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รายงานวิจัยฉบับสมบูรณ์

การศึกษาสารทุติยภูมิในเชื้อรากลุ่ม Ascomycetes

The study of secondary metabolites from selected Ascomycetes fungi

โดย

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Executive summary

Fungi are prolific producers of secondary metabolites. In 1971 Turner determined that there were almost 1,000 fungal products but this had more than doubled by the next decade (Turner,1971; Turner and Aldridge,1983). To date more than 10,000 different metabolites have been reported from fungi (Newman and Cragg,2012) and fungi are still be the top runner candidate on natural product discovery which leads to potential drugs. There were more than 500 fungal and mushroom isolates in the collection at the Centre of Excellent for Fungal Research at Mae Fah Luang University, Chiang Rai. Nevertheless, most of these fungal and mushroom have not been studies on their secondary metabolites production.

The aim of this research was to screen for the active fungal isolates by testing on their antibacterial activities via antimicrobial (AM) quick screen assay. The AM quick screen assay is a rapid and economize method to determine the activities of fungal crude extracts. Only 10 µg of each crude extract were used in the assay, each of crude extract was added into a well of 96 wells microtitre plate followed by the addition of 200 µl of bacterial strain into each well. After overnight incubation at 37 °C, 30 µl of resazurin solution was added into each well and observe on the discoloration of resazurin. Resazurin is a growth indicator and the presence of bacterial or fungi is indicated by oxygen emission which reacts with resazurin generate pink colour. Hence, viable cells can reduce resazurin (blue colour) into resorufin (pink colour), while the dead cells cannot reduce resazurin and remain blue colour. The UV absorption of each sample is read at 600 nm and compared against the UV absorption of the control media (O'Brien, Wilson *et al.*,2000). Then the absorption values were calculated into the percentage of cell viability (Lang et al. 2006). Crude extracts which gave the value if cell viability less than 20% were considered as "active extract". In the other hand, extracts that gave the percentage of cell viability between 21-50% were considered as "moderate active", and will considered as "inactive" when the cell viability is > 50%.

In this experiment, 35 fungal isolates were chosen for the study. These fungi were cultured on two different media and 70 extracts were yielded. Of these, 42 fungal extracts showed 80% inhibition activity against at least 1 strain of bacteria.

Extracts from fungus species *Diaporthe pterocarpi*, showed good activities against many bacterial strains in the AM quick screen, and hence this fungus was subject for up-scale cultivation in order to get the appropriate amount of crude extracts for separation and purification of pure compound in NMR analysis. Three factions of the main compounds (B0009-B, B0009C and B0009-D) were yield from purification by column chromatography and these three fractions were subjected for NMR analysis. The ^{1}H NMR spectrum showed that fraction B0009-B could be an unsaturated fatty acid, while fractions B0009-C and B0009-D were a mixtures. Further purification is needed for these two fractions.

The results from this research showed that fungi are the good source for natural products discovery. At the Institute of Excellence in Fungal Research, Mae Fah Luang University has many novel fungal species and these are promising to be a good source for discovery on the novel secondary metabolites. Furthermore, our research group has a good collaboration with HELMHOLTZ; the center for infection research in Germany, which an institute on a development of medicine from natural products especially from fungi and mushrooms.

At the start point on the natural products research in MFU, we could screen for the active fungal and mushroom isolates and send the active crude extracts to HELMHOLTS for the chemical analysis such as screening the crude extracts by high pressure liquid chromatography (HPLC), mass spectroscopy (MS) and NMR analysis. For the long term research, however, it is worth to build up our own library for the HPLC profile so we could eliminate the known compound from the novel or unknown one.

Abstract

Fungi are prolific sources of novel and biologically active compounds. At the Centre of Excellent for Fungal Research at Mae Fah Luang University, we held more than 500 fungal and mushroom isolates in our collection. Of these isolates, some are novel species and some are already been described. Nevertheless, most of these isolates have not been examined for their secondary metabolites production.

A study was concerned on secondary metabolites production mainly on 35 fungal isolates from genus *Chaetomium* sp., *Xylaria* sp., *Alternaria* sp., *Collectotrichum* sp., *Diaporthe* sp., *Phomopsis* sp. and *Pestalotiopsis* sp. A total of 70 crude extracts were obtained. Of these, 42 culture extracts of these fungus species showed significant activity at least in the antimicrobial (AM) quick screen assay.

Of these active extracts, one from *Diaporthe pterocarpi* showed good antibacterial activities against many bacteria. This extract was subjected to pure compound isolation and three main compounds were subsequently isolated. The ^{1}H spectrum showed that one of the three main compounds could be an unsaturated fatty acid and other two compounds were subjected for further analysis.

Keywords: Ascomycota, antimicrobial screening, *Diaporthe* sp., *Phomopsis* sp., resazurin testing.

บทคัดย่อ

ในการศึกษาทางด้านผลิตภัณฑ์ทางธรรมชาติในปัจจุบันพบว่าเห็ดและเชื้อราเป็นแหล่งผลิตสารออกฤทธิ์ทางชีวภาพที่สำคัญต่าง ๆมากมาย ทั้งนี้ ที่ศูนย์ความเป็นเลิศทางด้านเชื้อรา ณ มหาวิทยาลัยแม่ฟ้าหลวง ได้มีการรวบรวมสายพันธุ์เห็ดและเชื้อราสายพันธุ์ต่าง ๆ ทั้งสายพันธุ์ที่ถูกค้นพบแล้ว และสายพันธุ์ใหม่ไว้มากมายกว่า 500 ชนิด แต่เห็ดและเชื้อราเหล่านี้ส่วนใหญ่ยังไม่ได้ถูกนำมาศึกษาทางด้านสารออกฤทธิ์ทางชีวภาพแต่อย่างใด ทางคณะผู้วิจัยจึงมีความสนใจที่จะนำเชื้อราและเห็ดที่ถูกเก็บไว้เหล่านี้มาศึกษาเกี่ยวกับการผลิตสารออกฤทธิ์ทางชีวภาพที่สำคัญ เพื่อเป็นการขยายองค์ความรู้และต่อยอดงานวิจัยที่มีอยู่

ในการศึกษานี้ เชื้อราทั้งหมดจำนวน 35 สายพันธุ์ จากจีนัส *Chaetomium* sp., *Xylaria* sp., *Alternaria* sp., *Collectotrichum* sp., *Diaporthe* sp., *Phomopsis* sp. และ *Pestalotiopsis* sp. ได้ถูกนำมาศึกษาเกี่ยวกับการผลิตสารออกฤทธิ์ทางชีวภาพเบื้องต้นโดยเน้นไปในการออกฤทธิ์ยับยั้งการเจริญเติบโตของแบคทีเรียชนิดต่าง ๆจำนวน 8 สายพันธุ์ ในการวิเคราะห์แบบ quick scree จากการทดลองนี้ได้จำนวนสารสกัดหยาบทั้งหมด 70 สาร ในจำนวนนี้ มีสารสกัดหยาบ 42 สารที่ออกฤทธิ์ยับยั้งการเจริญเติบโดเชื้อแบคทีเรีย 80% และจากการวิเคราะห์เบื้องต้น สารสกัดจากเชื้อรา *Diaporthe pterocarpi* ออกฤทธิ์ยับยั้งการเจริญเติบโตของแบคทีเรียได้หลายชนิด เชื้อราตัวนี้จึงถูกนำมาเลี้ยงในขนาดขยายปริมาณเพื่อที่จะได้สารสกัดหยาบจำนวนมากพอในการวิเคราะห์ทางเคมี

จากการวิเคราะห์และแยกสารสกัดหยาบจากเชื้อรา*Diaporthe pterocarpi* พบว่าในสารสกัดหยาบมีสารหลักอยู่ 3 ชนิด และเมื่อนำมาวิเคราะห์หาโครงสร้างทางเคมีของสารเหล่านี้พบว่าสารตัวแรกเป็นไขมัน ส่วนอีกสองตัวที่เหลือยังไม่เป็นสารที่บริสุทธิ์ ซึ่งได้นำกลับมาเพื่อทำการทำให้บริสุทธิ์ในลำดับถัดไป

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Abbreviations

%	percent
°C	degrees Celsius
λ_{max}	maximum wavelength in nm
µg	microgram
µL	microlitre
µm	micrometer
$CDCl_3$	deuterated chloroform
CD_3OD	deuterated methanol
COSY	correlation spectroscopy (in NMR)
CRP	chloramphenicol
DCM	dichloromethane
DMSO-d6	deuterated dimethyl sulfoxide
e.g.	example given
EtOAc	ethyl acetate
g	gram
h	hour
HMBC	heteronuclear multiple bond correlation spectroscopy (in NMR)
H_2O	water
HPLC	high performance liquid chromatography
HSQC	heteronuclear single quantum coherence (in NMR)
i.e.	*id est* (that is)
MeOH	methanol
mg	milligram
mL	millilitre
mm	millimeter
MIC	minimum inhibitory concentration
min	minute
MPA	mycophenolic acid
MYPA	Malt Yeast Peptone Agar
No.	number
OD	Optical density

