xylaria* sp.9 | MM4 |
| TRMI5 | | *Xylaria* sp.10 | MM2 |
| TRMI6 | | Mycelia sterilia | |
| TRMI22 | | Mycelia sterilia | |
| TRPD1 | *Peltophorum dasyrachis* | *Xylaria* sp.11 | X10 |
| TRPD11 | | *Xylaria* sp.12 | N1 |
| TRPD12 | | *Pestalotiopsis* sp. | N2 |
| TRPD13 | | *Phomopsis* sp. | N3 |
| TRPD14 | | *Xylaria* sp.13 | X11 |
| TRPD15 | | Mycelia sterilia | |
| TRAC1 | *Anthocephalus chinensis* | *Xylaria* sp.14 | X12 |
| TRAC3 | | *Xylaria* sp.15 | X13 |
| TRAC6 | | *Xylaria* sp.16 | KT1 |
| TRAC9 | | *Xylaria* sp.17 | KT2 |
| TRAC10 | | *Pestalotiopsis* sp. | KT3 |
| TRAC11 | | *Phomopsis* sp. | KT4 |
| TRAC13 | | *Pestalotiopsis* sp. | KT5 |
| TRAC14 | | *Phyllosticta* sp. | KT6 |
| TRAC16 | | *Trichoderma* sp. | KT7 |
| TRAC23 | | Mycelia sterilia | KT8 |
| TRAC25 | | Mycelia sterilia | KT9 |
| TRAC26 | | Mycelia sterilia | KT10 |
| TRCP1 | *Cinnamomum porrectum* | *Xylaria* sp.18 | X15 |
| TRCP3 | | *Xylaria* sp.19 | X16 |
| TRCP6 | | *Xylaria* sp.20 | X17 |
| TRCP9 | | *Xylaria* sp.21 | X18 |
| TRCP15 | | *Xylaria* sp.22 | TT2 |
| TRCP17 | | *Pestalotiopsis* sp. | TT3 |
| TRCP18 | | *Phyllosticta* sp. | TT4 |
| TRCP23 | | *Daldinia* sp. | |
| TRCP28 | | Mycelia sterilia | |
| TRCP35 | | Mycelia sterilia | |
| TRCP36 | | Mycelia sterilia | |

**Table 4.1** Details of host plants and endophytic fungi isolated in this study (continued)

| Isolate | Host | Taxa | Code |
|---|---|---|---|
| TRDL1 | *Denarocalamus latifloma* | *Xylaria* sp.23 | X19 |
| TRDL4 | | *Xylaria* sp.24 | X20 |
| TRDL6 | | *Xylaria* sp.25 | X21 |
| TRDL8 | | *Xylaria* sp.26 | PM1 |
| TRDL9 | | *Xylaria* sp.27 | PM2 |
| TRDL10 | | *Xylaria sp.*28 | PM3 |
| TRDL11 | | *Nigrospora* sp. | PM4 |
| TRDL15 | | *Nigrospora* sp. | PM5 |
| TRDL20 | | *Daldinia* sp. | |
| TRDS1 | *Dracaena sanderiana* | *Xylaria* sp.29 | X22 |
| TRDS2 | | *Xylaria* sp.30 | X23 |
| TRDS5 | | *Xylaria* sp.31 | X24 |
| TRDS7 | | *Xylaria* sp.32 | X25 |
| TRDS8 | | *Xylaria* sp.33 | X26 |
| TRDS9 | | *Nigrospora* sp. | PL1 |
| TRDS10 | | *Nigrospora* sp. | PL2 |
| TRDS11 | | Mycelia sterilia | PL3 |
| TRDS17 | | Mycelia sterilia | PL4 |
| TRDS20 | | Mycelia sterilia | PL5 |
| TRDS26 | | Mycelia sterilia | |
| TRBR1 | *Baccaurea ramiflora* | *Xylaria* sp.34 | MF1 |
| TRBR5 | | *Xylaria* sp.35 | MF2 |
| TRBR6 | | *Xylaria* sp.36 | MF3 |
| TRBR7 | | *Xylaria* sp.37 | MF4 |
| TRBR9 | | *Xylaria* sp.38 | MF5 |
| TRBR10 | | *Xylaria* sp.39 | MF6 |
| TRBR11 | | *Xylaria* sp.40 | MF7 |
| TRBR12 | | *Xylaria* sp.41 | MF8 |
| TRBR13 | | *Daldinia* sp. | |
| TRBR17 | | Mycelia sterilia | |

## 4.2 Antimicrobial activity of endophytic *Xylaria* spp.

All isolates of endophytic *Xylaria* spp., 41 isolates in total, were cultured in Malt extract broth under static condition at room temperature. After 30 days, the

cultures were filtererd and extracted with ethyl acetate. The solvent phase was evaporated by rotary evaporation. Crude extracts of 38 isolates were dissolved in 10% DMSO for testing of their biological activity.

For the primary screening, 41 crude extracts were tested for antimicrobial activity against reference pathogenic microorganisms, including two Gram positive bacteria, *Staphylococcus aureus* (ATCC 6538-P), *Bacillus subtilis* (ATCC 6633) and two Gram negative bacteria, *Pseudomonas aerogenosa* (ATCC 9027), *Escherichia coli* (ATCC 8739) and the yeast, *Candida albicans* (ATCC 90028) by paper disc diffusion method. The result is shown in Table 4.2 revealed that most crude extracts inhibited the growth of Gram positive bacteria more than Gram negative bacteria and yeast. Crude extracts which shown great antimicrobial activity with inhibition zones upper 0.9 mm. was chosen for further study.

These results are also in agreement with the results from a report of Chareprasert *et al.* (2006) which reported that endophytic fungi from *Tectona grandis* L.and *Samanea saman* Merr. leaves inhibited the growth of Gram positive bacteria with stronger activity than against Gram negative bacteria (77). Sutjaritvorakul *et al*. (2011) also reported that endophytic fungi isolated from 4 species of Dipterocapous trees at Viengsa district Nan provine, Thailand, inhibited the growth of Gram positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis* more than Gram negative like *Pseudomonas aerogenosa* and *Escherichia coli* (73).

**Table 4.2** Summary of antimicrobial activity of crude extracts from endophytic *Xylaria* spp. isolated in this study

| Isolate | Code | Test microorganism and inhibition zone (cm) | | | | |
|---|---|---|---|---|---|---|
| | | *B.subtilis* | *S.aureus* | *P.aeruginosa* | *E.coli* | *C.albican* |
| TRDA1 | X1 | 0.83 | 0.9 | 0.8 | 0.85 | 0.92 |
| TRDA3 | X2 | 1 | 1.15 | 0.87 | 0.9 | 0.93 |
| TRDA5 | X3 | 1 | 1.2 | 0.9 | 0.87 | 0.9 |
| TRAH1 | X6 | 1 | 0.94 | 0.85 | 0.83 | 0.87 |
| TRAH3 | KN2 | 0.95 | 0.92 | 0.87 | 0.9 | 0.9 |
| TRAC1 | X12 | 1.13 | 1.3 | 0.85 | 1.02 | 0.9 |
| TRAC3 | X13 | 0.8 | 0.92 | 0.72 | 0.87 | 0.9 |
| TRAC6 | KT1 | 1.15 | 1.23 | 0.83 | 0.9 | 0.97 |
| TRCP1 | X15 | 0.97 | 1.13 | 0.93 | 0.93 | 1 |

**Table 4.2** Summary of antimicrobial activity of crude extracts from endophytic *Xylaria* spp. isolated in this study (continued)

| Isolate | Code | Test microorganism and inhibition zone (cm) | | | | |
|---|---|---|---|---|---|---|
| | | *B.subtilis* | *S.aureus* | *P.aeruginosa* | *E.coli* | *C.albican* |
| TRCP6 | X17 | 1.3 | 1.37 | 0.92 | 0.95 | 0.97 |
| TRCP9 | X18 | 1.08 | 1.1 | 0.9 | 1 | 0.85 |
| TRCP15 | TT2 | 1.18 | 1.18 | 0.9 | 0.93 | 0.9 |
| TRMI1 | X7 | 1 | 1.02 | 0.93 | 0.98 | 0.92 |
| TRMI3 | X8 | 1.2 | 1.1 | 1.02 | 0.98 | 0.85 |
| TRMI4 | MM4 | 0.82 | 1.07 | 0.82 | 0.83 | 1.05 |
| TRMI5 | MM2 | 0.85 | 1.12 | 0.82 | 0.9 | 1.05 |
| TRPD11 | N1 | 0.83 | 1.08 | 0.88 | 0.9 | 1 |
| TRPD14 | X11 | 0.7 | 1.12 | 0.85 | 0.87 | 0.87 |
| TRDL8 | PM1 | 1.58 | 1.87 | 1.17 | 1.13 | 1.18 |
| TRDS5 | X24 | 1.32 | 1.28 | 1.02 | 1.03 | 0.98 |
| TRBR6 | MF3 | 1.13 | 1.12 | 0.85 | 0.83 | 0.93 |
| TRDL1 | X19 | 1.33 | 1.33 | 1.03 | 1.03 | 0.92 |
| TRDL9 | PM2 | 0.93 | 0.75 | 0.73 | 0.7 | 0.83 |
| TRDS2 | X23 | 1 | 1.22 | 0.92 | 0.88 | 0.97 |
| TRBR5 | MF2 | 0.9 | 0.98 | 0.9 | 0.9 | 0.95 |
| TRBR1 | MF1 | 0.8 | 1.18 | 0.77 | 0.83 | 1 |
| TRBR9 | MF5 | 0.92 | 1.22 | 0.92 | 0.95 | 0.98 |
| TRBR12 | MF8 | 0.9 | 1.12 | 0.87 | 0.88 | 0.9 |
| TRDS7 | X25 | 0.97 | 1.23 | 0.9 | 0.88 | 0.83 |
| TRDS1 | X22 | 1.25 | 1.2 | 0.95 | 1 | 0.98 |
| TRDL6 | X21 | 1.17 | 1.15 | 0.92 | 1.02 | 0.95 |
| TRBR11 | MF7 | 1.1 | 1.02 | 0.82 | 0.9 | 0.95 |
| TRBR10 | MF6 | 1.17 | 1.05 | 0.75 | 0.85 | 0.8 |
| TRDL4 | X20 | 1.45 | 1.6 | 0.92 | 1.03 | 1 |
| TRDL10 | PM3 | 1.1 | 1.08 | 0.85 | 0.88 | 0.87 |
| Positive control | | 2.03 | 2.02 | 1.48 | 1.48 | 2.73 |
| DMSO | | | 0.6 | | 0.6 | 0.7 |

