



**BIOACTIVE COMPOUNDS OF ENDOPHYTIC FUNGI ISOLATED
FROM *Cinnamomum loureiroi*, *Polyscias fruticosa*,
Barleria prionitis, AND ESSENTIAL OIL
FROM *Cuminum cyminum***

CHUTIMA TANAPICHATSAKUL

**DOCTOR OF PHILOSOPHY
IN
APPLIED CHEMISTRY**

**SCHOOL OF SCIENCE
MAE FAH LUANG UNIVERSITY**

2021

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2021

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Chutima Tanapichatsakul



Thesis Title	Bioactive Compounds of Endophytic Fungi Isolated from <i>Cinnamomum loureiroi</i> , <i>Polyscias fruticosa</i> , <i>Barleria prionitis</i> , and Essential Oil from <i>Cuminum cyminum</i>
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ABSTRACT

This research aimed to investigate the secondary metabolites and biological activities of endophytic fungi and the selected plant. The endophytic fungi were isolated from selected plants: *Cinnamomum loureiroi*, *Polyscias fruticosa*, *Barleria prionitis*, while the selected plant is *Cuminum cyminum*. The obtained results are divided into four parts according to their different activities.

Firstly, 11 endophytic fungi were isolated from healthy *C. loureiroi* leaves. All endophytic fungi of *C. loureiroi* were cultured for one month prior to analysis of their antioxidant and antibacterial activity against *Bacillus cereus* and *Staphylococcus epidermis*, and the chemical composition. Crude extracts of MFLUCC15-1130 and MFLUCC15-1131 showed the lowest minimum inhibitory concentration at 3.91 µg/mL. Significant antioxidant activity was detected in the crude extract of fungus MFLUCC15-1130 and MFLUCC15-1131 with half-maximal inhibitory concentration (IC₅₀) of 22.92 ± 0.67 and 37.61 ± 0.49 µg/mL, respectively. The phylogenetic study

suggested MFLUCC15-1130 and MFLUCC15-1131 endophytes is identified as were identified as *Neopestalotiopsis* sp. and *Diaporthe* sp., respectively. The chemical composition of both crude extracts analyzed by gas chromatography-mass spectrometry (GC-MS) revealed eugenol, myristaldehyde, lauric acid, and caprylic acid as major components.

Secondly, 34 endophytic fungi isolates were isolated from the healthy leaves of *P. fruticosa*. *In vitro* antagonism of endophytic fungi isolates against the pathogenic fungus, *Athelia rolfsii* on tomatoes plant using a dual culture assay. The efficiency of different solvents for the extraction was determined using ethyl acetate, methanol, and hexane. Each crude extract was tested for antifungal activity. Especially, the hexane crude extract was showed the highest inhibition percentage. The phylogenetic study suggested MFLUCC17-0313 endophytes is identified as *Diatrype palmicol*. The bioactive compound was further purified using bioassay guidance. The bioactive compound was identified as 8-methoxynaphthalen-1-ol. The minimum inhibition concentration of 8-methoxynaphthalen-1-ol was 250 µg/mL against *A. rolfsii* and represented no phytotoxic effect on tomato leaves.

Thirdly, 34 endophytes were isolated from healthy leaves of *B. prionitis*. Antibacterial properties of all crude extracts were evaluated using disk-diffusion assay against human pathogenic Gram-positive and Gram-negative bacterial (*Staphylococcus aureus*, *S. epidermidis*, *Streptococcus pyogenes*, *Enterococcus faecium*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium*). The ethyl acetate crude extract of mycelia of isolate MFLUCC19-0492 was shown a significant inhibited growth all of bacteria with the lowest minimum inhibitory concentrations (MIC) values (0.39 mg/mL). The phylogenetic study suggested MFLUCC19-0492 endophytes is identified as *Diatrypella* sp. The cytotoxic activity was investigated by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) and 2,3-bis (2-methoxy-4-nitro-5-

sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) assays against Molt-3, HuCCA-1, A549 and HepG2 compared to doxorubicin and etoposide, reference drugs. The ethyl acetate crude extract of mycelia showed significantly inhibited the Molt-3, HuCCA-1, A549, and HepG2 with 100, 66, 71, and 54.17%, respectively. On the other hand, the ethyl acetate crude extract of mycelia of isolate MFLUCC19-0492 displayed cytotoxic activity.

Lastly, the essential oil derived from *C. cyminum* was tested their antifungal activity against *Aspergillus aculeatus*. The chemical composition of *C. cyminum* essential oil were also identified by GC-MS. The main components were cuminaldehyde (33.94%) and α -terpinen-7-al (32.20%). *C. cyminum* oil exhibited significant inhibition percentage of 95.08% against *A. aculeatus*. The effects of the reduction on conidia germination was detected in *C. cyminum* oil and cuminaldehyde treatment at a concentration of 1,000 and 100 $\mu\text{g/mL}$, respectively. *In vivo* assay, the results showed the decrease of the severity (0.42%) and incidence (1.67%) probability of *A. aculeatus* on grape tested at 1,000 $\mu\text{g/mL}$ of *C. cyminum* oils than other treatments. In addition, grape treated with *C. cyminum* oil had slightly decreased weight loss and retained fruit firmness. The quality of grape fruit treated with *C. cyminum* oil was delayed including total soluble solids, total phenolic content, and antioxidant activity.