SDA	Sabouraud Dextrose Agar
SDB	Sabouraud Dextrose Broth
Sol^{n}	solution
Temp	temperatures
TTC	tetracycline
w/o	without

Chapter 1: Introduction

1.1 Background

Fungi are the second largest group of organisms in the world after insects. Despite various diseases on plants and animals caused by fungi, many research found that fungi are a good source to retrieve their secondary metabolites as a source for medicinal usage (Zain, Amani *et al.*,2009). In 1971 Turner determined that there were almost 1,000 fungal products but this had more than doubled by the next decade (Turner,1971; Turner and Aldridge,1983). To date more than 10,000 different metabolites have been reported from fungi (Zain, Amani *et al.*,2009; Newman and Cragg,2012). Some of the fungal metabolites that have been known for a long time are finding new and unexpected uses in medicine todayA classic example on the use of fungi as medicine is penicillin discovery by Alexander Flaming in 1928. Another example is mycophenolic acid (**Figure 1.1**) was isolated from the fungus *Penicillum brevicompactum* by Gorsio in 1896 and then was developed as an immunosuppressive agent in 1995. This compound was also used as a base for a synthetic pro-drug, mofetil (**Figure 1.2**) and is now commercially available (Pearce,1995)

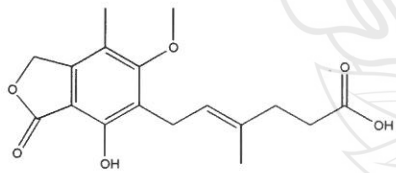


Figure 1.1: Mycophenolic acid

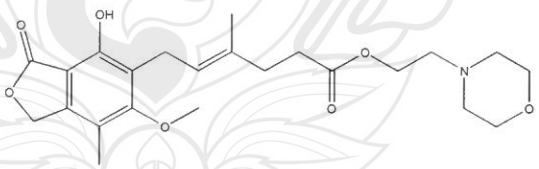


Figure 1.2: Mofetil

For the recent natural products researches, there are multidisciplinary approaches for drug discovery today. This included the old tradition by retrieve natural products from its own natural source, or the new approaches such as manipulation on the

biosynthesis pathways of the target organisms, or a combinatorial between synthesis and natural product compounds (semisynthetic drug) (Newman and Cragg,2012).

In our fungal research group at the Centre of Excellent for Fungal Research at Mae Fah Luang University, there were more than 500 of the fungal and mushroom collections both known and novel species. Nevertheless, most of these fungi have not been studied on their secondary metabolites production. In this research we aim to study on the secondary metabolites production from Ascomycetes as it is the largest group in Kingdom Fungi and deem to produce novel bioactive compounds (Hawksworth and Kalin-Arroyo,1995; Murphy and Horgan,2005). Extracts from these Ascomycetes are worth to look on their medicinal properties such as antimicrobial and cytotoxic activities. Furthermore, active extracts can be further applied on the inhibition for bacteria and fungi that cause several plant diseases.

1.2 Objectives

- To find out the method to enhance the secondary metabolites production in Ascomycetes.
- To assay on the antimicrobial properties and other medicinal properties from the crude extracts, isolate pure compounds from the active crude extract.
- To elucidate the structure of the novel compound(s) (If it is discovered).

1.3 Consequence from this research

- Active fungal extracts can be applied in agriculture. For example, it can be used to test against many bacteria and fungi that caused plant disease.

1.4 Scope of present study

Thirty five Ascomycete isolates from the collection are selected for a study on their secondary metabolites production. These fungal cultures are subjected to grow on different media in order to compare metabolites production from different media. Crude extracts from these fungi are initially test against bacteria (both Gram positive and Gram negative). Active extracts are further subjected for purification for the pure compounds and analyzed for their chemical structure.

1.5 Definitions

Natural products are organic compounds that are formed by living organisms. These naturally occurring compounds can be divided into three broad categories; Primary metabolites, Structural components and Secondary metabolites (Hanson,2003). In this report, the term of "Natural products" is refers to secondary metabolites only.

Chapter 2: Literatures review

2.1 Natural products overview

Natural products are organic compounds that are formed by living organisms and can be divided into three broad categories (Hanson,2003);

- Primary metabolites, this type of compounds play a role in the metabolism and reproduction of the cell such as amino acids and sugars.

- Cell's structural components, this type of compounds form the structural of the cell such as cellulose in plant cell, peptidoglycan in bacteria or chitin in fungi.

- Secondary metabolites, unlike the first two categories which are essential for life, these types of compounds served roles related to survival function for the organisms that produce them (Demain and Fang,2000).

The production of secondary metabolites is believed that is affected by external stimuli such as environmental stress, competition, communication and infection (Strohl,2000).

2.2 Natural products discovery

Natural products have long been used by humans for thousands of years as traditional medicines and as natural poisons (Larsen, Smedsgaard *et al.*,2005). The most well known and widely used drug for its antipyretic and anti-inflammatory properties is aspirin (**Figure 2.1**) (Diarmuid,2005). Other example of natural product from plant with an analgesic property is morphine (**Figure 2.2**) (Aldrich,1996).

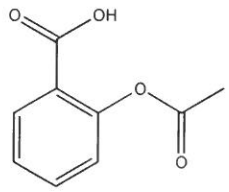


Figure 2.1: Aspirin

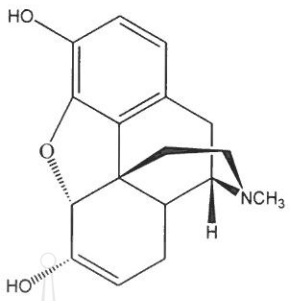


Figure 2.2: Morphine

Natural products research and fungal metabolite studies were revolutionized by the discovery of penicillin in 1928 by Alexander flaming from *Penicillium chrysogenum* (Tulp and Bohlin,2004). Penicillin G (**Figure 2.3**), the original metabolite isolated, is still a front line for the base of antibiotic drugs in the market today.

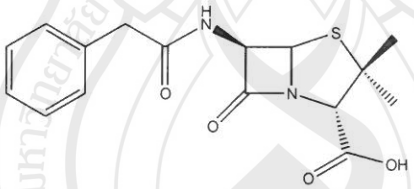


Figure 2.3: Penicillin G

A paradigm shift of natural products discovery start when their properties is not only limit to the antibacterial but also other fascinating properties such as anticancer, immunosuppressive, anti-diabetic and anti-malaria drugs (Harvey,2000; Dickschat,2011).

2.3 Ascomycota

Phylum Ascomycota is the largest phylum of fungi with over 30,000 member species (Hawksworth and Kalin-Arroyo,1995). The distinct characteristic of Ascomycota is the production of sexual spores (ascospores) inside the sac-shaped cells (asci) which may or may not be produced within a fruiting body (ascoma). Hence, they are commonly known as sac fungi (Blackwell and Spatafora,2004).

Despite the pathogenic aspect of Ascomyctes, many of Ascomycetes have been utilized in the food processing such as *Saccharomyces cerevisiae* or the baker's yeast, used to make bread, beer and wine. Apart from the food processing aspect, they also play a role in the production of valuable commodities such as antibiotics, enzymes, vitamins and pharmaceutical compounds (Murphy and Horgan,2005). One example of a commercial medicine isolated from Ascomycetes is one of the ergot alkaloids called ergotamine (**Figure 2.4**) isolated from fungus *Claiceps purpurea*. This compound and caffeine (**Figure 2.5**) are major components for the antimigraine drug "cafegot" (Diener, Jansen *et al.*,2002).