Nine crude extracts which showed significant antimicrobial activity are X-12, X-16, X-17, X-18, X-19, X-20, X-21, X-22 and X-24. They were chosen to determine the minimum inhibitory concentration (MIC) at the final concentration of 256 µg/ml. The result is shown in Table 4.3

**Table 4.3** MIC of 9 crude extracts against test microorganisms

| Code / Bacteria | X-12 | X-16 | X-17 | X-18 | X-19 | X-20 | X-21 | X-22 | X-24 |
|---|---|---|---|---|---|---|---|---|---|
| *E. coli* | ← >256 µg/ml → | | | | | | | | |
| *P. aeruginosa* | >256 | >256 | >256 | 64 | 128 | 128 | 256 | 256 | 64 |
| *B. subtilis* | 256 | >256 | >256 | >256 | 256 | >256 | >256 | 256 | 256 |
| *S. aureus* | 256 | 256 | 256 | 256 | 256 | 256 | >256 | 256 | 128 |

## 4.3 Morphology of representative of endophytic *Xylaria* spp. on PDA

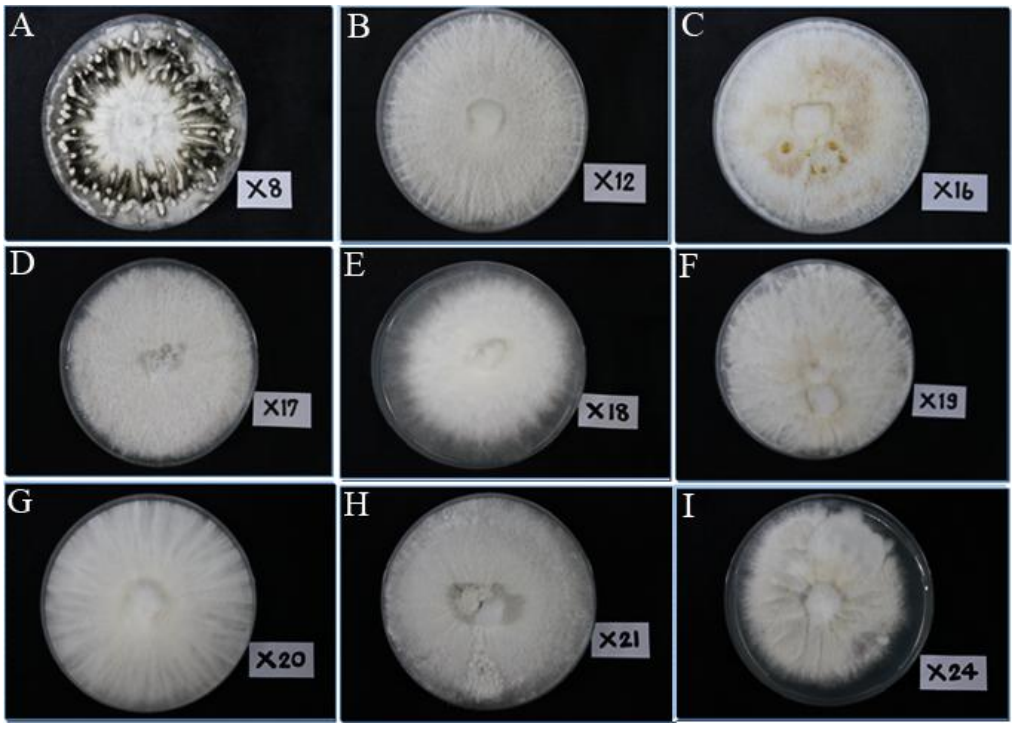



**Figure 4.2** Colony morphology of representative isolates of endophytic *Xylaria* spp. on PDA A) a colony morphology of X-8 which produced black stromata with a white head on PDA plate for 2 weeks. B-I) colony morphology of X-12, X-16, X-17, X-18, X-19, X-20, X-21 and X-22 which showed white mycelia on PDA plate for 2 weeks.

Morphology of the nine representative isolates of endophytic *Xylaria* spp. were observed for colony characteristics, colony color, color pigment production and the growth rate on media every week for 2 weeks. Colony characteristics of all isolates on PDA at 2 weeks is shown in Figure 4.2 (A-I). All mycelia are white with a regular margin except X8 which produced black stromata with a white head. Moreover, X-16 produced orange pigment. After 4 weeks, some isolates slowly turned black around the center and some developed black stromata with a white head such as X-8, X-12, X-16, X-19 and X-22 (Figure 4.3)

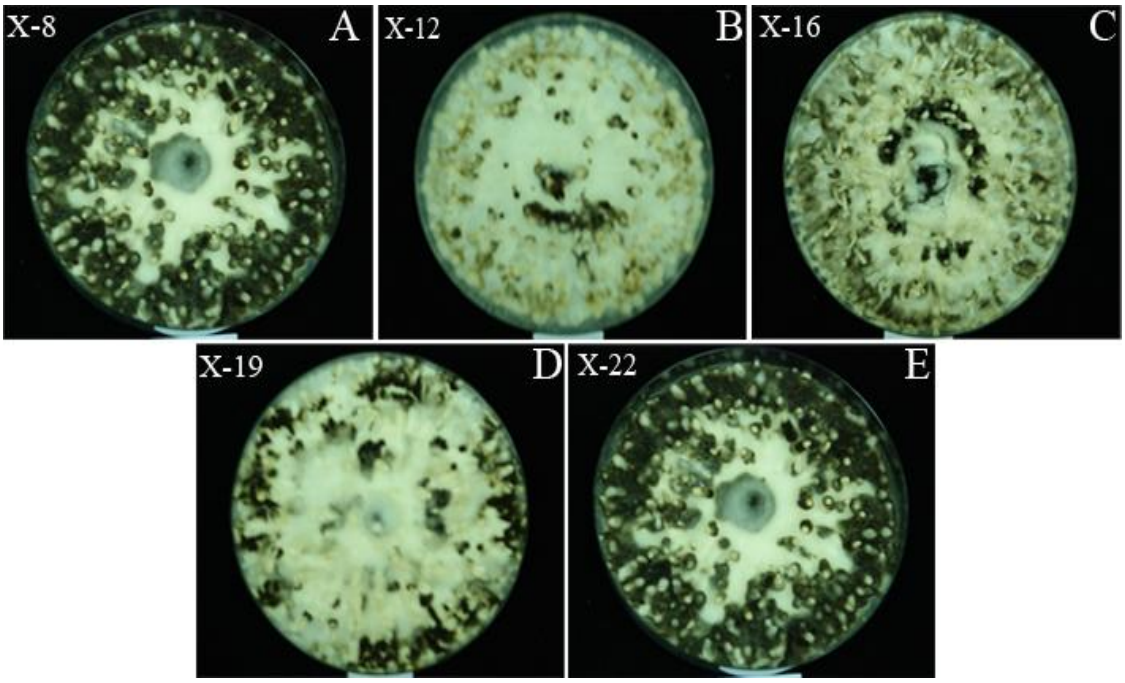



**Figure 4.3** Stromata of representative isolates of endophytic *Xylaria* spp. grown on PDA for 4 weeks (A-E) colony morphology of X-8, X-12, X-16, X-19 and X-22 which produced black stromata on PDA plate for 4 weeks.

### 4.4 Anti-cancer activity against human cervical cancer cell line

Selected nine crude extracts were tested for cytotoxicity against CaSki cell line by MTT assay. The results showed that X-12, X-17 and X-21 exhibited strong anticancer activity with the $IC_{50}$ of 20.71±1.17, 10.05±0.28 and 23.65±1.11 µg/ml, respectively (Table 4.4). Subsequently, three crude extracts (X-12, X-17 and X-21) were preliminary analyzed by $^{1}H$ NMR spectroscopy. The $^{1}H$ NMR profiles of the 3 crude extracts revealed potential signal compounds which may have biologically activity such as aldehyde, phenol and aromatic carbon (Figure 4.5). However, $^{1}H$ NMR spectra of crude extract X-17 revealed an interesting characteristic of $^{1}H$ NMR signals more than the other two. Therefore, crude extract from isolate X-17 was chosen for further experiments.