Keywords: Antibacterial, Antifungal, Endophytic fungi, Essential oil, *Cinnamomum loureiroi*, *Polyscias fruticosa*, *Barleria prionitis*, *Cuminum cyminum*

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	(3)
ABSTRACT	(5)
LIST OF TABLES	(11)
LIST OF FIGURES	(13)
ABBREVIATIONS AND SYMBOLS	(16)
 CHAPTER	
1 INTRODUCTION	1
1.1 Overview of the Research	1
1.2 Objectives	2
1.3 Scope of Research	2
 2 LITERATURE REVIEW	 3
2.1 Plant of this work	3
2.2 Background of instruments	29
 3 PRODUCTION OF EUGENOL FROM FUNGAL ENDOPHYTES	
<i>Neopestalotiopsis</i> sp. AND <i>Diaporthe</i> sp. ISOLATED FROM	
<i>Cinnamomum loureiroi</i> LEAVES	38
3.1 Introduction	39
3.2 Materials and Methods	41
3.3 Results	47
3.4 Discussion	58
3.5 Conclusion	61
3.6 Supporting Information	62

TABLE OF CONTENTS (continued)

CHAPTER	Page
4 ANTIFUNGAL ACTIVITY OF 8-METHOXYNAPHTHALEN-1-OL ISOLATED FROM THE ENDOPHYTIC FUNGUS <i>Diatrype palmicola</i> MFLUCC 17-0313 AGAINST THE PLANT PATHOGENIC FUNGUS <i>Athelia rolfsii</i> ON TOMATOES	63
4.1 Introduction	64
4.2 Materials and Methods	66
4.3 Results	73
4.4 Discussion	81
4.5 Conclusion	83
4.6 Supporting Information	84
5 ANTIBACTERIAL AND CYTOTOXIC ACTIVITIES OF ENDOPHYTIC FUNGUS <i>Diatrypella</i> SP. MFLUCC 19-0492	89
5.1 Introduction	90
5.2 Materials and Methods	91
5.3 Results	97
5.4 Discussion	103
5.5 Conclusion	105
6 <i>In vitro</i> AND <i>In vivo</i> ANTIFUNGAL ACTIVITY OF <i>Cuminum cyminum</i> ESSENTIAL OIL AGAINST <i>Aspergillus aculeatus</i> CAUSING BUNCH ROT OF POSTHARVEST GRAPES	106
6.1 Introduction	107
6.2 Materials and Methods	109
6.3 Results	116
6.4 Discussion	133

TABLE OF CONTENTS (continued)

CHAPTER	Page
6.5 Conclusion	136
7 CONCLUSIONS	137
REFERENCES	138
APPENDIX	171
CURRICULUM VITAE	176



LIST OF TABLES

Table	Page
3.1 Antioxidant activity of endophytic fungal extracts and standard drugs, expressed as half maximal inhibitory concentration (IC ₅₀) values using 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay	45
3.2 Volatile compounds detected in crude fungal extracts of <i>Neopestalotiopsis</i> sp. MFLUCC15-1130 and <i>Diaporthe</i> sp. MFLUCC15-1131 using gas chromatography-mass spectrometry (GC–MS)	56
4.1 Phytotoxicity of compounds toward <i>S. lycopersicum</i> leaf disks at different concentrations after 18 h	78
4.2 Inhibition percentage of the all endophytic fungi against <i>A. rolfsii</i> from dual culture assay	88
5.1 Antibacterial activity of crude extracts of endophytic fungi isolated from <i>B. prionitis</i> leaves (50 mg/mL) and chloramphenicol (5 mg/mL) against <i>S. aureus</i> and <i>E. coli</i>	98
5.2 Antibacterial activity of crude extracts of ANG4 and chloramphenicol against tested human pathogens	100
5.3 Percentage of cytotoxicity of crude extracts of ANG4 (30 µg/mL), doxorubicin and etoposide against four human cancer cell lines	101
6.1 Antifungal activity of <i>C. cyminum</i> , <i>Z. bungeanum</i> Maxim., <i>C. sativum</i> , <i>Z. montanum</i> (J. Koenig) Link ex A. Dietr., and <i>Z. rhetsa</i> essential oils at different concentrations against <i>A. aculeatus</i> using contact phase assay	118
6.2 Volatile compounds of <i>C. cyminum</i> essential oil	123

LIST OF TABLES (continued)

Table	Page
6.3 Antifungal activity of cumin aldehyde and α -terpinen-7-al at different concentrations against <i>A. aculeatus</i> using contact phase assay	124
6.4 Quality of grape berries obtained from all treatments after 10 days	129
6.5 Quality of grape berries obtained from all treatments after 20 days	131



LIST OF FIGURES

Figure	Page
2.1 The leaves of <i>C. loureiroi</i> (A) The dry barks of <i>C. loureiroi</i> (B)	4
2.2 Polyphenolic compounds of cinnamon essential oil	6
2.3 The chemical structures of some important compounds isolated from cinnamon	7
2.4 <i>P. fruticosa</i> (L.) Harms	10
2.5 The leaves of <i>B. prionitis</i>	14
2.6 The dry seeds of cumin (A) The aerial parts of cumin plant (B)	22
2.7 Extraction methods of chemical composition from plants	24
2.8 Example of some compounds found in <i>C. cyminum</i>	25
2.9 Example of some phenols and flavonoids found in <i>C. cyminum</i>	26
2.10 Diagram of a permeably packed column and its idealisation, in which gas and adsorbed phases are represented separately	29
2.11 Schematic Diagram of GC Instrument	30
2.12 Schematic Diagram of GC-MS System	31
2.13 Schematic Diagram of HPLC System	33
2.14 Schematic Diagram of MS System	35
2.15 Energy Levels for a Nucleus with Spin Quantum Number	36
2.16 Two-dimensional J-resolved NMR spectra of phenylpropanoids of <i>B. rapa</i> leaves in the range of δ 6.25–6.50 (a); δ 5.32– 5.42 (b). The spectra were measured in methanol-d ₄	37
3.1 Antibacterial activities of crude fungal extracts and chloramphenicol against <i>B. cereus</i> (A), <i>S. aureus</i> (B), <i>S. epidermidis</i> (C), <i>E. coli</i> (D), <i>P. aeruginosa</i> (E), and <i>S. typhimurium</i> (F), expressed as an inhibition zone diameter and MIC	51