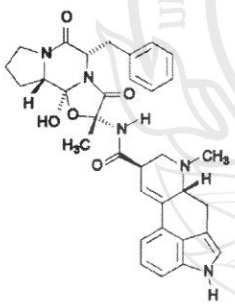


Figure 2.4: Ergotamine

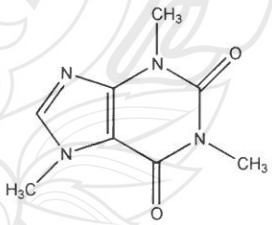
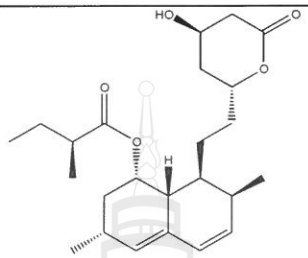
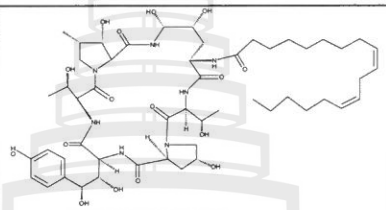
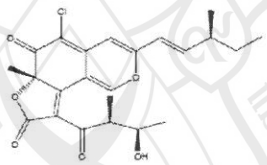
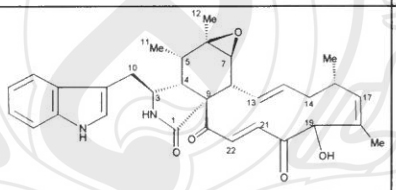
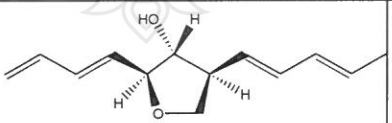
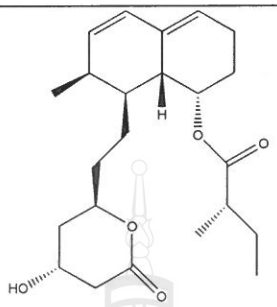
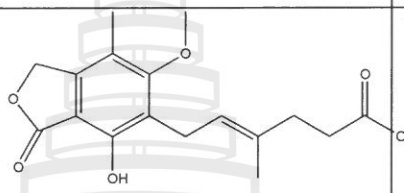
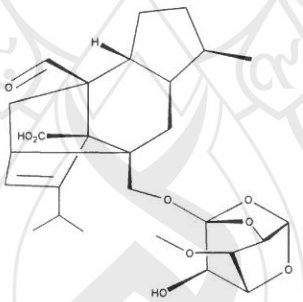
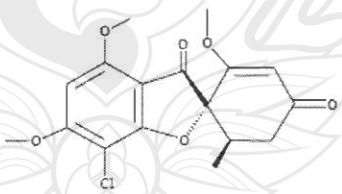


Figure 2.5: Caffeine

Vast varieties of natural products and their properties isolated from Ascomycetes are summarized in **Table 2.1**.

Table 2.1: Examples of active compounds isolated from Ascomycota

Fungal species	**Compound**	**Chemical structure**	**Activity**
Aspergillus terreus	Lovastatin (Butler,2004)		cholesterol-lowing agents
Aspergillus nidulans	Echinocandin B (Murphy and Horgan,2005)		Antifungal agent
Chetomium sp.	Chaetoviridin A (Takahashi, Koyama *et al.*,1990)		Anticancer agent
	Chaetoglobosin A (Sekita, Yoshihira *et al.*,1973)		Anticancer agent
	Aureonitol (Abraham and Arfmann,1992)		Anticancer agent

Fungal species	Compound	Chemical structure	Activity
Penicillium citrinum	Mevastatin (Alberts, Chen *et al.*,1980)		cholesterol-lowing agents
Penicillium brevicompactum	Mycophenolic acid (Pearce,1995)		immunosuppressive agent
Xylaria sp.	Xylarin (Murphy and Horgan,2005)		against mycobacterim tuberculosis
	Griseofluvin (Oxford, Raistrick *et al.*,1939)		Anticancer agent

Finding new secondary metabolites is a prerequisite for drug development in both novel pharmaceuticals or develops the drug for resistance pathogens. While the need for new bioactive compounds is increasing, the yield of finding new bioactive compounds is declining (Dickschat,2011). Part of the problem stem is the redundancy on the source of bioactive metabolites. Fungi are good candidates for the source of

secondary metabolites as they are the second largest group of organism after insects. However, less than 5% of the fungal species have been described and of this 5% only a fraction have been examine on production of natural products.

Chapter 3: Methodology

3.1 Culturing methods

3.1.1 Fungal collections and starter cultures preparation

In this study, 35 fungal isolates from seven genus (see details in **Table 3.1**) were obtained from the collection which kept in 10% glycerol at -80 °C were transferred to Malt Yeast Peptone Agar (MYPA; **Appendix I**) and incubated at 25 °C for seven days. These cultures were used as starters for inoculation on the solid phase fermentation.

Table 3.1: Fungal isolates used in this study

No.	Fungal name	Fungal code
1	*Chaetomium globosum*	N/A
2	*Xylaria* sp.	N/A
3	*Alternaria* sp.	KSU-C2
4	*Collectotrichum* sp.	N/A
5	*Diaporthe* sp.	DPH-001, DPH-002, DPH-003, DPH-014
6	*Diaporthe siamensis*	DPH-004
7	*Diaporthe pterocarpi*	DPH-006
8	*Diaporthe thunbergii*	DPH-008
9	*Diaporthe pterocarpicola*	DPH-013
10	*Phomopsis* sp.	DPH-015, DPH-101, DPH-104, DPH-106, DPH-111, DPH-131A, DPH-140, OP 013, OP 084, OP 111, OP 118, OP 139
11	*Pestalotiopsis* sp.	S19, SS011, S09, S16, NTCL 013, NTCL 041-1, NTCL 077, NTCL 015, DLR 131A, BN 1006, 11-0471

3.1.2 Solid phase fermentation

Two agar media were used in the solid phase fermentation; ¼ Sabouraud Dextrose Agar (¼SDA; **Appendix I**) and MYPA. Inoculum was prepared by cutting the edge of the colony, 4 mm in diameter. The inoculum disc was inverted onto the centre of an 88 mm Petri dish containing 20 ml of either SDA or MYPA. Inoculate plates were incubated 25 °C for 30 days prior to extraction. For each isolate, five replicates were carried out.

3.2 Preparation of culture extracts

3.2.1 Solid phase extraction

Agar from plates with fungal cultures were macerated with 30 mL HPLC grade ethyl acetate (EtOAc) using a blender. This extraction process was carried out three times. The agar slurry was left sitting overnight for the first EtOAc extraction, and eight hours each for the second and third extractions. All three EtOAc extracts were combined and transferred to a pre-weighed vial and air dried to yield a crude extract. Crude extracts were prepared to a concentration of 1 mg/mL in HPLC grade methanol for bioassays. The procedure for the solid phase extraction process is summarised in **Figure 3.1.** The totals of 70 crude extracts were obtained from the solid phase extraction and were subjected for antimicrobials quick screen assay.

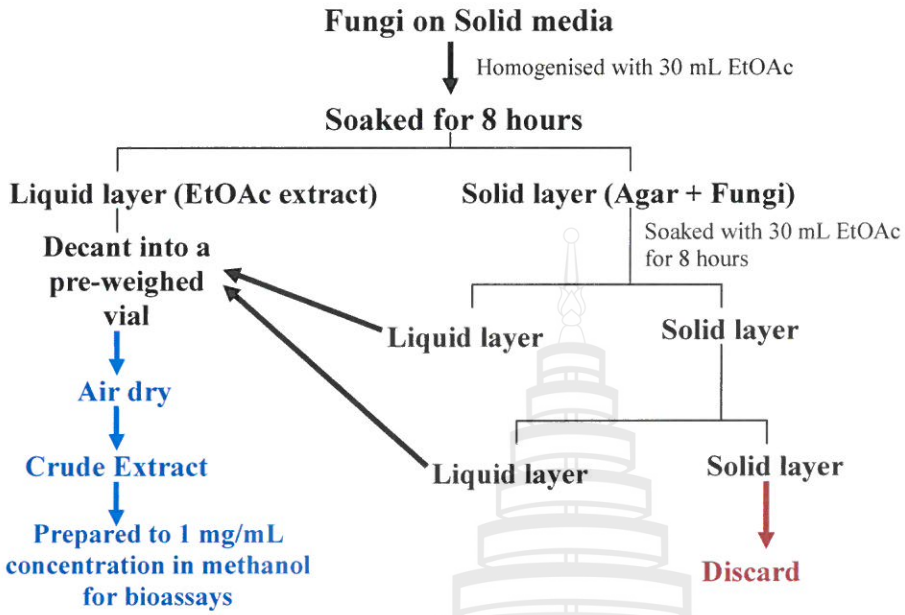


Figure 3.1: Solid phase extraction method

3.2.2 Crude extracts preparation

Crude extracts were prepared to 1 mg/ml concentration in HPLC grade methanol (MeOH) prior to antimicrobial screening.

3.3 Bioassays

Crude extracts were initially screened in a preliminary antimicrobial (AM) quick screen assay against eight strains of bacteria as listed in **Table 3.2.**

Table 3.2: Bacterial strains used in antibacterial assay

No.	Bacterial code	Bacterial strain	Gram stain
1	TISTR 687	*Bacillus cereus*	Gram-positive
2	TISTR 008	*Bacillus subtilis*	Gram- positive
3	TISTR 884	*Micrococcus luteus*	Gram- positive
4	TISTR 1466	*Staphylococcus aureus*	Gram- positive
5	TISTR 781	*Pseudomonas aeruginosa*	Gram-negative
6	TISTR 780	*Escherichia coli*	Gram-negative
7	TISTR 292	*Salmonella* Typhimurium	Gram-negative
8	PMST 8845	*Serratia marcescens*	Gram-negative

3.3.1 Samples preparation in Microtitre (MTT) plate

A 10 µl aliquot of crude extracts (as mentioned in 3.2.2) was pipette into individual well of a 96-wells microtitre plate (Rows A-G for crude extracts, Row H for controls) prior to a preliminary quick screen assay (see **Figure 3.2**). After extracts and controls were loaded, the MTT plate was subjected to air dried prior to inject any bacterial cells into each well. Experiment was carried in duplicate.