The crude extract X-17 was extract from endophytic *Xylaria* sp. associated with *Cinnamomum porrectum*.

*C. porrectum* locally known as Theptaro, belonging to the family of cinnamom (Lauraceae). There are many reports about its aroma characteristic and exhibit medicinal properties from their many parts. *C. porrectum* oil also has an antibacterial and antifungal activity. Phongpaichit *et al*. (2006) reported that they extracted oil from the root of *Cinnamomum porrectum* which were tested for its antimicrobial activity. The results revealed moderate activity against human pathogens including bacteria, yeasts and dermatophytes (78).

**Table 4.4** Anti-cancer activity of 9 crude extracts against CaSki cell line

| Isolates code | $IC_{50}$ (µg/ml) |
|---|---|
| X12 | 20.71±1.17 |
| X16 | 64.89±2.59 |
| X17 | 10.05±0.28 |
| X18 | 32.37±7.06 |
| X19 | 65.99±4.55 |
| X20 | 88.64±7.50 |
| X21 | 23.65±1.11 |
| X22 | 84.08±4.05 |
| X24 | 81.18±3.14 |

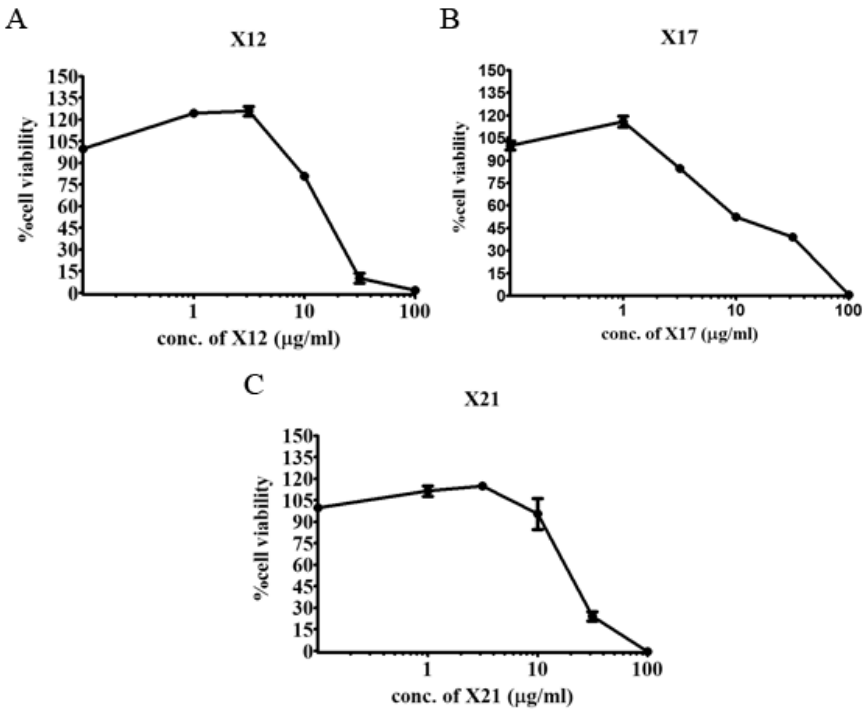



**Figure 4.4** The effect of representative crude extracts on viability of CaSki cell line. CaSki cell line was treated with crude extract X-12 (A), X-17 (B) and X-21 (C) at various concentrations for 4 days. Cell viability was assayed by MTT assay. The results showed mean±SD and represented two independent experiments.

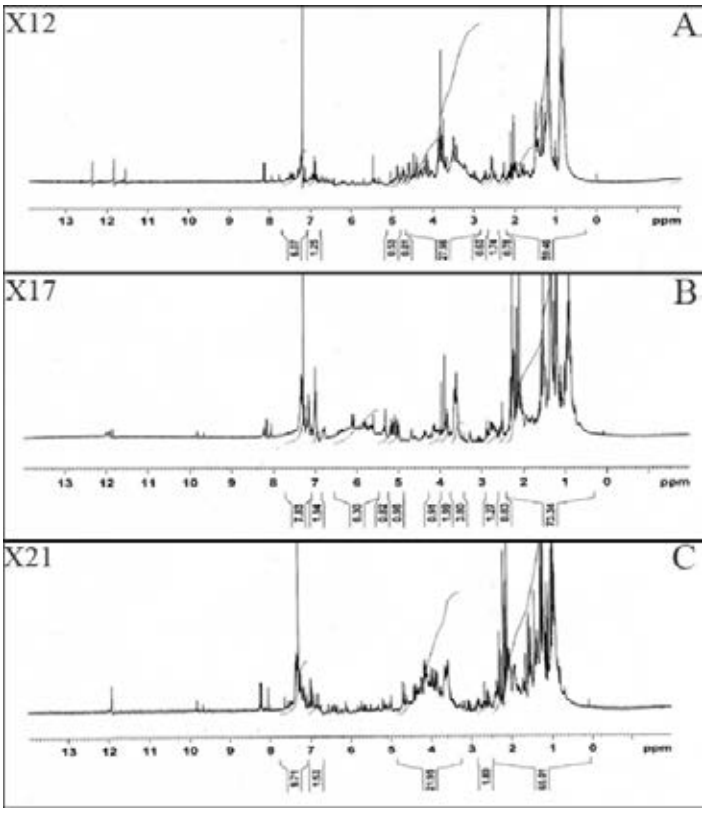



**Figure 4.5** $^{1}$H NMR spectra of three crude extracts from 30 days old cultures. The NMR spectra were recorded in $CDCl_3$. A**)** Spectrum of X-12 crude extract B) Spectrum of X-17 crude extract C) Spectrum of X-21 crude extract

### 4.5 Cytotoxicity of crude extract X-17 against CaSki cell line

Crude extract X-17 was tested for cytotoxicity against CaSki cell line by using MTT assay. Crude extract X-17 showed cytotoxic activity against CaSki with the $IC_{50}$ of 10.05±0.28 µg/ml and caused morphological changes, such as cell shrinkage and loss of cell adhesion (Figure 4.6). Moreover, detailed study on the anti-tumor activity of crude extract X-17 against CaSki was carried out by analysis of cell cycle profile and apoptosis.

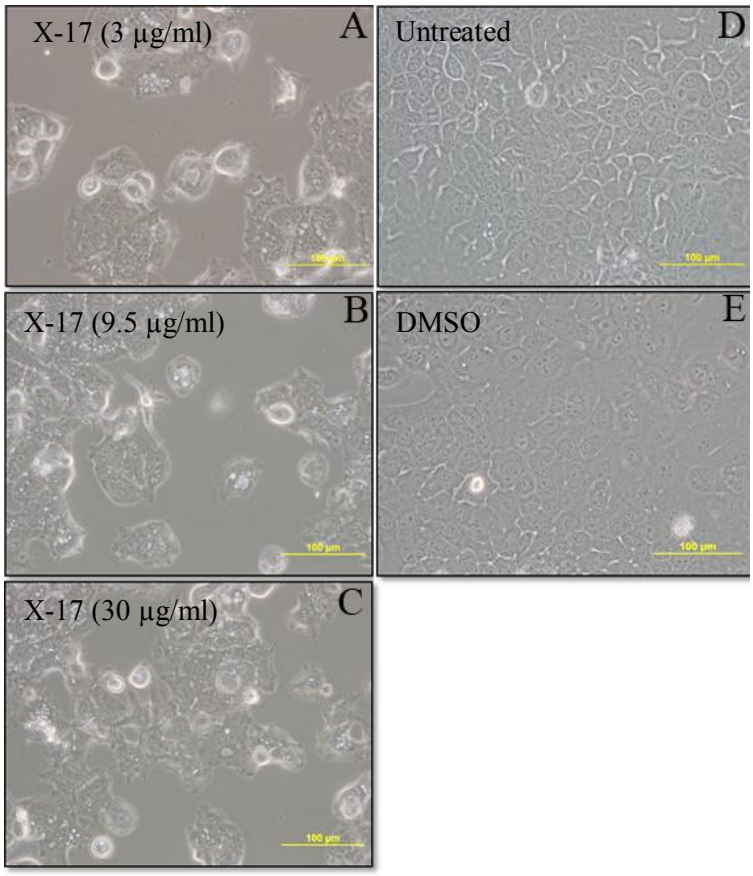



**Figure 4.6** Cellular morphology of CaSki cell line treated with crude extract X-17. CaSki cell line was treated with crude extract X-17 at 3 µg/ml (A), 9.5 µg/ml (B) and 30 µg/ml (C). The morphology was different from untreated (D) and DMSO treatment (E).

Besides testing for its cytotoxicity against CaSki cell line, a macrophage cell line, RAW 264.7, was subjected to treatment with X-17 and tested for cytotoxicity. After treatment of RAW 264.7 with crude extract X-17 for 24 h, the cell viability was measured by MTT assay. The result showed that X-17 negatively affected the cell survival of RAW 264.7 with the $IC_{50}$ of 79.0±2.77 µg/ml (Figure 4.7).