LIST OF FIGURES (continued)

Figure	Page
3.2 Antifungal activities of crude fungal extracts against <i>C. albicans</i> (A) and <i>A. niger</i> (B), expressed as percentage of radical growth inhibition and MIC	52
3.3 Phylogenic tree of <i>Neopestalotiopsis</i> sp. MFLUCC15-1130	53
3.4 Phylogenic tree of <i>Diaporthe</i> sp. MFLUCC15-1131	54
3.5 Colonies and morphologies of endophytic fungi isolated from <i>C. loureiroi</i>	62
4.1 Inhibition percentage of the all endophytic fungi against <i>A. rolfsii</i> from dual culture assay	74
4.2 Dual culture plate assay between some endophytic fungi against the pathogen <i>A. rolfsii</i>	76
4.3 Chemical structure of 8-methoxynaphthalen-1-ol	77
4.4 Symptoms caused by compounds toward <i>S. lycopersicum</i> leaf disks at different concentrations after 18 h	79
4.5 Phylogenic tree of <i>Diatrype palmicola</i> MFLUCC17-0313	80
4.6 Endophytic fungi were isolated from <i>P. fruticosa</i> leaves. All endophytic fungi were cultured in PDA at room temperature for two weeks	84
4.7 Antifungal activity of crude hexane extract (EXT), amphotericin B (drug), hexane solvent (Hex) and control against <i>A. rolfsii</i> for seven days using disk diffusion assay	85
4.8 Antifungal activity of some fractions and control against <i>A. rolfsii</i> for seven days using disk diffusion assay	85
4.9 Antifungal activity of pure compounds at different concentrations and amphotericin B (drug) against <i>A. rolfsii</i> for seven days using disk diffusion assay	86

LIST OF FIGURES (continued)

Figure	Page
4.10 Symptoms caused by compounds toward <i>S. lycopersicum</i> leaf disks at different concentrations after 18 h	87
5.1 Colony of endophytic fungi isolated from <i>B. prionitis</i> leaves	97
5.2 Phylogenetic tree of endophytic fungus <i>Diatrypella</i> sp. MFLUCC 19-0492	102
6.1 Diameter of <i>A. aculeatus</i> pathogen after treating (■) and without treating (■) with <i>C. cyminum</i> essential oil on medium agar plates	122
6.2 Effect of different concentrations of <i>C. cyminum</i> essential oil (A), cuminaldehyde (B), and α -terpinen-7-al (C) on conidia germination of <i>A. aculeatus</i>	125
6.3 <i>In vivo</i> assay of the antifungal activity of the <i>C. cyminum</i> essential, cuminaldehyde, and α -terpinen-7-al against <i>A. aculeatus</i> infections after 10 days	126
6.4 <i>In vivo</i> assay of the antifungal activity of the <i>C. cyminum</i> essential, cuminaldehyde, and α -terpinen-7-al against <i>A. aculeatus</i> infections after 20 days	127
6.5 Severity (■) and incidence (■) percentage of <i>A. aculeatus</i> in grape berries treated with different concentrations of <i>C. cyminum</i> essential oil (A and D), cuminaldehyde (B and E), and α -terpinen-7-al (C, F) after 10 days and 20 days, respectively	128

ABBREVIATION AND SYMBOL

ATCC	American type culture collection
°C	Degree of Celsius
CFU/mL	Colony forming unit per milliliter
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DNA	Deoxyribonucleic acid
DMST	Department of medical sciences Thailand culture collection
eV	Electron volt
et al.	And others
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
HPLC	High Performance Liquid Chromatography
IC ₅₀	Half Maximal Inhibitory Concentration
ITS	Internal transcribed spacer
MFLUCC	Mae Fah Luang University culture collection
MS	Mass Spectrometry
mL	Milliliter
mg/mL	Milligram per Milliliter
µg/mL	Microgram per Milliliter
µL	Microliter
mM	Millimolar
MIC	Minimum inhibiting concentration
MAFFT	Multiple alignment using fast Fourier transform
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NIST	National institute of standards and technology data base

ABBREVIATION AND SYMBOL (continued)

μL	Microliter
mM	Millimolar
MIC	Minimum inhibiting concentration
MAFFT	Multiple alignment using fast Fourier transform
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NIST	National institute of standards and technology data base
NMR	Nuclear Magnetic Resonance
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
RAxML	Randomized Axelerated maximum likelihood
RI	Retention index (retention indices)
spp.	Species (plural)
sp.	Species (singular)
sp. nov	Species nova (new specie)
TISTR	Thailand institute of scientific and technological research culturecollection
%v/v	Percent volume by volume
%w/v	Percent weight by volume

CHAPTER 1

INTRODUCTION

1.1 Overview of the Research

The search for new and more effective compounds from natural products is being needed for further developing drug discovery. Medical plants have extensively been used for pharmaceutical and therapeutic use and are promising candidates over the last decades. Recently, phytochemistry related to the secondary metabolites isolated from medicinal plants is outstanding for biological activity, including flavonoids, terpenoids, lignans, phenolic compounds, alkaloids, polyketides, isocoumarins, lactones, and peptides.