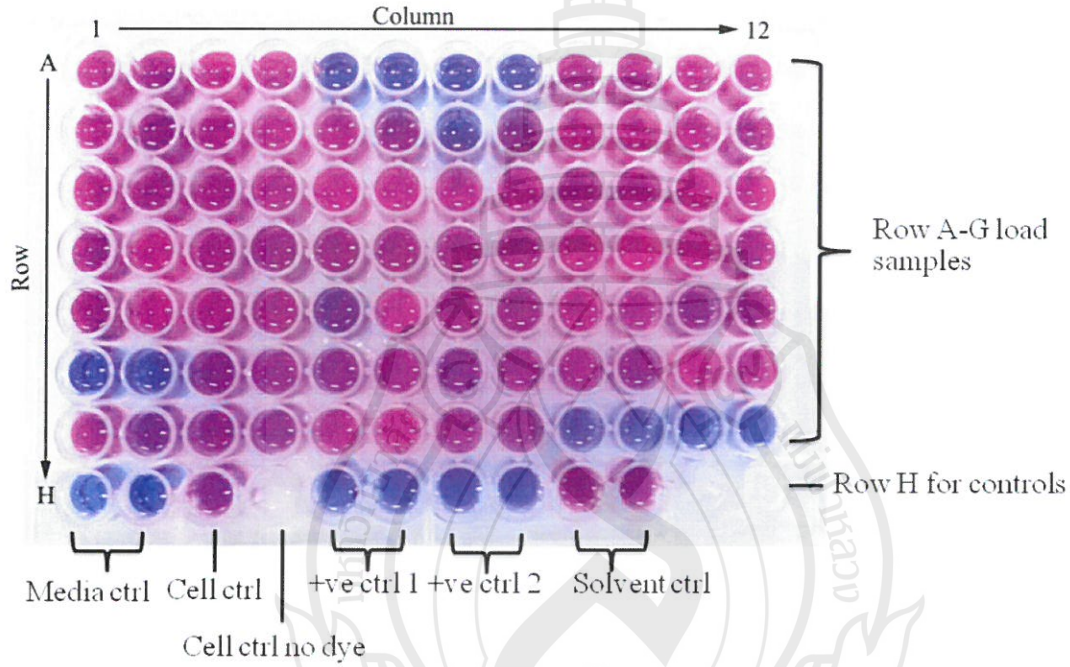


Figure 3.2: AM quick screen plate: blue/purple indicates dead or unhealthy cells and pink indicates viable cells

The control media, culture, standard antibiotics and solvent in Row H were summarized in **Table 3.3.**

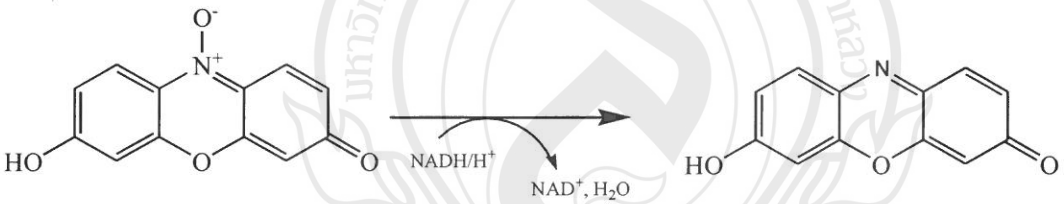
Table 3.3: Control experiments in antibacterial assay

Wells	**H1-2**	**H3**	**H4**	**H5-6**	**H7-8**	**H9-10**
Types of control	Media control	Bactria w/o antibiotic (add dye)	Bactria w/o antibiotic (no dye)	standard antibiotic 1 (tetracycline 4 µg)	standard antibiotic 2 (chloramphe -nicol 5 µg)	solvent control (methanol)

3.3.2 Antimicrobial quick screen

The antimicrobial quick screen was followed the protocol optimized by Marine Chemistry group at the University of Canterbury, New Zealand (Lang, Mitova *et al.*,2006) with a combination of the Procedure manual from Institute of Tropical Medicine, Mycobacteriology Unit Antwerp, Belgium (Martin and Palomino,2010). Bacterial suspensions are prepared in Nutrient Broth (N B) (**Appendix I**). The density of cells is read at 610 nm and adjusted to an optical density (OD) of 0.04 for Gram-positive bacteria; and 0.05 OD for Gram-negative bacteria. In each well, 200 µL of the bacterial suspension is incubated with each crude extract for 24 h at 37 °C.

The cell viability was determined colourimetrically by the addition of 30 µL of 0.02 % resazurin into each well and wait for 20 minute for discolouration. Resazurin is a growth indicator and the presence of bacterial or fungi is indicated by oxygen emission which reacts with resazurin generate pink colour. Hence, viable cells can reduce resazurin (blue colour) into resorufin (pink colour), while the dead cells cannot reduce resazurin and remain blue colour (**Figure 3.3**).



Resazurin (Blue color) Resorufin(Pink color)

Figure 3.3: Resazurin reaction with the presence of living microorganisms.

The UV absorption of each sample is read at 600 nm and compared against the UV absorption of the control media (O'Brien, Wilson *et al.*,2000). Results are given as a percentage of cell viability (Lang, Mitova *et al.*,2006). Crude extracts which had a result less than 20%; i.e. 80% of bacterial cells were killed by the crude extract, are noted as strong activity and were subjected for upscale in order to obtain more crude extracts for further study.

3.4 Isolation and Identification of active compounds

Selected active fungal isolates were subjected for upscale cultivation to yield an adequate amount of crude extracts for a separation by column chromatography. Pure compounds were subjected to Nuclear Magnetic Resonance (NMR) spectroscopy for the structure elucidation.

3.4.1 Thin layer chromatography (TLC)

Crude extracts were once again prepared at the concentration of 1 mg/mL in HPLC grade methanol and were spot on a 10 cm. Thin Layer Chromatography (TLC) plate with the solvent system of Ethyl acetate: Hexane (30:60). This method was for screening on compounds in each crude extract.

3.4.2 Column chromatography

Crude extracts eluent were separate by column chromatography (30 cm height) packed with silica gel with the solvent system of Ethyl acetate: Hexane (30:60).

3.4.3 Nuclear magnetic resonance (NMR) spectroscopy

Pure compounds which obtained from column chromatography were subjected for NMR spectroscopy. ^{1}H NMR experiment was recorded on a Bruker spectrometer at 25 °C, operating at 400 MHz. Chemical shifts are expressed in part per million (ppm) on the δ scale, and were referenced to the appropriated solvent peaks:

$CDCl_3$ referenced to $CHCl_3$ at δ_H 7.25 ppm (^{1}H) and $CDCl_3$ at δ_C 77.0 ppm (^{13}C)

CD_3OD referenced to CHD_2OD at δ_H 3.30 ppm (^{1}H) and CHD_2OD at δ_C 49.3 ppm (^{13}C);

DMSO-d_6 referenced to $CD_3(CHD_2)SO$ at δ_H 2.50 ppm (^{1}H) and $(CD_3)_2SO$ at δ_C 39.6 ppm (^{13}C).

Chapter 4: Results and Discussion

4.1 Antimicrobial activities screening of culture extracts

A total of 35 fungal isolates were cultured on MYPA and ¼SDA for 30 days (five replicates). The inoculation plates were checked for contaminations for every 3 days for 30 days prior to extraction. The resultant culture extracts with ethyl acetate (EtOAc) to yield a total of 70 fungal crude extracts. All 70 extracts were assayed in the quick screen against eight bacteria which were: four Gram-positive bacteria; *Bacillus cereus, Bacillus subtilis, Micrococcus luteus and Staphylococcus aureus,* and four Gram-negative bacteria; *Pseudomonas aeruginosa, Escherichia coli, Salmonella* Typhimurium and *Serratia marcescens.*

On the antimicrobial quick screen assays, 10 mg of each crude extract was used to test against each bacterial strain. Normally, we can determine the activities of crude extracts in the quick screen assay by the discoloration of the chromagen; in this case resazurin. As resazurin is a growth indicator and the presence of living cells would reacted with the blue resazurin and generate the resorufin which is pink colour.(Sarker, Nahar *et al.*,2007). **Figure 4.1** showed the assay plate number 3 of the crude extracts against *Salmonella* Typhimurium, the pink colour at wells A3-4 implied that the fungal extract was inactive (duplicate experiment), while fungal extract in wells G11-12 was active as the indicator was presence in distinct blue colour.