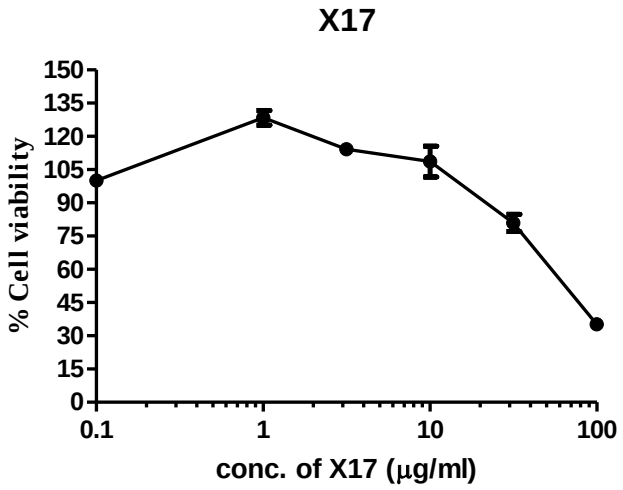



**Figure 4.7** The cytotoxicity of crude extract X-17 against RAW264.7 cell line

RAW 264.7 cell line was treated with crude extract X-17 at various concentrations for 4 days. Cell viability was assayed by MTT assay. The results showed mean±SD and represented two independent experiments.

### 4.5.1 Apoptosis test

CaSki cell line was treated with crude extract X-17 at various concentrations (3, 9.5 and 30 µg/ml) for 96 h and stained with Hoechst 33342 fluorescence DNA dye to detect apoptotic cells. From the images of fluorescence microscope, the CaSki cell line treated with X17 extract exhibited changes in nuclei morphology such as DNA fragmentation, chromatin condensation and formation of apoptotic bodies similar to when cells were treated with anti-cancer drug etoposide that used as positive control (Figure 4.8; C, D, E and F). On the other hand, untreated cells and cells treated with DMSO did not show apoptotic characteristics (Figure 4.8 A and B). Therefore, crude extract X-17 induced apoptosis in CaSki cell line.

The results that cells exhibited changes in nuclei morphology when treated with crude extract X-17 are in agreement with the results obtained from effect of trichostatin A in gastric cancer cell lines, which exhibited nuclear chromatin condensation. From under fluorescence microscope, the cells treated with TSA were stained highly condensed, bright nucleus while the cells in control group were stained average slightly blue (79).

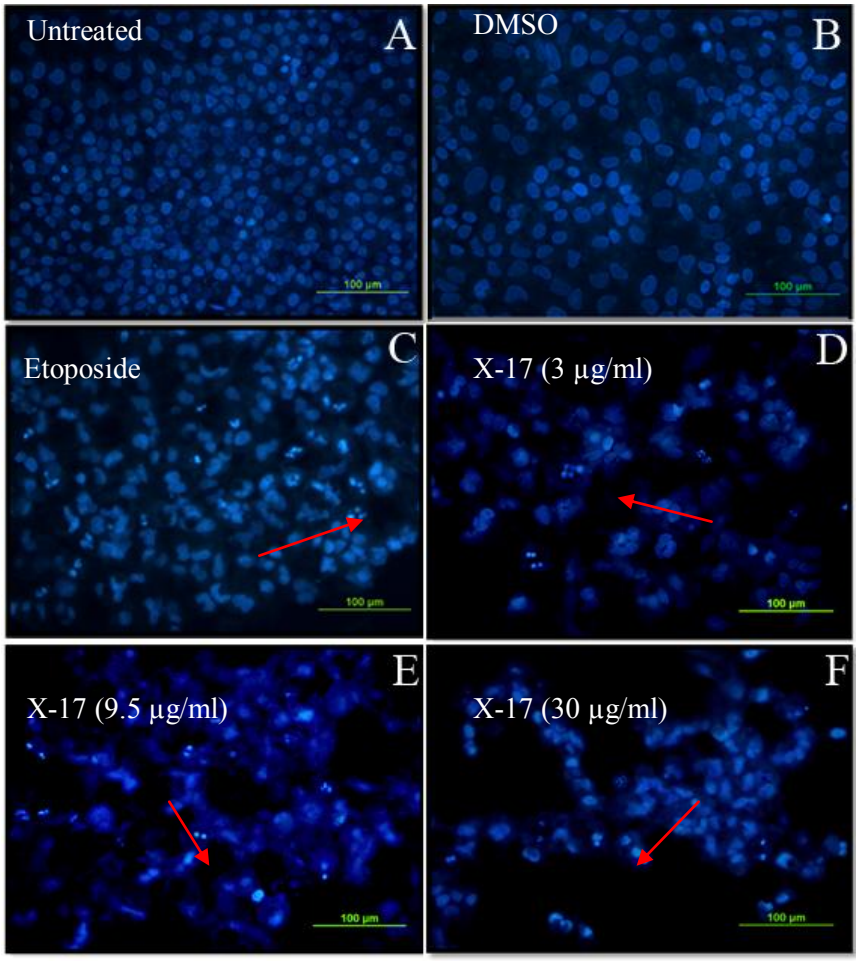



**Figure 4.8** Crude extract-X17 induced apoptosis in CaSki cell line.

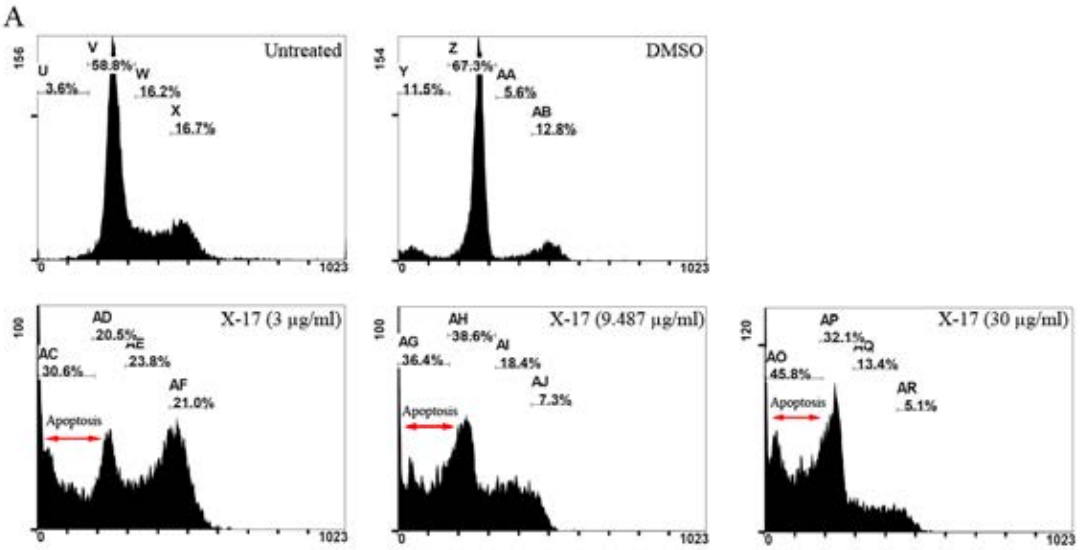
CaSki cell line was treated with crude extract X-17 at various concentrations 3 µg/ml (D), 9.5 µg/ml (E) and 30 µg/ml (F) for 96 h and stained with Hoechst 33342 fluorescence DNA dye. Treated cells showed nuclear fragmentation and apoptotic bodies as indicated with the red arrows. As a result, they similar to cell were treated with etoposide that used as positive control (C), and different from untreated (A) and DMSO treatment (B).

### 4.5.2 Effect of X-17 on cell cycle profile of CaSki cell line

To study the effect of crude extract X-17 on cell cycle profile of CaSki cell line, cells were treated with crude extract X-17 at three different concentrations, i.e. 3, 9.5 and 30 µg/ml for 48 h. and cell cycle profile was analyzed by flow cytometry using propidium iodine staining.

As shown in Figure 4.9A, the percentage of cells in the sub G1 phase increased in a concentration of the crude extract X-17 dependent manner (Figure 4.9B), compared to cells treated with DMSO. In addition, the percentage of cells in the G1 phase was significantly decreased by crude extract X-17 treatment (Figure 4.9C). These data strongly suggest that crude extract X-17 induced apoptosis in this cell line via a G1 arrest.

The apoptotic cells with degraded DNA appear as cells with hypodiploid DNA content and are represented in "sub-G1" peaks on DNA histograms.




The result from Xiao-Ming *et al.* (2008) on the effect of bioactive compounds on cell cycle is consistent with the results from this study. In their experiments, they treated human gastric epithelial cell lines BGC-823 and SGC-7901 with trichostatin A (TSA) for 3 days and stained with propidium iodine staining. Effects on cell cycle were examined by FACS analysis. The results showed that TSA induced apoptosis in human gastric epithelial cell line, which was demonstrated by the rise in the percentage of cells in sub-G1 phase (79).