Fungal endophytes represent a complex community of microorganisms colonizing asymptotically internal reside of host plants without causing apparent disease symptoms. Several reports have shown that their sources defend themselves by direct production of bioactive secondary metabolites, which are involved in protecting against environmental stress. The potential secondary metabolites have a broad range of biological activities such as antifungal, anti-inflammatory, antimicrobial, antioxidant, antimicrobial, and anticancer properties, which have a wide range of biotechnological applications in the pharmaceutical, food, and agriculture industries. Furthermore, essential oils have possessed biological activities including, antibacterial, antifungal, antioxidant, anti-rheumatic, antiseptic, and antihelminthic properties. Over 90% of global essential oil production is developed in the food industry for flavoring or flavor, fragrance application, and agricultural industries. Most of these bioactive compounds have the biological potential to be discovered, this research investigated new sources of antimicrobial activity in Thailand which may be useful for applying in various applications.

1.2 Objectives

This research aimed to investigate endophytic fungi and also plants having potential of antimicrobial activity including antibacterial and antifungal activity against human and plant pathogens. The obtained crude extracts and essential oil were also tested their antioxidant and cytotoxicity. The chemical composition and bioactive compounds were analyzed using modern techniques in chemistry.

1.3 Scope of Research

This research divides into four parts according to four different samples including, *C. loureiroi*, *P. fruticose*, *B. prionitis*, and *C. cyminum*. Their chemical constituents and antimicrobial activity screening of crude extracts of fungal endophytes, isolated from *C. loureiroi*, *P. fruticosa*, *B. prionitis* leaves, and essential oil of *C. cyminum*. Firstly, fungal endophytes of *C. loureiroi* were isolated and identified their genera through morphology. All crude extract was further screened their antibacterial against *Bacillus cereus* and *Staphylococcus epidermis*. The chemical composition of crude extracts was characterized by Gas Chromatography - Mass Spectrometry (GC-MS). Second, fungal endophytes of *P. fruticose* leaves were isolated and tested *in vitro* antagonism against pathogenic fungus, *Athelia rolfsii* on tomato plants. Third, fungal endophytes of *B. prionitis* of healthy leaves were evaluated for antibacterial activity against human pathogenic Gram-positive and Gram-negative. The cytotoxicity was also investigated in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 2,3-bis-2-methoxy-4-nitro-5-sulfonylphenyl]-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) assays against T-lymphoblastic Molt-3, cholangiocarcinoma cells HuCCA-1, adenocarcinomic human alveolar basal epithelial cells A549, and liver cancer cells HepG2. Lastly, *C. cyminum* was subjected to hydrodistillation for extracting essential oil and then analyzed by GC-MS. Antibacterial activity of *C. cyminum* was screened against *A. aculeatus* which causes bunch rot disease on postharvest grapes.

CHAPTER 2

LITERATURE REVIEW

The literature reviews described the important role of selected plants (*Cinnamomum loureiroi*, *Polyscias fruticosa*, *Barleria prionitis*, and *Cuminum cyminum*) including, botanical description, medicinal application using, chemical constituents and its biological possessing. The isolation techniques were reviewed including, Gas Chromatography (GC), Gas Chromatography Mass Spectrometry (GC-MS), High Performance Liquid Chromatography (HPLC), Nuclear magnetic resonance spectroscopy (NMR), and Mass spectrometry (MS) which are conducted for isolation, purification, and elucidation of bioactive compound.