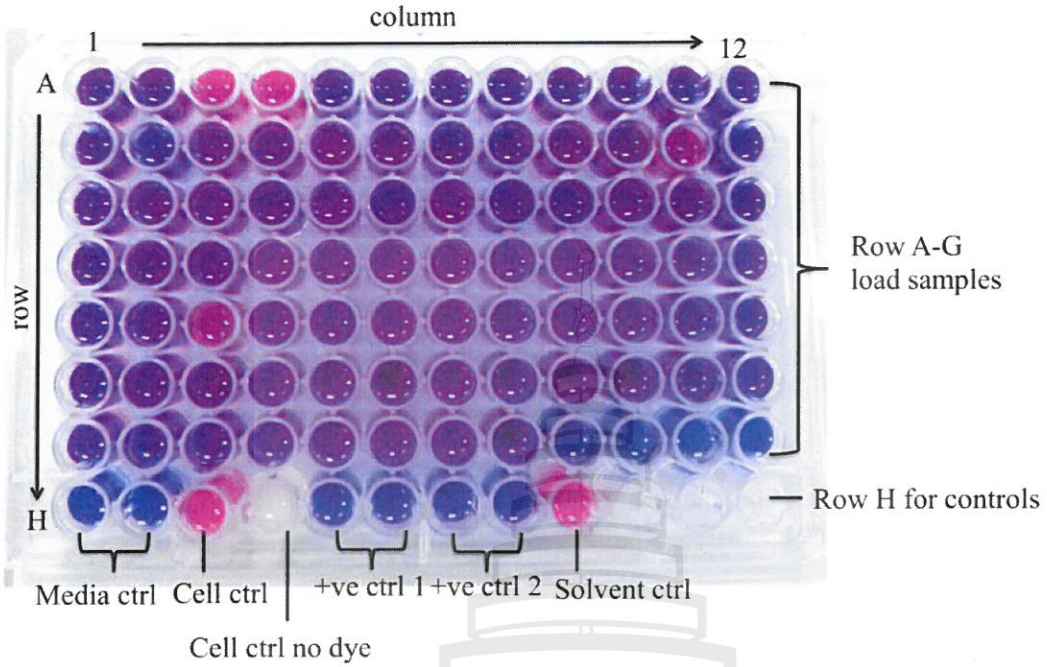


Figure 4.1: Microtitre plate resultant in antimicrobial quick screen on *Salmonella* Typhimurium, blue colour mean extract is active against bacterial while pink colour interpreted as inactive.

The determination of cell viability by the naked eyes, however, was not applied in some case of the assays when the discoloration was in between blue and pink colour. Moreover, the discoloration of resazurin was not qualitatively value, as showed in **Figure 4.1** many wells were in purple colour. Therefore, the percentage of cell viability is needed in order to give the qualitative value on the activities of the fungal extract. The UV absorption of each sample is read at 600 nm and compared against the UV absorption of the control media (O'Brien, Wilson *et al.*,2000). Results are given as a percentage of cell viability (Lang, Mitova *et al.*,2006)The activities were represented in percentage of cells viability, which can be calculated as the following equation;

$$100 - \left(\frac{\text{[sample OD - cell control (with dye) OD]}}{\text{[media control OD - cell control (with dye) OD]}} \text{ X } 100 \right)$$

Crude extracts which gave the value if cell viability less than 20% were considered as "active extract". In the other hand, extracts that gave the percentage of cell viability between 21-50% were considered as "moderate active", and will considered as "inactive" when the cell viability is > 50%. The calculation values of the percentage cell viability of extracts in plate number 3 (**Figure 4.1**) was showed in **Table 4.1**.

Table 4.1: Percentage of cell viabilities of *Salmonella* Typhimurium after treated with fungal extracts.

	1	2	3	4	5	6	7	8	9	10	11	12
A	10.4	9.5	90.2	80.0	4.8	5.5	8.7	8.6	9.2	12.1	8.5	10.6
B	8.2	4.4	7.7	14.8	11.4	9.2	9.2	10.2	9.9	9.6	56.1	10.6
C	2.1	9.3	7.2	10.2	4.2	8.3	7.2	12.0	14.0	10.3	9.5	9.8
D	9.0	9.0	13.5	8.4	8.0	12.3	8.1	13.1	13.5	11.7	17.0	10.0
E	5.6	8.6	39.8	11.4	3.9	9.6	7.7	6.6	9.0	5.6	9.4	9.0
F	9.0	7.4	8.5	7.4	6.7	4.1	7.2	5.6	7.2	9.1	10.9	10.4
G	17.5	9.3	11.2	11.9	14.0	11.9	10.1	6.9	-2.4	-2.4	-5.1	-1.3
H	2.098		0.72	0.13	-0.8	-4.2	-1.7	-3.3				

The calculation results were conformed to the discoloration in **Figure 4.1**, for example, in wells A3-4 which is pink colour and the percentage of cell viabilities were 90.2 and 80.0%. The best inhibition activities from this plate assay were found in wells G9-12, where no growth observed (negative value of the percentage of cell viability).

The summary number of extracts which exhibited the activities against each bacterium was given in **Table 4.2**.

Table 4.2: Summary of the number of extracts activities

Activities	Number of extracts showed activities against each bacteria							
	B. cereus	*B. subtilis*	*M. luteus*	*S. aureus*	*P.aerug inosa*	*E.coli*	*Sa.*typhi murium	*Ser. macescens*
Active	19	14	1	11	4	0	37	0
Moderate	2	0	6	0	2	7	0	1
Inactive	49	56	63	59	64	63	33	69

From the data in **Table 4.2**, fungal extracts were deemed to be good candidates for the source of bioactive compounds as they showed a potent inhibition against many bacterial strains. In this research fungal extracts, however, did not showed potent activities against *E.coli* and *Serratia marcescens*. The activities of each extract against each bacterium are given in **Appendix II**. Furthermore, if we look closely at the activities of each extract (see **Table 4.3**), it showed different patterns towards each bacterial strain. It can imply that, the compounds in crude extracts were specific active against each bacterial strain.

Table 4.3: Activities of crude extracts from fungal *Diaporthe* sp.against bacterial strains in the quick screen assay.

Fungal code	Media	*Cell viability <20% = 1 , 21-50% = 0.5, >50% = 0							
		Extract Code	*B. cer*	*B. sub*	*M. luteus*	*St. aureus*	*P.aeru*	*E.coli*	*Sa.*Typh	*Ser. mace*
DPH-002	MYPA	B0003	1	1	0	1	0	0	1	0.5
	¼SDA	B0004	1	1	0	0	0	0	1	0
DPH-003	MYPA	B0005	0	0	0	0	0	0	1	0
	¼SDA	B0006	0	0	0	0	0	0	1	0
DPH-004	MYPA	B0007	0	1	1	0	0	0	1	0
	¼SDA	B0008	0	0	0.5	0	0	0	1	0
DPH-006	MYPA	B0009	0	0	0.5	0	0	0	1	0
	¼SDA	B0010	1	1	0.5	1	0	0	1	0
DPH-008	MYPA	B0011	0	0	0	0	0	0	1	0
	¼SDA	B0012	0	0	0.5	1	0	0	0	0

*Note: The cell viabilities of each bacterium were change into a score.

Moreover, some extracts which obtained from the same fungus but was cultivated on different medium yielded different patterns of inhibition towards different bacterial strain. For example, from **Table 4.3**, extracts from the fungus *Diaporthe pterocarpi* (DPH-006); extract B0009; which was obtained from isolate cultivated on MYPA medium, and B0010; which was obtained from isolate cultivated on ¼SDA medium, showed different activities pattern against various bacterial strains. Furthermore, extract of this fungus obtained from ¼SDA medium showed better activities than extract from MYPA medium. More experiment such as HPLC (High Pressure Liquid Chromatography) analysis, however, is needed for a comparison on the pure compounds presence in each extract. This would not only confirm on the

compounds presence in the crude extracts, but also the amount of each pure compounds presence in the extract as well.

4.2 Purification and structure elucidation on selected active crude extracts

From the results above, fungal species *Diaporthe pterocarpi* (DPH-006) which yield crude extracts number B0009 and B0010 shows a potent activity against various bacterial strains. This fungus was subjected for upscale production in both MYPA and ¼SDA. Crude extracts (B0009 and B0010) were screening on a TLC plate and found three main major compounds. These three main compounds were identical in both extracts, hence, both extract were combine together (total of 147.6 mg) and named B0009A prior for separation the main compounds in a column chromatography.

After separation, three main compounds were yielded; B0009-B (26.3 mg), B0009-C (22.7 mg) and B0009-D (6.3 mg). Compound B0009-B, B0009-C and B0009-D were examined by NMR spectroscopy and the ^{1}H spectrums obtained as showed in **Figure 4.2 – 4.4** respectively.