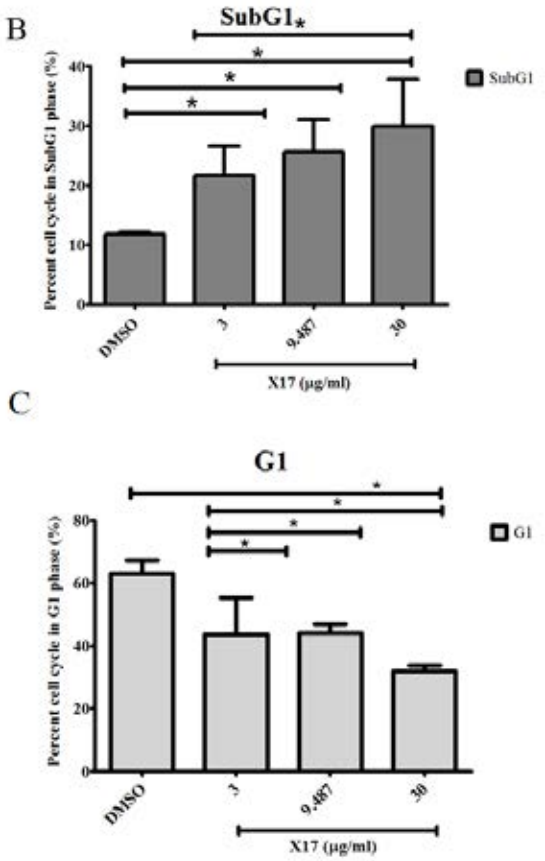



**Figure 4.9** Effect of X-17 on cell cycle. (A) Representative histograms of cell cycle profile of CaSki cell line treated with crude extract X-17 at three different concentrations, 3, 9.5 and 30 µg/ml for 48 h.

(B-C) Percent cell cycle in Sub G1 and G1 phases were compared among different concentrations of X17. At the same time, percent cell cycle in G1 phase was decreased.

*indicated the statistically significant differences between conditions at $p<0.05$

### 4.6 Anti-inflammatory activity of crude extract X-17

Next, Crude extract X-17 was tested for anti-inflammatory activity against nitric oxide production in LPS and IFN γ-stimulated macrophage cell line RAW 264.7. After treating cells with crude extract X-17 and cells were stimulated with LPS and IFNγ, the amount of nitric oxide decreased as the concentration of the extract increased, indicating that the extracts suppressed nitric oxide production (Figure 5.1). The result of cytotoxicity, however, showed that the $IC_{50}$ against nitric oxide production was 10.42±1.231 µg/ml with the $IC_{50}$ for cell viability of 9.022±0.9872 µg/ml. Therefore, decreasing amount of nitric oxide is probably the result of cell death. Thus, treatment with crude extract X-17 did not directly affect the nitric oxide production.

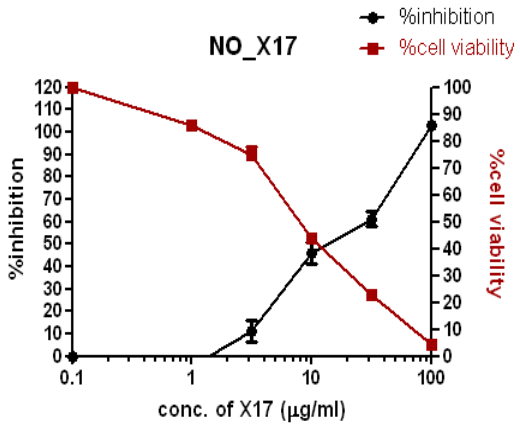



**Figure 5.1** Anti-inflammatory activity and cytotoxicity of crude extract X-17.

RAW 264.7 cell line was pretreated with crude extract X-17 or vehicle control DMSO for 1 h. After the pretreatment, cells were stimulated with LPS and IFN γ for 24 h before measuring the amount of nitric oxide production, and cell viability. The cytotoxicity of crude extract X-17 was evaluated by MTT assay. The results showed mean±SD and represented two independent experiments.

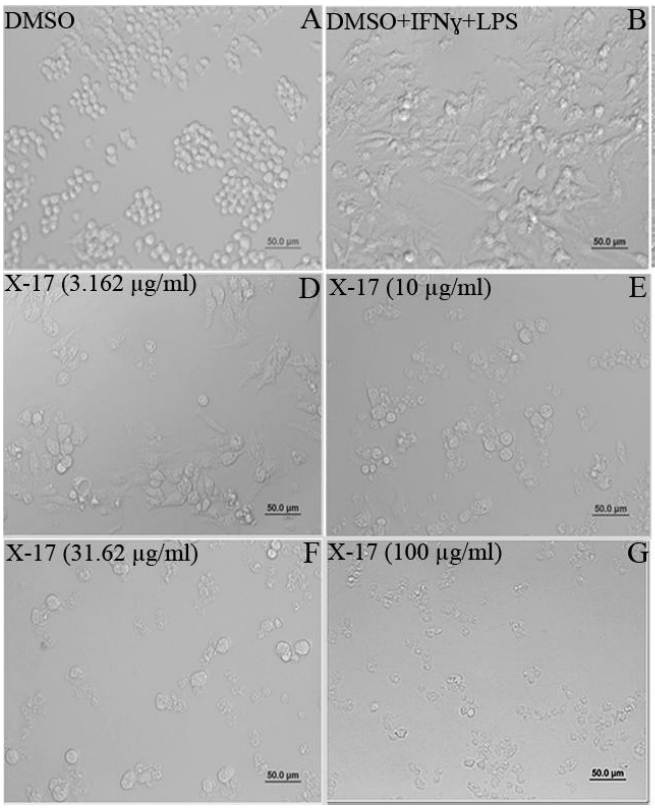



**Figure 5.2** Cellular morphology of RAW 264.7 cell line after treatment with X-17 crude extract at various concentrations: 1 µg/ml (C), 3.162 µg/ml (D), 10 µg/ml (E), 31.62 µg/ml (F) and 100 µg/ml (G). Cells were stimulated with LPS and IFN γ for 24h, and compared with cells treated with DMSO that were stimulated (B) and unstimulated (A) with LPS and IFN γ.

### 4.7 Identification of endophytic fungus isolate X-17

The fungus was then identified based on the basic of morphological and molecular characteristics.

#### 4.7.1 Morphological identification

Morphological characteristics of endophytic fungus isolate X-17 on PDA was shown in Figure 5.2. On PDA plate, endophytic fungus isolate X-17 did not produce spore or conidia. Thus, cultural characteristics were used for principal identification. For 1 week, mycelium was initially white in colour and looked like a feather which is a specific characteristic of genus *Xylaria* (Figure 5.3A). Colony growth of endophytic *Xylaria* sp. X-17 on PDA reached margin of the plate in 2 weeks (Figure 5.3B). After cultivation for 4-6 weeks on PDA plate, mycelium became black in colour and produced immature anamorph stage (Figure 5.3C).

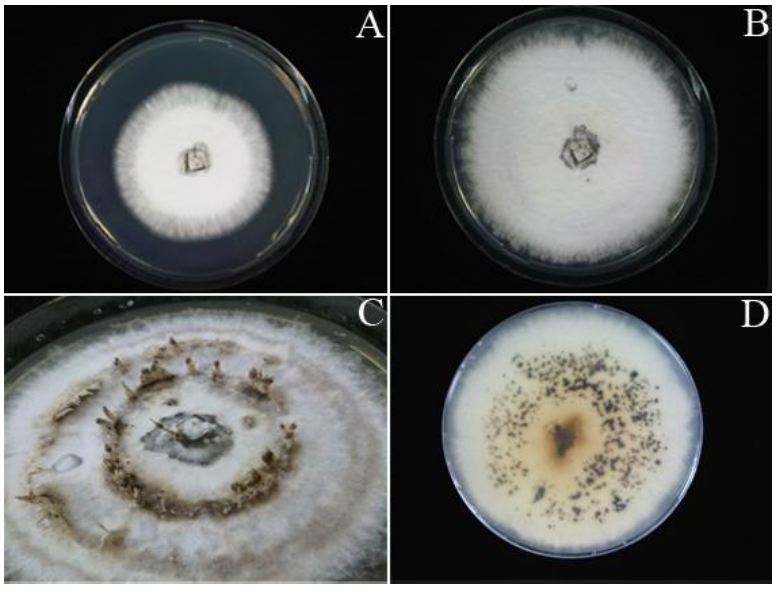



**Figure 5.3** Colony morphology of endophytic *Xylaria* sp. isolate X-17 on PDA plate. A) Mycelium of isolate X-17 which was initially white colony for one week. B) Colony morphology of isolate X-17 C) Stromata of isolate X-17 on PDA plate for 4-6 weeks D) Colony morphology of isolate X-17 when observed in reverse

**Figure 5.4** Characteristic of endophytic *Xylaria* sp. isolate X-17 on MEB after cultivation

Moreover, the mycelium characteristics of endophytic *Xylaria* sp. isolate X-17 were observed under the light microscope. The results showed mycelium with septate hyphae as shown in Figure 5.5

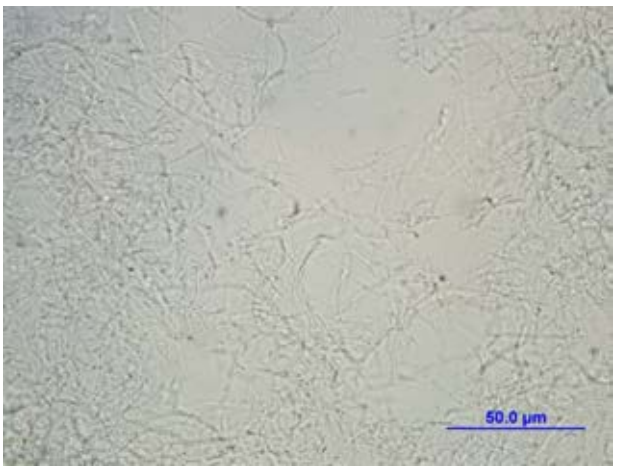



**Figure 5.5** Mycelial characteristic of endophytic *Xylaria* sp. isolate X-17 on slide culture

**4.7.2 Molecular identification**

The rDNA of ITS region of endophytic *Xylaria* sp. isolate X-17 was amplified by PCR using universal primers ITS1 and ITS4 as forward and reverse, respectively. The nucleotide sequence result was analyzed and compared to ITS sequences available in GenBank DNA database by using BLAST search tool. As a result, the