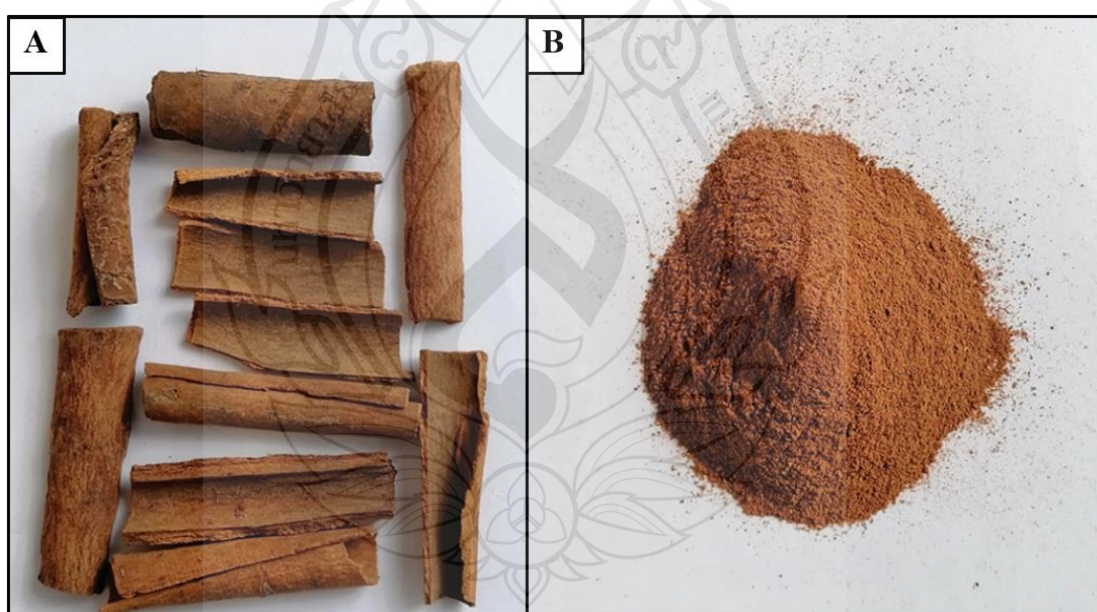
2.1 Plant of this work

2.1.1 *Cinnamomum loureiroi*

2.1.1.1 Botanical Description of *C. loureiroi*

Cinnamon is considered as an important genus of tropical evergreen aromatic trees and composed approximately 300 species throughout the world. Cinnamon belongs to the Lauraceae family. The cinnamon tree is consisting of strong branches, smooth, and yellow colour. Their leaves are dark green on top and light green at the bottom and its present the inconspicuous yellow flowers without any odour. The fruit is a tiny purple berry with a single seed, long that ripens to black, partly surrounded by a cup-like perianth. The taste of cinnamon is spice derived from the dried outer bark and scraping the inner bark. The dried inner bark of the coppiced shoots of cinnamon is one of the cultivations for harvesting (Rawat et al., 2019). Cinnamon is obtained from the dried bark of plant part derived from various species, including *Cinnamomum cassia*, *Cinnamomum burmannii*, *Cinnamomum zeylanicum*,

or related plant (Lee et al., 2010). There are four species are used domestically and industrially in the preparation of food, cosmetic, fragrance, and pharmaceuticals such as *Cinnamomum zeylanicum*, *Cinnamomum loureiroi*, *Cinnamomum burmannii*, and *Cinnamomum aromaticum* (Chen et al., 2014). *C. loureiroi* is synonyms Vietnamese cinnamon and cultivated mainly in Northern regions of Vietnam, Southern China, and Southern Thailand. The young bark of *C. loureiroi* is provided a traditional flavoring for food industries with a cassia-like flavour. *C. loureiroi* is most popular, and its preset sweet, spice, and freshness. Although, over 30% of oil content of *C. loureiroi* is greater than other varieties and it is much more powerful, and the overall of *C. loureiroi* and Chinese cassia cinnamon is similar flavor (Jiang et al., 2008). The quality of cinnamon depends on several factors such as cultivation, botanical source, seasonal climate conditions, harvesting, and production which lead to different taste and chemical constituents (Avula et al., 2015).



Source Kamber et al. (2020)

Figure 2.1 The leaves of *C. loureiroi* (A) The dry barks of *C. loureiroi* (B)

2.1.1.2 Medicinal Application

Cinnamon has been used over thousand years old both as a culinary spice and medicinal purposes including, wound healing, flatulence, aperitif, aromatic, leukorrhea, vaginitis, erectile, conjunctivitis, leukorrhea, hypertensive, tonic, antidiabetic, antinociceptive, and diuretic (Khan et al., 2014). The first application of this genus, the leaves of *C. rhynchophyllum* and *C. soegengii* were used for stomach ache and food poisoning (Samy, 2005). Similarly, the mucilage of *C. iners* is the second species that was treated for mosquito coils, scented joss sticks, and formica treatment, while the leaf juice of *C. iners* was used as a poultice by the Sakai ethnic group for rheumatism, generally used in Peninsular Malaysia (Burkill, 1996). The application for prevention fatigue and chest pain remedy is from the decoction of the third species, *C. javanicum* (Burkill, 1996). Moreover, the treating fever after childbirth was prevented by decoction of the roots of *C. iners*, *C. porrectum*, and *C. subcuneatum*. While, the pain relief on rheumatic joints were also protected by the roots of *C. subcuneatum* (Burkill, 1996). The last application species, the seed oil derived from *C. porrectum* can used for rheumatism. Besides, the other parts, the bark and fruits are also used as a scent for soap (Burkill, 1996).

2.1.1.3 Chemical Constituents and its Biological activity

In the past few decades, more than 160 phytochemical and pharmacology of cinnamon have been isolated and identified such as ferulic acids, caffeic, gallic, vanillic, protocatechuic and p-coumaric along with the polyphenols (Figure 2.2). Regarding to the volatile components, the chemical composition derived from cinnamon is depended on the plant part and also plant extraction. O-methoxycinnamaldehyde, cinnamic acid, cinnamaldehyde, cinnamate, cinnamyl acetate, α -thujen, terpineol, α -cubebene, eugenol, and coumarin are different classes of compound have been documented in Figure 2.3 (Mohamed et al., 2020). Over 70% of essential oil obtained from the bark cinnamon is Cinnamaldehyde that is the majorities and present antibacterial activity (Doyle & Stephens, 2019). Likewise, benzyl benzoate, β -caryophyllene, cinnamyl acetate, linalool and eugenyl acetate are classified as hydrocarbons and oxygenated compounds that are the minor volatile compounds in cinnamon. Nevertheless, eugenol is one of the great importance as the majorities derived from the cinnamon leaf oil, flower and fruit parts, and also

(E)-cinnamyl acetate and caryophyllene are present in these cinnamon parts (Kumar et al., 2019). According to a study, a few reports mention about the study on the diversity and bioactive compounds of fungal endophytes associated with *C. loureiroi*.