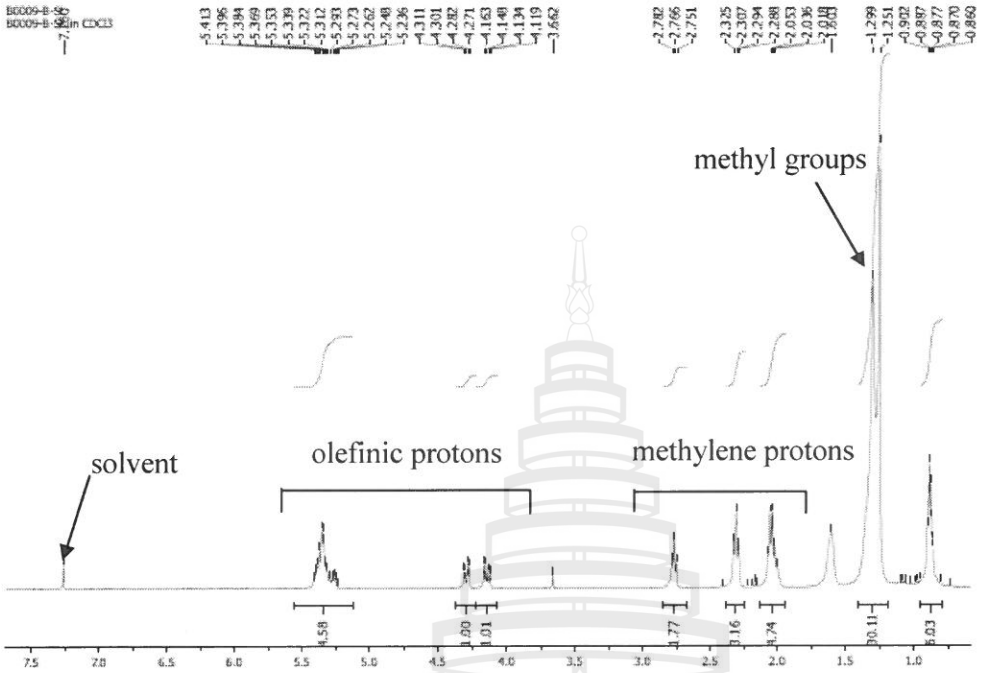


Figure 4.2: The ^{1}H NMR spectrum of B0009-B in $CDCl_3$, recorded at 400 MHz.

From the ^{1}H spectrum obtained, fraction B0009-B showed the presence of methyl groups at δ_H 1.26 and methylene protons at δ_H 2.03-2.30. The olefinic protons exhibited at δ_H 4.1-4.3 and δ_H 5.25-5.5 (**Figure 4.2**). From the ^{1}H NMR spectrum, this compound could be an unsaturated fatty acid. This should further confirm by an Infrared (IR) spectroscopy to confirm the functional group of the compound, the ^{13}C spectrum to determine the number of carbon in the compound as well as confirmed the molecular weight by Mass Spectroscopy (MS) analysis. In this study, however, after separation did not obtain enough amount of pure compound to run further NMR analysis such as correlation spectroscopy (COSY), heteronuclear multiple bond correlation spectroscopy (HMBC) and heteronuclear single quantum coherence (HSQC). These chemical analyses data would help the elucidation on the chemical structure. Up scaling on the fungal cultivation would be necessary for the future work, in order to obtain enough amount of crude extract for separation and purification on the pure compounds for the further chemical analyses and biological assays of each pure compound.

The ^{1}H NMR spectrum of fractions B0009-C and B0009-D (**Figure 4.3 and Figure 4.4**), however, were impure compounds.

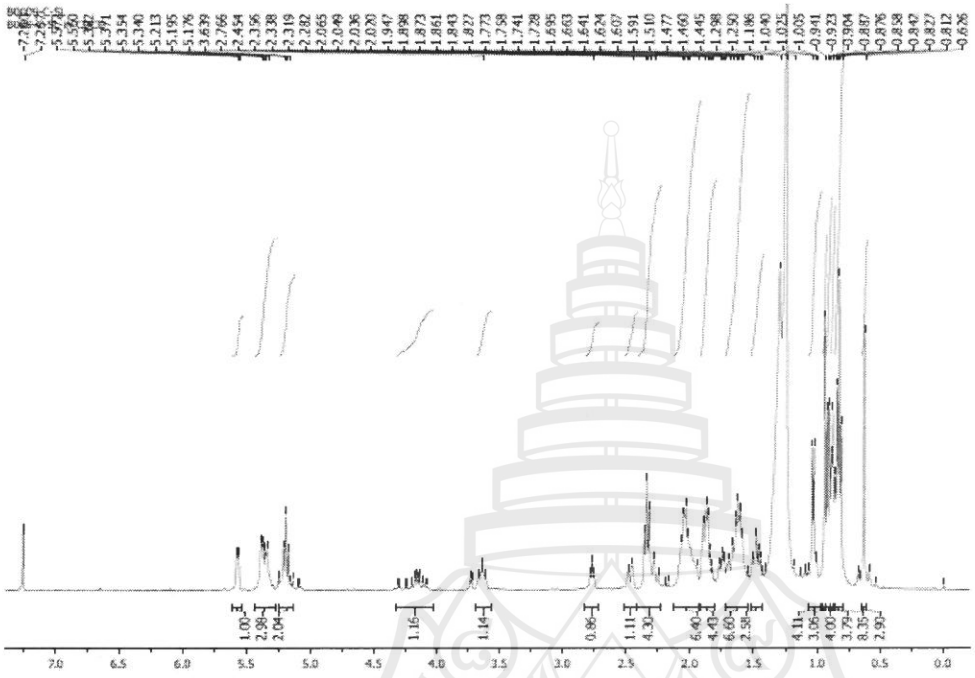


Figure 4.3: The ^{1}H NMR spectrum of B0009-C in $CDCl_3$, recorded at 400 MHz.

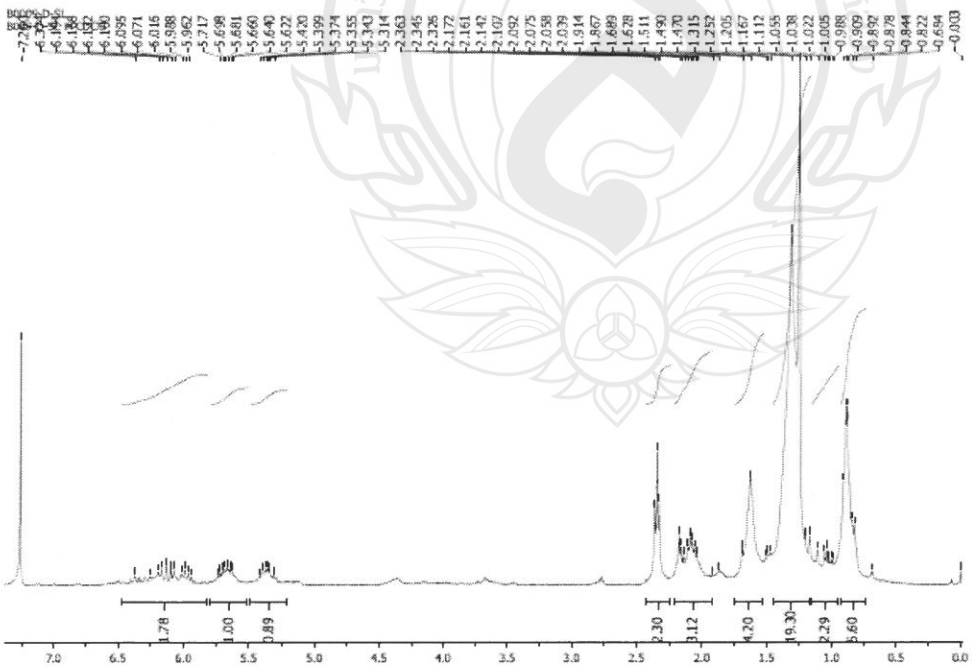


Figure 4.4: The ^{1}H NMR spectrum of B0009-D in $CDCl_3$, recorded at 400 MHz.

4.3 Discussion

The results from antimicrobial (AM) quick screen assays showed that of the 70 extracts, 28 extracts displayed no activities in any AM quick screen assays and were considered as inactive extracts. Furthermore, extracts which displayed the inhibition of the cell viabilities >50% were also considered as inactive. This implication might not all correct as these extracts might possess activities at the higher concentration or possess other than assays that were utilized at Mae Fah Luang University (Davies and Ryan,2012).

The quick screening is a rapid method for selection on the active fungal isolates. Those fungal extracts which were active against bacteria (<20% cell viability) were subjected for pure compounds isolation in each crude extracts. Furthermore, those fungi which produced active crude extracts were considered as good candidates for up scaling to yield the fungal extract and for manipulation on metabolites production. Minimal Inhibition Concentration (MIC) experiment is needed for further studies on both active extracts and on pure compounds; this is to define the lowest concentration of the extracts that will inhibit the visible growth of bacterial strains.

In this experiment, two media were used for cultivation each fungal isolate, and the extracts yielded from each fungal isolates showed different patterns against bacteria. One assumption is that the shifting on the secondary metabolites production of microorganisms was occurred when the cultivation parameters; such as media composition, pH value, and temperature were altered (Bode, Bethe *et al.*,2000; Grond, Papastavrou *et al.*,2002; Christian, Compton *et al.*,2005). It would be best to confirm the on metabolite productions pattern when the fungal were grown on different by HPLC analysis.