ITS sequence of endophytic *Xylaria* sp. isolate X-17 was closely related to *Xylaria laevis* isolate 419 NCBI with 99% homology to the best matching sequences.

```
                10         20         30         40         50
X-17_ITS  TCTGTTTAGT ATTGAATTCT GAACCTATAA CTAAATAAGT TAAAACTTTC

                60         70         80         90        100
X-17_ITS  AACAACGGAT CTCTTGGTTC TGGCATCGAT GAAGAACGCA GCGAAATGCG

               110        120        130        140        150
X-17_ITS  ATAAGTAATG TGAATTGCAT AATTCAGTGA ATCATCGAAT CTTTGAACGC

               160        170        180        190        200
X-17_ITS  ACATTGCGCC CATTAGTATT CTAGTGGGCA TGCCTGTTCG AGCGTCATTT

               210        220        230        240        250
X-17_ITS  CAACCCTTAA GCCCTCGTTG CTTAGTGTTG GGAGCCTACG GTACCCGTAG

               260        270        280        290        300
X-17_ITS  CTCCTCAAAG TTAGTGGCGG AGTCGGTTCA CACTCTAGAC GTAGTAATTT

               310        320        330        340        350
X-17_ITS  TATCTCGCCT ATCAGTTGGA CCGGTCCCCT GCCGTAAAAC ACCCCAATTT

               360        370        380        390        400
X-17_ITS  CTAAAGGTTG ACCTCGGATC AGGTAGGAAT ACCCGCTGAA CTTAAGC
```

**Figure 5.6** Nucleotide sequences of the partial 18S rRNA gene

### 4.8 Purification of bioactive compounds from crude extracts of endophytic *Xylaria* sp. isolate X-17

For scale up cultivation, endophytic *Xylaria* sp. isolate X-17 was cultured in 500 ml Erlenmeyer flask (x100) with each flask containing 200 ml of MEB (20L) under static condition at room temperature for 30 day. Culture was filtered, extracted with EtOAc and obtained 6.82 g of EtOAc crude extract.

The EtOAc crude extract (6.82 g) was purified through a Sephadex LH-20 column and eluted with 100% MeOH. The similar fractions were combined on the basis of TLC profile resulting in nine fractions (P1 to P9). After that, all fractions were tested for anti-tumor activity against CaSki cell line by MTT assay as shown in Table 4.5

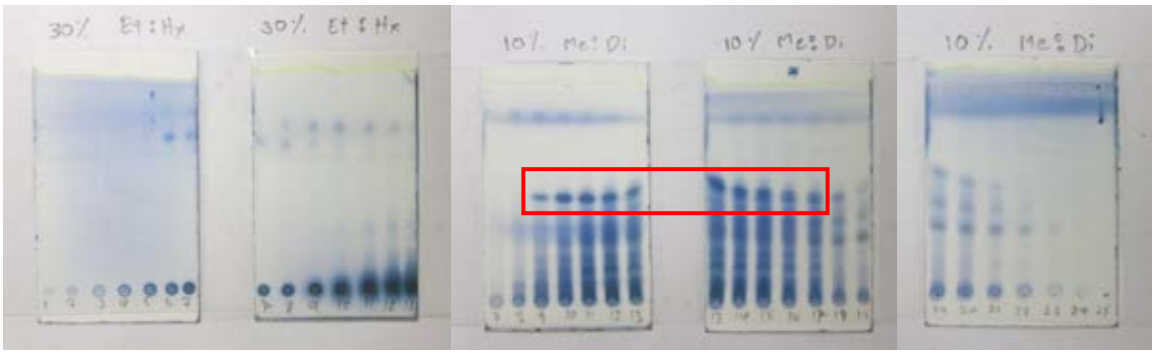


**Figure 5.7** TLC profile of crude extract X-17

**Table 4.5** The combined fraction and $IC_{50}$ value from crude extract X-17

| Fraction Code | Fraction No. | Weight(mg) | $IC_{50}$ |
|---|---|---|---|
| P1 | 6 | 23.8 | inactive |
| P2 | 7 | 34.6 | inactive |
| P3 | 8 | 21.9 | 91.56±3.47 |
| P4 | 9-12 | 1128.9 | 79.84±2.32 |
| P5 | 13-17 | 3685.1 | 33.01±5.32 |
| P6 | 18-22 | 628.8 | 29.24±0.48 |
| P7 | 23-24 | 27.6 | inactive |
| P8 | 25-26 | 12 | inactive |
| P9 | 27-33 | 18.6 | inactive |
| CrudeX17 | | 6820 | 17.53±1.04 |

As a result, fraction P5 yielded the most extract and exhibited intense blue characteristic coloration more than other fractions (Figure 4.12). Thus, fraction P5 was chosen for rechromatographed over silica gel and eluted with MeOH: $CH_2Cl_2$ in gradient condition (2%, 4%, 6%, 8% and 10%) to obtain 5 fractions (P5S1 to P5S5). Fraction P5S2 was rechromatographed over silica gel eluted with 10% MeOH: $CH_2Cl_2$ to give Compound 1 that confirmed by $^1H$ NMR. As a result compound 1 is a Cytochalasin D (appendix A)

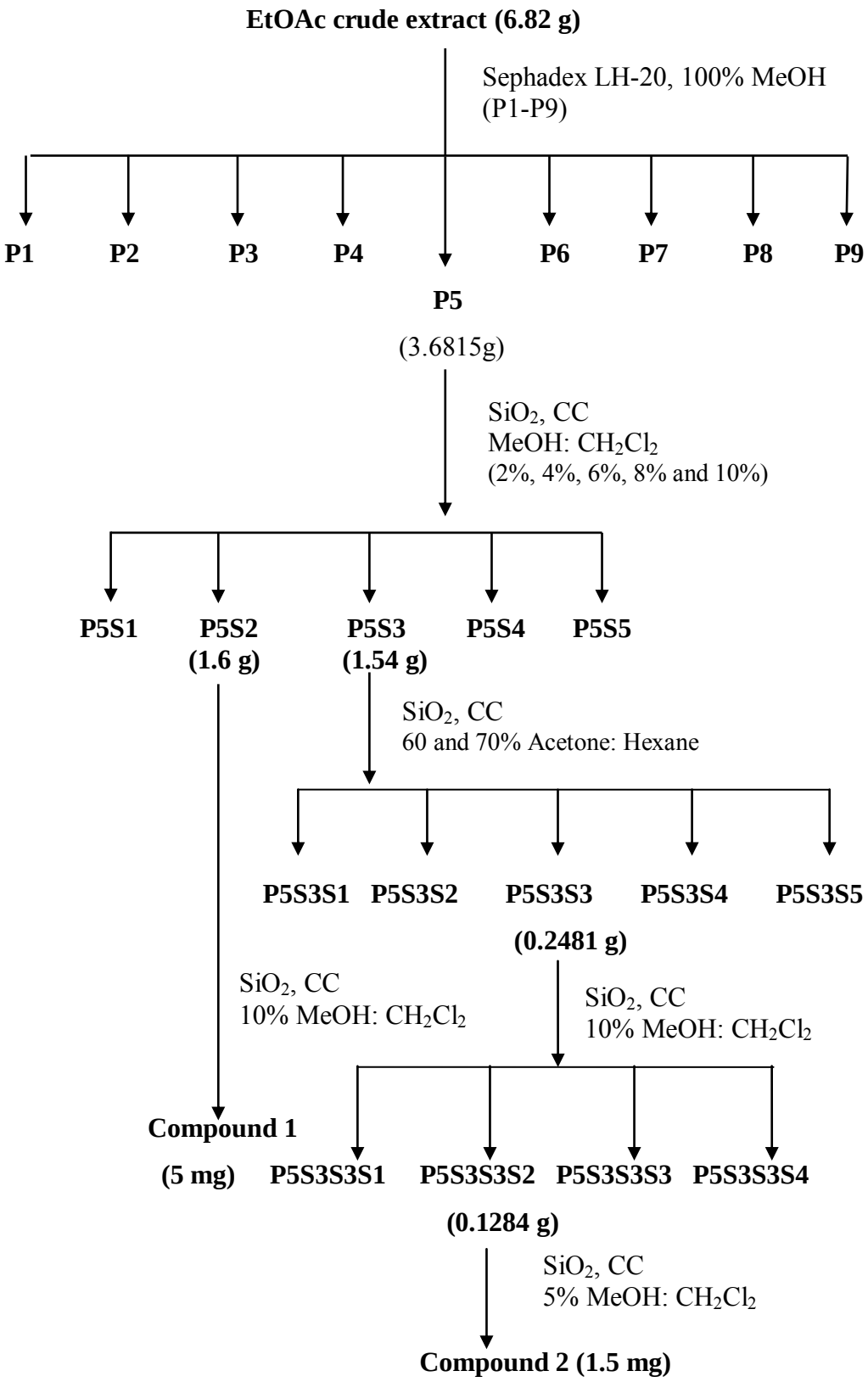
Fraction P5S3 was chromatographed on a $SiO_2$ column. Elutes of increasing polarity of 60 and 70% Acetone: Hexane to give 5 fractions (P5S3S1 to P5S3S5) and then fraction P5S3S3 was rechromatographed over silica gel eluted with 10% MeOH: $CH_2Cl_2$ to give 4 fractions (P5S3S3S1 to P5S3S3S4). Fraction P5S3S3S2 was subjected to $SiO_2$ column using 5% of MeOH in $CH_2Cl_2$ as eluent to obtain compound

2 that confirmed by $^{1}$H NMR. As a result compound 2 is a 2-*p*-tolylethanol (appendix A)

The isolation of bioactive compounds from crude extract isolate X-17 is summarized in Scheme 3.2.