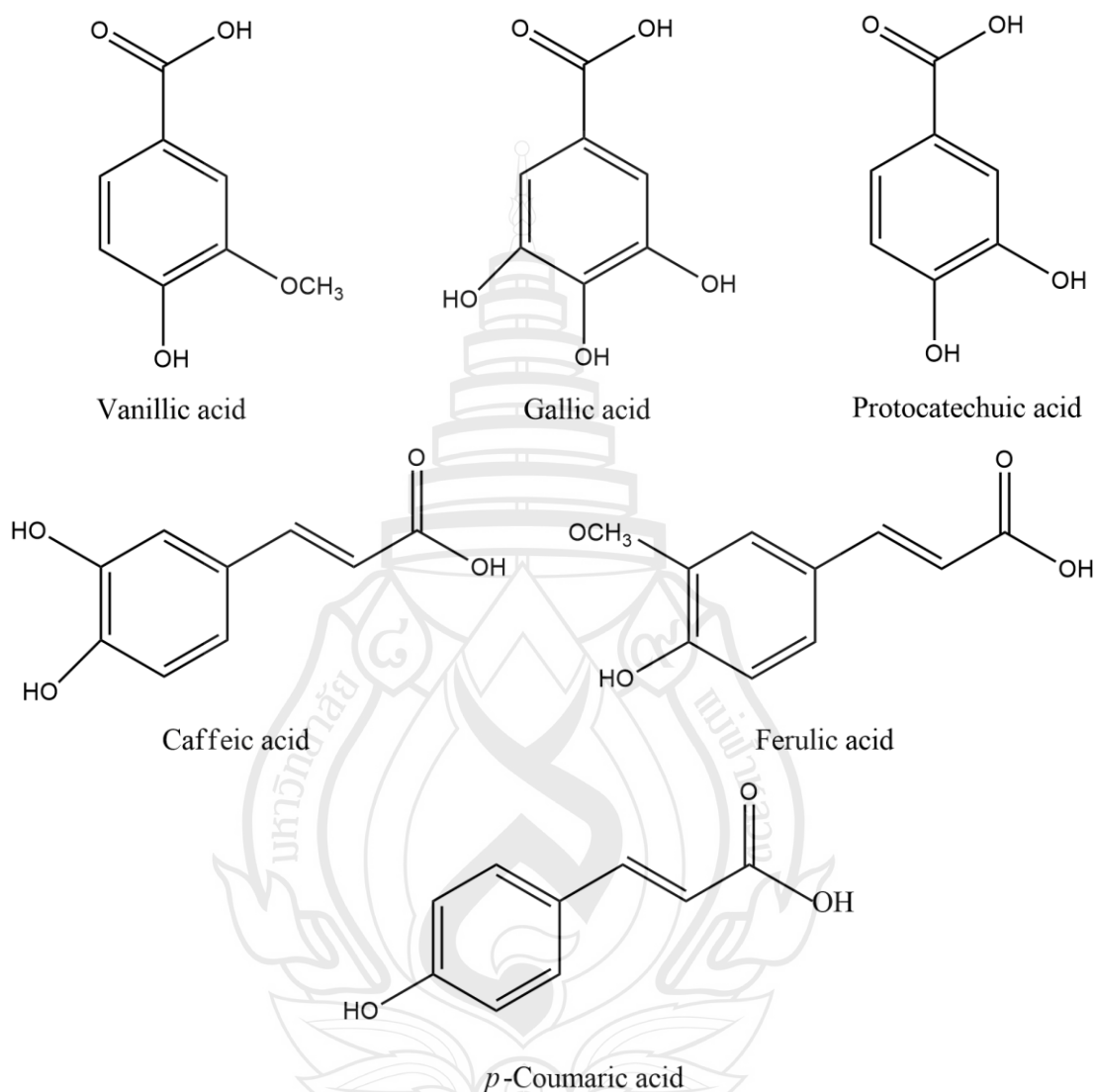


Figure 2.2 Polyphenolic compounds of cinnamon essential oil

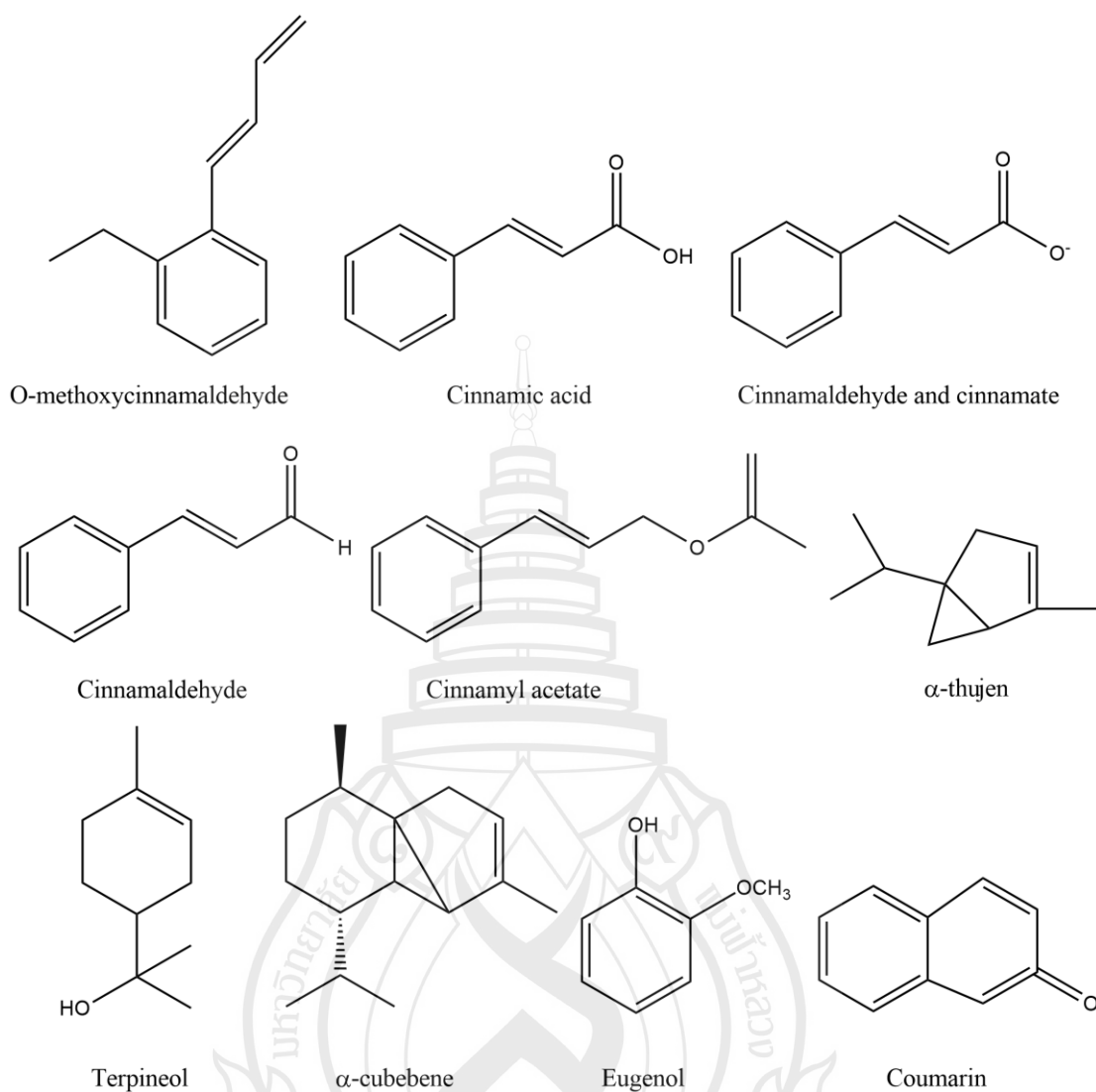
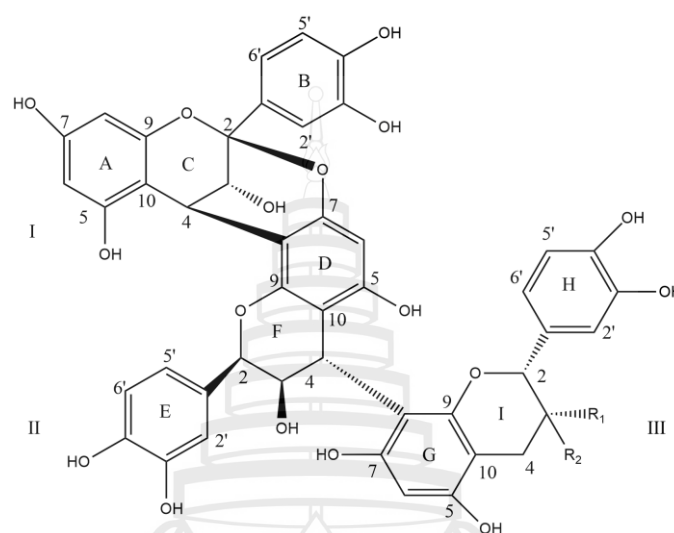


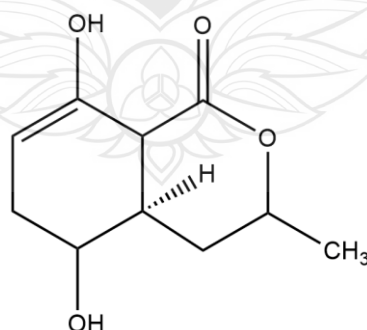
Figure 2.3 The chemical structures of some important compounds isolated from cinnamon

In 2011, Killday and co-worker isolated the two trimeric proanthocyanidins compounds from *C. cassia* bark, namely cinnamtannin B-1 (**1**) and cinnamtannin D-1 (**2**). These compounds exhibited significant inhibition against cyclooxygenase-2 (COX-2) at micromolar concentrations.



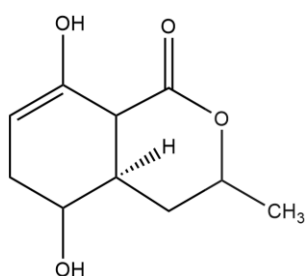
(**1**) $R_1 = OH, R_2 = H$, (**2**) $R_1 = H, R_2 = OH$

In 2012, Santiago and co-worker isolated 5-Hydroxyramulosin (**3**), produced by an endophytic fungus obtained from *C. mollisimum*. This compound indicated the presence of on major