The main compound; B0009-B, which was the major compound from the crude extract exhibited a strong microbial activity (~20% cell viability) against *Staphylococcus aureus* and moderate activity (~50% cell viability) against *Micrococcus leuteus* in the antimicrobial quick screen assays. This compound deems to be a major compound related to the antimicrobial activities in the crude extract. Nevertheless, the

determination on the minimum inhibitory concentration (MIC) is needed to verify the activities of this pure compound. For compounds B0009-C and B0009-D which were a mixture, a further purification is needed to obtain a pure compound for further NMR analysis and antimicrobial screening test.

Chapter 5: Conclusion and Future works

The aim of this investigation mainly was to discover which of the fungal isolates was producing bioactive compounds, especially on antibacterial activity. Fungal extracts were subjected to test on their antimicrobial activities against eight bacterial strains; *Bacillus cereus*, *Bacillus subtilis*, *Micrococcus luteus*, *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Salmonella* Typhimurium and *Serratia marcescens.* Of the 70 fungal crude extracts, 42 extracts showed activity against at least one bacterial strain from eight strains in the AM quick screen assays. Only 28 extracts (40% of total extracts) displayed no activities in any AM quick screen assays.

Extracts from fungus species *Diaporthe pterocarpi*, showed good activities against many bacterial strains in the AM quick screen, and hence this fungus was subject for up-scale cultivation in order to get the appropriate amount of crude extracts in purification for NMR analysis. Three factions of the main compounds (B0009-B, B0009C and B0009-D) were yield from purification by column chromatography and these three fractions were subjected for NMR analysis. The ^{1}H NMR spectrum showed that fraction B0009-B could be an unsaturated fatty acid, while fractions B0009-C and B0009-D were a mixtures. Further purification is needed for these two fractions.

From the AM quick screen assay, fungi in phylum Ascomycota are good candidates in the production of valuable commodities such as antibiotics. For the further studies, the work on determining of the Minimal Inhibitory Concentration (MIC) or the assay to determine the concentration required to inhibit the growth of bacterial cultures by 50% (IC_{50}) of crude extracts is needed. For those active crude extracts, an isolation of pure compounds in the crude extract and AM quick screen is needed to define which compound is active in the test. Furthermore, it would be essential for each active pure compound to test on the MIC; this is to define the lowest amount of pure compound needed to inhibit the growth of each bacterial strain. (Davies and Ryan,2012) The studies on the manipulating metabolites production from active

fungal isolates are another option for the future work; this could elevate metabolites production of the fungus by influencing the biosynthetic pathways.

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Appendix I

Media used in cultivation and bioassay

Media for cultivation method

Malt yeast peptone agar (MYPA)

12.0 g malt extract (Oxoid), 1.8 g peptone (Oxoid), 0.6 g yeast extract (Oxoid) and 9.0 g bacteriological agar (Oxoid) were dissolved in 600 mL distilled water. The medium pH was measured and recorded as pH 6.1 (±0.1) prior to sterilization

¼ strength Sabouraud dextrose agar (¼ SDA)

3.9 g Sabouraud dextrose agar (Oxoid), 0.6 g yeast extract (Oxoid) and 9.0 g bacteriological agar (Oxoid) were dissolved in 600 mL distilled water. The medium pH was measured and recorded as pH 5.7 (±0.1) prior to sterilization.

Media for bioassay

Nutrient agar (NA)

8.0 g Nutrient powder (oxiod) and 9.0 g bacteriological agar (Oxoid) were dissolved in 600 mL distilled water prior to sterilization.

Nutrient broth (NB)

8.0 g Nutrient powder (oxiod) were dissolved in 600 mL distilled water prior to sterilization.

Appendix II

Summary of activities of crude extracts against bacterial strains in the quick screen assay

Note: The percentage of cell viability were interpreted in mark as followed: Cell viability <20% = 1 , 21-50% = 0.5, >50% = 0

Fungal code/ species	Media	Code	Activities mark interpreted from % cell viability; cell viability <20% = 1 , 21-50% = 0.5, >50% = 0							
			B. cereus	*B.subtilis*	*M. luteus*	*St. aureus*	*P.aeruginosa*	*E.coli*	*Sa.typhimurium*	*Ser. macescens*
C. globosum	MYPA	A0001	0	0	0	0	0	0	0	0
	SDA	A0002	0	0	0.5	0	0	0	0	0
Xylaria	MYPA	A0003	0	0	0	0	0	0	0	0
	SDA	A0004	0	0	0	0	0	0	0	0
Alternaria	MYPA	A0005	1	0	0	0	0.5	0	0	0
	SDA	A0006	0	0	0	0	0	0	0	0
Collectrotrichum	MYPA	A0007	0	0	0	0	0	0	0	0
	SDA	A0008	0	0	0	0	0	0	0	0
S19	MYPA	A0013	0	0	0	0	0	0	0	0
	SDA	A0014	0	0	0	0	0	0	0	0
SS011	MYPA	A0015	0	0	0	0	0	0	0	0
	SDA	A0016	0	0	0	0	0	0	0	0
DPH131-A	MYPA	A0017	0	0	0	0	0	0	0	0
	SDA	A0018	0	0	0	0	0	0	0	0
S09	MYPA	A0020	0	0	0	0	0	0	0	0

Fungal code/ species	Media	Code	Activities mark interpreted from % cell viability; cell viability <20% = 1 , 21-50% = 0.5, >50% = 0							
			B. cereus	*B.subtilis*	*M. luteus*	*St. aureus*	*P.aeruginosa*	*E.coli*	*Sa.typhimurium*	*Ser. macescens*
	SDA	A0021	0	0	0	0	0	0	0	0
S16	MYPA	A0022	0	0	0	0	0	0	0	0
	SDA	A0024	0	0	0	0	0	0	0	0
NTCL013	MYPA	A0025	0	0	0	0	0	0	0	0
	SDA	A0026	0	0	0	0	0	0	0	0
NTCL-041-1	MYPA	A0027	0	0	0	0	0	0	0	0
	SDA	A0028	0	0	0	0	0	0	0	0
NTCL 077	MYPA	A0029	0	0	0	0	0	0	0	0
	SDA	A0030	0	0	0	0	0	0	0	0
DPH-140	MYPA	A0039	0	0	0	0	0	0	0	0
	SDA	A0040	0	0	0	0	0.5	0	0	0
NTCL-015	MYPA	A0041	0	0	0	0	0	0	0	0
	SDA	A0042	0	0	0	0	0	0	0	0
DPH-104	MYPA	A0057	0	0	0	0	0	0	0	0
	SDA	A0058	0	0	0	0	0	0	0	0
DPH-001	MYPA	B0001	0	0	0	0	0	0	1	0
	SDA	B0002	0	0	0	0	0	0	0	0
DPH-002	MYPA	B0003	1	1	0	1	0	0	1	0.5
	SDA	B0004	1	1	0	0	0	0	1	0
DPH-003	MYPA	B0005	0	0	0	0	0	0	1	0
	SDA	B0006	0	0	0	0	0	0	1	0
DPH-004	MYPA	B0007	0	1	1	0	0	0	1	0
	SDA	B0008	0	0	0.5	0	0	0	1	0
DPH-006	MYPA	B0009	0	0	0.5	0	0	0	1	0

Fungal code/ species	Media	Code	Activities mark interpreted from % cell viability; cell viability <20% = 1 , 21-50% = 0.5, >50% = 0							
			B. cereus	*B.subtilis*	*M. luteus*	*St. aureus*	*P.aeruginosa*	*E.coli*	*Sa.typhimurium*	*Ser. macescens*
DPH-008	SDA	B0010	1	1	0.5	1	0	0	1	0
	MYPA	B0011	0	0	0	0	0	0	1	0
	SDA	B0012	0	0	0.5	1	0	0	0	0
DPH-013	MYPA	B0013	0.5	0	0	1	0	0	1	0
	SDA	B0014	1	0	0	0	1	0	1	0
DPH-014	MYPA	B0015	0	0	0.5	0	1	0	1	0
	SDA	B0016	0	0	0	0	1	0	1	0
DPH-015	MYPA	B0017	1	0	0	1	1	0	1	0
	SDA	B0018	0	0	0	1	0	0	1	0
DPH-101	MYPA	B0019	0	0	0	0	0	0	1	0
	SDA	B0020	1	0	0	0	0	0	1	0
DPH-106	MYPA	B0021	1	0	0	0	0	0.5	1	0
	SDA	B0022	0.5	1	0	1	0	0	1	0
DPH-111	MYPA	B0023	0	0	0	1	0	0	1	0
	SDA	B0024	0	0	0	0	0	0	1	0
OP-013	MYPA	B0025	0	0	0	1	0	0	1	0
	SDA	B0026	1	0	0	0	0	0	0	0
OP-084	MYPA	B0027	1	1	0	0	0	0	1	0
	SDA	B0028	1	0	0	0	0	0.5	1	0
OP-111	MYPA	B0029	1	0	0	0	0	0.5	1	0
	SDA	B0030	1	0	0	0	0	0	1	0
OP-118	MYPA	B0031	1	1	0	1	0	0	1	0
	SDA	B0032	1	1	0	0	0	0	1	0
OP-139	MYPA	B0033	1	1	0	0	0	0	1	0