Cytochalasin D (CytD) belongs to a group of fungal metabolites that was capable of permeating cell membrane, binding to actin and altering its polymerization. As a result, cytochalasin D was used as a tool to study motility-related phenomena in a variety of cell types (81). Rubtsova *et al.* (1998) reported that Cytochalasin D disrupted actin microfilaments in normal rat embryo fibroblasts, leading to accumulation of p53 in the cells and activation of p53-dependent transcription (80). Moreover, Huang *et al*. (2012) also reported that pegylated liposomal CytD (CytD-PEGL) could reduce its side effect when it was used as a cytotoxic agent in cancer therapy. Morover, CytD-PEGL was selectively accumulated in tumor tissues to a greater extent than natural CytD *in vivo*. In addition, CytD-PEGL could induce apoptosis and inhibited tumor angiogenesis. Thus, this research suggests that CytD-PEGL might be a potential alternative agent for cancer therapy (82).

2-*p*-tolylethanol is a member belongs to fragrance structural group Aryl Alkyl Alcohols and is a primary alcohol. It occurs in a nature which observed in mushrooms (83). 2-*p*-tolylethanol is a fragrance ingredient used in many fragrance mixtures. It was found mostly in fragrances for cosmetics, fine fragrances, shampoo, toilet soap and other toiletries. Moreover, it was found in non-cosmetic products such as household cleaners and detergents.




**Scheme 3.2** Purification scheme of bioactive compounds from crude extracts of selected endophytic *Xylaria* sp. isolate X-17.

### 4.9 Cytotoxicity of pure compound

Compound 1 and 2 were identified as cytochalasin D and 2-*p*-tolylethanol, and the pure compounds were tested for cytotoxic activity against CaSki cell line using MTT assay. The results were shown in Figure 5.7. Cytochalasin D exhibited strong cytotoxicity against CaSki cell line with an $IC_{50}$ of 49.06±13.68 μM. On the contrary, 2-*p*-tolylethanol showed no effect against viability of CaSki cell line.

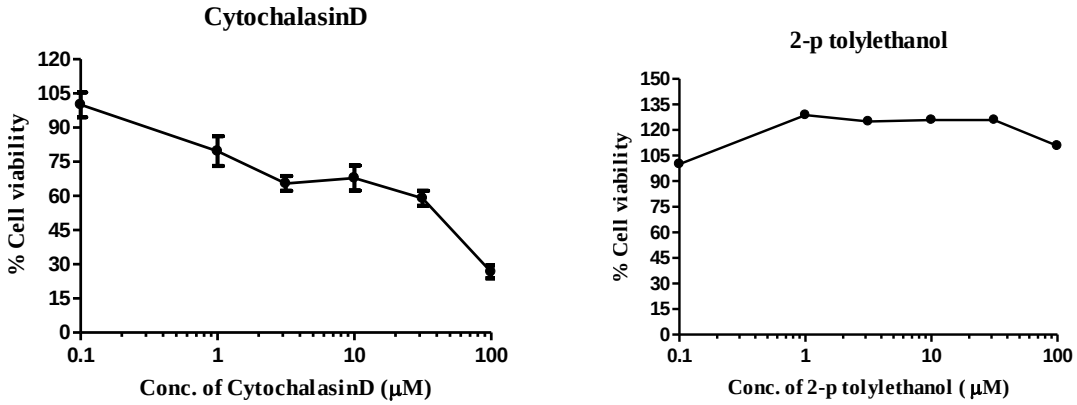



**Figure 5.8** The effect of compound treatment on viability of CaSki cell line.

CaSki cell line was treated with compound 1 and 2 at various concentrations for 4 days. Cell viability was assayed by MTT assay. The results showed mean±SD and represented two independent experiments.

# CHAPTER V

# CONCLUSIONS

The objectives of this study were to isolate endophytic *Xylaria* spp. and to screen for bioactive compounds derived from metabolites of isolated endophytes, including antimicrobial, anti-inflammation and anticancer activities. Eighty three endophytic fungi were isolated from plant leaves collected at Trad Agroforesty Research and Training station, Trad province, Thailand, including 41 *Xylaria* spp., 5 *Daldinia* spp., 1 *Fusarium* spp., 1 *Collectotrichum* spp., 4 *Pestalotiopsis* spp., 2 *Phomopsis* spp., 2 *Phyllosticta* spp., 1 *Trichoderma* spp., 4 *Nigrospora* spp. and 22 Mycelia sterilia.

For the primary screening, an antimicrobial activity was used as criteria. Nine crude extracts of endophytic *Xylaria* spp. showed good activity against the growth of five indicator microbes, especially stronger against Gram positive bacteria than Gram negative bacteria and yeast.

Crude extract from X-17 that is associated with *Cinnamomum porrectum* was chosen to test for anti-cancer activity against human cervical cell line, CaSki, based on the $^{1}$H NMR profile. Crude extract X-17 exhibited anti-cervical cancer with the $IC_{50}$ of 17.53±1.04 µg/ml and it was found to induce cell cycle arrest and apoptosis in CaSki. Testing for anti-inflammation activity indicated that X-17 has no activity but it caused cell death in macrophage cell line.

Two pure compounds derived from crude extract of endophytic *Xylaria* X-17 were identified as cytochalasin D and 2-*p*-tolylethanol.

Cytochalasin D and 2-*p*-tolylethanol were tested for anticancer activity. The results of cytochalasin D exhibited cytotoxic activity against CaSki cell line with an $IC_{50}$ value 49.06±13.68 µg/ml. On the contrary, 2-*p*-tolylethanol has no activity against cervical cancer cell line.

Identification of endophytic isolate X-17 was based on morphology and ITS sequencing analyses which lead to the identification as *Xylaria laevis*.