compound. Furthermore, 5-Hydroxyramulosin showed antimicrobial activity against *Bacillus subtilis* and *Aspergillus niger*, and also showed cytotoxic activity against murine leukemia cells.

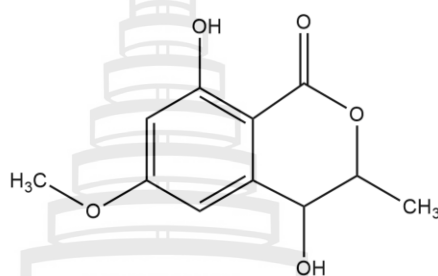


(**3**)

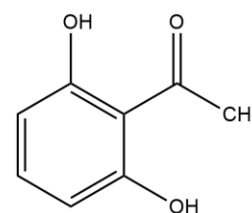
In 2014, three known compounds, namely 4-hydroxymellein (4), 4,8-dihydroxy-6-methoxy-3-methyl-3,4-dihydro-1H-isochromen-1-one (5) and 1-(2,6-dihydroxyphenyl) ethanone (6), were isolated from endophytic fungus and reported by Santiago and co-worker. 4-hydroxymellein is classified as polyketide, were shown antifungal and cytotoxicity against *B. subtilis*, and murine leukemic cells, respectively. In addition, 4,8-dihydroxy-6-methoxy-3-methyl-3,4-dihydro-1H-isochromen-1-one is classified as benzopyran, showed moderate antimicrobial activity against *B. subtilis*, and murine leukemic cells.