Fungal code/ species	Media	Code	Activities mark interpreted from % cell viability; cell viability <20% = 1 , 21-50% = 0.5, >50% = 0							
			B. cereus	*B.subtilis*	*M. luteus*	*St. aureus*	*P.aeruginosa*	*E.coli*	*Sa.typhimurium*	*Ser. macescens*
	SDA	B0034	1	1	0	0	0	0.5	1	0
11-0471	MYPA	B0035	1	0	0	0	0	0.5	1	0
	SDA	B0036	0	1	0	0	0	0.5	1	0
BN-1006	MYPA	B0037	0	1	0	0	0	0	1	0
	SDA	B0038	1	1	0	0	0	0	1	0
DRL-131A	MYPA	B0039	0	1	0	1	0	0	1	0
	SDA	B0040	0	0	0	0	0	0.5	1	0
	Viability	Total	70	70	70	70	70	70	70	70
	<20%		19	14	1	11	4	0	37	0
	21-50%		2	0	6	0	2	7	0	1
	>50%		49	56	63	59	64	63	33	69

ประวัตินักวิจัย

หัวหน้าโครงการวิจัย (สัดส่วนทำวิจัย 90%)

1. ชื่อ- สกุล (ภาษาไทย) นางสาวสุนิตา แจ่มยวง

(ภาษาอังกฤษ) Miss Sunita Chamyuang

2. รหัสประจำตัวนักวิจัยแห่งชาติ หรือ รหัสบัตรประจำตัวประชาชน

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3. ตำแหน่งปัจจุบัน หน่วยงานที่อยู่ที่ติดต่อได้สะดวก

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4. ประวัติการศึกษา

คุณวุฒิ	สาขาวิชา	สถาบัน	ปีที่จบ
ปริญญาเอก	Microbiology	The University of Canterbury	2010
ปริญญาตรี	Biological Science	The University of Canterbury	2005

5. ประวัติการทำงาน

ปีที่ทำงาน	สถานที่ทำงาน	ตำแหน่ง
2006-2010	The University of Canterbury	ผู้ช่วยสอน
2010-ปัจจุบัน	มหาวิทยาลัยแม่ฟ้าหลวง	อาจารย์

6. สาขาวิชาการที่มีความชำนาญพิเศษ (แตกต่างจากวุฒิการศึกษา) ระบุสาขาวิชาการ

Secondary metabolites from fungi

7. ประวัติการนำเสนอผลงานวิจัยทั้งภายในและภายนอกประเทศ

Conference presentation:

- Chamyuang. S (2006) "*Application Of Selective Methods In The Search For New Bioactive Natural Products From Fungi*" School of Biological Sciences, University of Canterbury, Christchurch New Zealand, October 2006. Departmental seminar
- Blunt, J., Chamyuang. S., Cole. A., Hickford. S., Lang. G., Mahyudin N. A., Mitova. M., Munro M., van der Sar. S., Sun. L. and Yusof, M. T. (2007). "*Simplifying Complexity: The HPLC-Bioactivity-NMR Approach to the Dereplication of Natural Product Extracts*". Proceedings of the Xth International Marine and Freshwater Mycology Symposium (IMFMS), Pulau Pinang, 2 – 6 December 2007. Oral Presentation
- Sun, .L., Mahyudin, N.A., Chamyuang, S., Blunt, J., Cole, A., Lang, G., Mitova, M., van der Sar, S. and Munro M. (2007) "*Less is More: Dereplication and Discovery Using CapNMR Techniques.*" 12th International Symposium on Marine Natural Products (MaNaProXII), Queenstown, New Zealand, 4 - 9 Feb 2007. Poster Presentation
- Chamyuang. S., Cole, A., Munro, M, Blunt, J. and Lin, S. (2008) "*Application of a selective method in the search for new bioactive natural products from fungi.*" Athens, Greece: 7th Joint Meeting of AFERP, ASP, GA, PSE and SIF, 3-8 Aug 2008. Poster Presentation
- Chamyuang. S. (2008) "*Studies on Bioactive metabolites from Fungi*" School of Biological Sciences, University of Canterbury, Christchurch New Zealand: Annual Biology Colloquium for Doctoral Students (ABCD), October 2008. Oral Presentation
- Chamyuang. S., Cole, A., Munro, M, Blunt, J. and Lin, S. (2008) "*Application of a selective method in the search for new bioactive natural products from fungi.*" Dunedin, New Zealand: Chemistry and the Biosphere Conference, 30 Nov-4 Dec 2008. Poster Presentation
- Blunt, J., Chamyuang. S., Cole. A., Mahyudin N. A., Munro M. and Sun. L. (2009) "*Marine Natural Products: From There to Here*" Porto, Portugal: 6PthP European Conference on Marine Natural Products, 19-23 July 2009. Oral Presentation
- Blunt, J., Chamyuang. S., Cole. A., Mahyudin N. A., Munro M. and Sun. L. (2009) "*Reducing Complexity: the Gentle Art of Dereplication*" Seoul, Korea: 2009 CMDD Synposium, 16-18 September 2009. Oral Presentation

ผู้ร่วมโครงการวิจัย (สัดส่วนทำวิจัย 10%)

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1996 – 1999 PhD in Biochemistry, Research School of Biosciences, University of Kent at Canterbury, UK; Project title "Evolution of CUG codon reassignment in *Candida* species" with Prof. Mick Tuite

1995 – 1996 MSc in Biotechnology, University of Kent, UK; Project title "Cloning of Ser-tRNA^{CAG} genes from various *Candida* species and expression in *Saccharomyces cerevisiae*" with Prof. Mick Tuite

1990 – 1994 BSc (First Class Hons.) in Biology, Department of Biology, Faculty of Science, Chiang Mai Univeristy, Thailand; Project title "Lactic acid production by starch-utilising lactic acid bacteria" with Assoc. Prof. Dr. Saisamorn Lumyong

Awards and Scholarships:

1995 – 1999 Postgraduate studentship sponsored by the DPST project to pursue MSc/PhD study aboard

1998 Travel grant from the Genetics Society of America (GSA), Bethesda, MD, USA (for Yeast Genetics and Molecular Biology Meeting); from the organising committee, University of Crete, Heraklion, Greece (for Evolutionary Biology Meeting); from Department of Biosciences, University of Kent, UK (for Translation UK)

1994 Prof. Dr. Dhab Nelanithi Foundation Award

1990 – 1994 Studentship under the DPST project, Chiang Mai University

Work experiences:

2000 – present Lecturer, Mae Fah Luang University

Academic committee for Undergraduate and Postgraduate Programme in Biotechnology, Mae Fah Luang University

2001 – 2002 Postdoctoral Fellow, Department of Applied Chemistry, Faculty of Engineering, Oita Univeristy, Japan

1995 – 1999 Teaching Assistance in the following undergraduate practical:

Nucleic Acids and Proteins, Enzyme Kinetics, Gene Cloning, Yeast Mutagenesis, Immunology, Microbiology, UKC, UK

Selected publications:

Dajanta K, Apichartsrangkoon A and Chukeatirote E. 2011. Free-amino acid profiles of thua nao, a Thai fermented soybean. Food Chemistry 125: 342–347.

Dajanta K, Apichartsrangkoon A and Chukeatirote E. 2011. Volatile profiles of thua nao, a Thai fermented soy product. Food Chemistry 125: 464-470.

Dajanta K, Chukeatirote E and Apichartsrangkoon A. 2011. Analysis and characterisation of amino acid contents of thua nao, a traditionally fermented soybean food of Northern Thailand. International Food Research Journal 18: 588-592.

Wikee S, Cai L, Noireung P, McKenzie EHC, Su YY, Chukeatirote E, Thi HN, Bahkali AH, Moslem MA, Abdelsalam K and Hyde KD. 2011. *Colletotrichum* species from Jasmine (*Jasminum sambac*). Fungal Diversity 46: 171–182.

Chukeatirote E, Dajanta K and Apichartsrangkoon A. 2010. *thua nao*, indigenous Thai fermented soybean: a review. Journal of Biological Sciences 10: 581-583.

Phengsintham P, Chukeatirote E, Abdelsalam KA, Hyde KD and Braun U. 2010. **Cercospora** and allied genera from Laos 2. Cryptogamie Mycologie 31: 161-181.

Chang-ngern P, Sardsud U, Pathom-aree W, Chantrasri P and Chukeatirote E. 2010. Diversity of moulds in fresh longan. Agricultural Science Journal 41 (1S): 322-324.

Jannok P, Apichartsrangkoon A and Chukeatirote E. 2010. Effect of ultra-high pressure on physical, chemical and microbiological qualities of pennywort juice. Food 40: 71-79. (in Thai)