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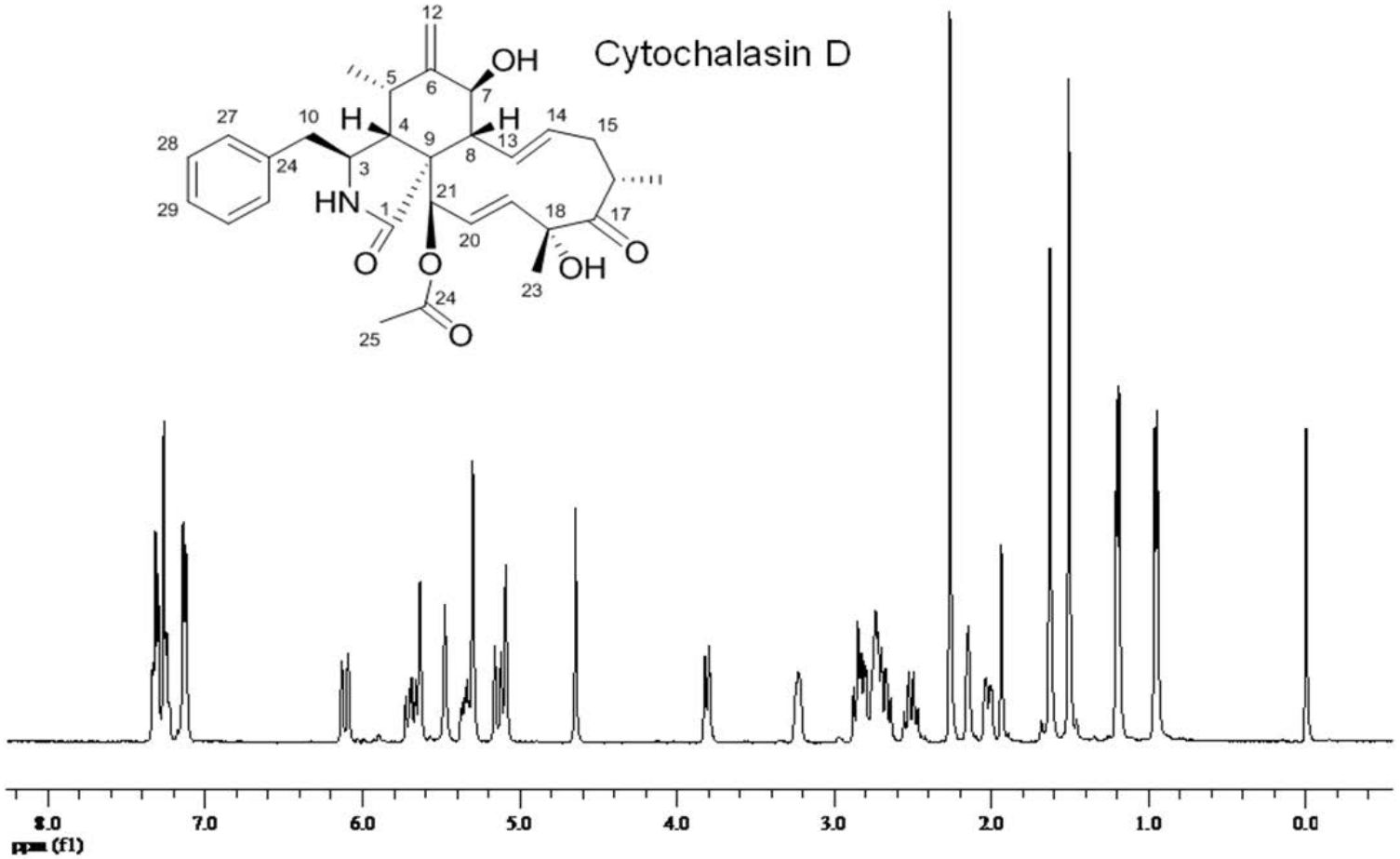
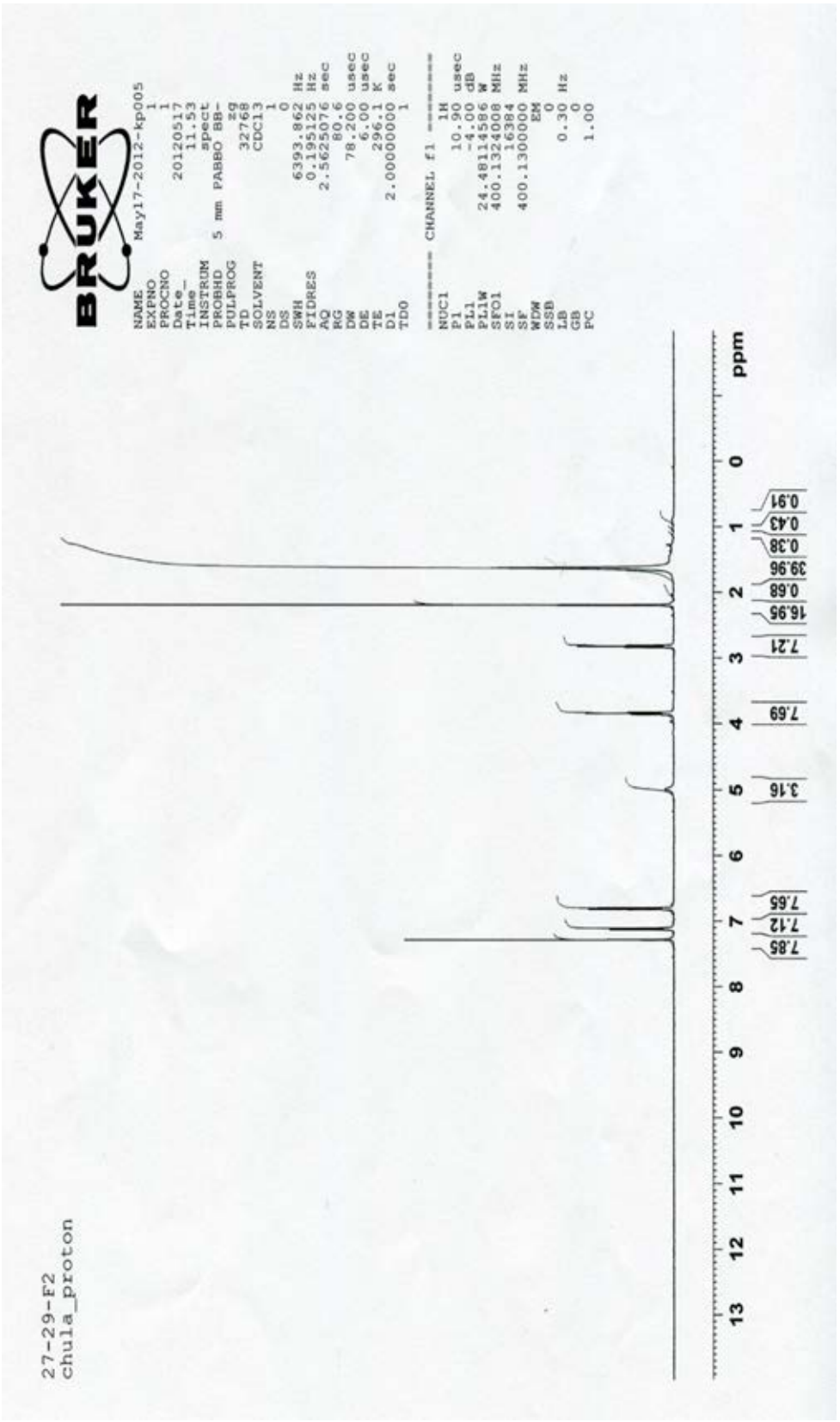
# APPENDICES

# APPENDIX A




400 MHz $^{1}$H NMR ($CDCl_3$) spectrum of cytochalasin D




$^1$H NMR ($CDCl_3$) spectrum of 2-*p* tolylethanol

# APPENDIX B

1. PDA (Potato Dextrose Agar)

| | | |
|---|---|---|
| Potato | 200 | g |
| Glucose | 20 | g |
| Agar | 20 | g |
| Water | 1,000 | ml |

2. NA (Nutrient Agar)

| | | |
|---|---|---|
| Beef extract | 1 | g |
| Peptone | 5 | g |
| NaCl | 8 | g |
| Agar | 18 | g |
| Water | 1,000 | ml |

3. NB (Nutrient Broth)

| | | |
|---|---|---|
| Beef extract | 1 | g |
| Peptone | 5 | g |
| NaCl | 8 | g |
| Water | 1,000 | ml |

4. MEB (Malt Extract Broth)

| | | |
|---|---|---|
| Malt extract | 2 | g |
| Peptone | 1 | g |
| Glucose | 20 | g |
| Water | 1,000 | ml |

5. MHB (Mueller-Hinton Broth)

| | | |
|---|---|---|
| Beef extract | 2 | g |
| Acid casein hydrolysate | 17.5 | g |
| Starch | 1.5 | g |
| Water | 1,000 | ml |

6. YMA ( Yeast Malt Extract Agar)

| | | |
|---|---|---|
| Malt extract | 10 | g |
| Yeast extract | 4 | g |
| Glucose | 4 | g |
| Agar | 18 | g |
| Water | 1,000 | ml |

7. Completed RPMI 1640 100 ml

| | | |
|---|---|---|
| RPMI 1640 | 90 | % |
| FBS | 10 | % |
| Penicillin | 100 | U/ml |
| Streptomycin | 0.4 | mg/ml |
| Sodium pyruvate | 1 | % |
| HEPES | 1 | % |

8. Complete DMEM 100 ml

| | | |
|---|---|---|
| DMEM | 90 | % |
| FBS | 10 | % |
| Penicillin | 100 | U/ml |
| Streptomycin | 0.4 | mg/ml |
| Sodium pyruvate | 1 | % |
| HEPES | 1 | % |

9. Freezing media 10 ml

| | | |
|---|---|---|
| Complete media | 90 | % |
| DMSO | 10 | % |

10. FBS inactivation

Before using, FBS must be inactivated at 56˚ C for 30 minute in water bath.

11. Penicillin and Streptomycin

Penicillin was prepared at final concentration 106 U/ml and Streptomycin was prepared at final concentration 50 mg/ml by diluting in sterile deionized water. The solution were filtered by using 0.22 μm syringe filter and then aliquoted and kept at -20˚ C

12. 1xPBS pH7.4 1,000 ml

| | | |
|---|---|---|
| NaCl | 8 | g |
| KCl | 0.2 | g |
| $Na_2HPO_4$ | 1.44 | g |
| $KH_2PO_4$ | 0.24 | g |

In 800 ml of deionized water and adjust pH to 7.4. Then make volume to 1,000 ml and sterile by autoclave at 121˚ C and pressure 15 psi for 15 minutes.

13. 70% Ethanol for propidium iodide staining 1,000 ml

| | | |
|---|---|---|
| Absolute ethanol | 700 | ml |
| Sterile deionized ultrapure water | 300 | ml |

14. 10 mg/ml RNase A 1 ml

| | | |
|---|---|---|
| RNaseA | 10 | mg |
| Sterile deionized water | 1 | ml |

15. 1 mg/ml propidium iodide 1 ml

| | | |
|---|---|---|
| Propidium iodide | 1 | mg |
| Sterile deionized water | 1 | ml |

16. Sulfanilamide solution 50 ml

Sulfanilamide 1% (w/v) was dissolved in 5% phosphoric acid and total volume was adjusted to 50 ml by deionized water

17. NED solution 50 ml

NED 0.1% (w/v) was dissolved in 50 ml deionized water.

18. Standard nitrite

Sodium nitrite 0.1 M was prepared as a stock solution. For working solution, 100 μM was prepared by diluting the stock solution in DMEM complete media to 1 ml total volume.

# Biography

Kunlanee Khajadpai was born in Nakhonratchasima, Thailand on July 8, 1987. She graduated from the Department of Microbiology, Faculty of Science, Chulalongkorn University with a Bachelors degree in 2010. In the same year, she enrolled in the Masters Program of Science in Industrial Microbiology at Chulalongkorn University.

## Academic presentation

Khajadpai, K., Sihanonth, P. and Palaga, T. Isolation of endophytic *Xylaria* spp. from Trad Province and bioactive compounds from their metabolites. The proceedings of The 3rd Annual International Conference for Research and Academic Presentation on "ASEAN Community and Regional Sustainable Development" on 28 July 2012 at Ubon Ratchathani Rajabhat University.