(4)

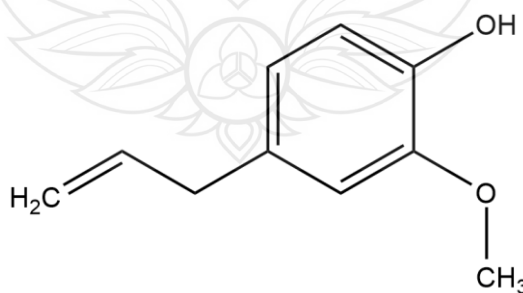


(5)



(6)

In 2021, Wijesinghe and co-worker isolated eugenol derived from the leaves of *C. verum*. Eugenol (7) was detected, and was the abundant compound in *C. verum* essential oil. *In vitro* antibacterial and antibiofilm activity were performed, this study showed that *C. verum* essential oil possess an antibacterial and antibiofilm potential against *P. aeruginosa*, *S. aureus* and *K. pneumoniae* without causing apparent toxicity to human cells.



(7)

2.1.2 *Polyscias fruticosa*

2.1.2.1 Botanical Description of *P. fruticosa*

P. fruticosa (L.) Harms belongs to the family Araliaceae, and is native to Central Malesia, Northern Australis, New Caledonia and New Guinea. *P. fruticosa* is widely distributed in southeastern Asia and the tropical islands for the Pacific region (Vo et al., 1998). The plant is a slow-growing, and able to grow up to 3–5 meter tall, evergreen shrub with spreading branches. Plant leaves are provided a dark green, glossy, alternate, and irregularly pinnately compound (Stone, 1965). The flower is pale yellow to white colour in a terminal inflorescence, relatively small, and a panicle of umbels, that was grown from August to September. The *P. fruticosa* fruit is a small, dry drupe with flattened laterally, and measured 4-5 mm long and 4.5-6 mm wide. They are popular as an ornamental plants and spice in tropical and also possessed culinary and medicinal purposes (Vo et al., 1998).



Soucre Koffuor et al. (2014)

Figure 2.4 *P. fruticosa* (L.) Harms

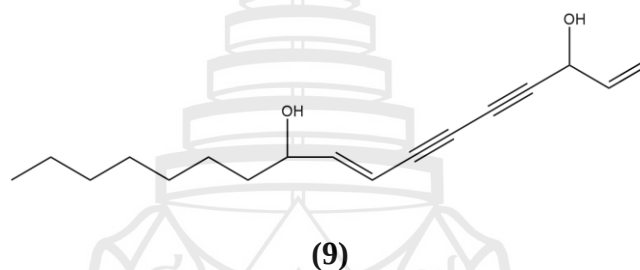
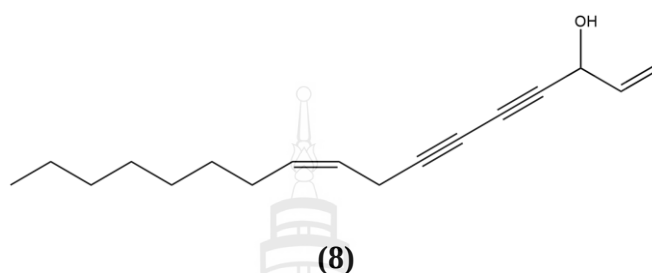
2.1.2.2 Medicinal Application

For a long history, *Polyscias* species are well-known for pharmacological purposes. The plant parts are used for treatment of ischemia, inflammation and increasing blood circulation in the brain (Do et al., 2000). Neurodegenerative diseases such as Parkinson's and Alzheimer's diseases can be assist using the plant leaves in term of folk medicines in Vietnam. Similarly, *P. fruticosa* have been traditionally used as a tonic and anti-inflammatory, (Ly et al., 2022; Tuyet & Phung, 2007). In traditional uses, Sugimoto et al. (2017) reported that *P. fulva* has been used to treat fever, mental illness, malaria, venereal infections, and obesity. In Asian countries, the leaves have been commonly used as a tonic, anti-inflammatory, antitoxin, and antibacterial, and also the root have been used a diuretic, febrifuge, anti-dysentery agent, and are applied for neuralgia and rheumatic pains (Huan et al., 1997). In addition, the decoction derived from the root and the alcohol extract are used as tonic, and also the leaves are consumed preferably in salads (Vo, 2012).

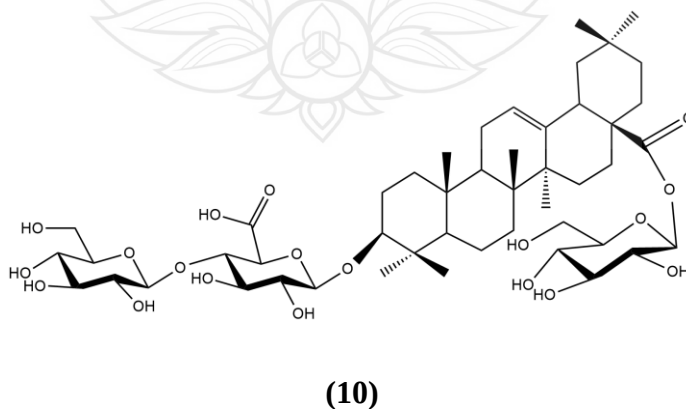
2.1.2.3 Chemical constituents and its biological activity

Studies of the phytochemical of *P. fruticosa* illustrated that the main chemical constituents were oleanolic acid-triterpene saponins and polyacetylenes (Lutowski et al., 1992; Vo et al., 1998). Various class of bioactive compounds including flavonoid, terpenoid, saponins, tannin, hydroxybenzoic acid and vanillic acid have been represented in this plant (Andarwulan et al., 2010). The biological activities of *P. fruticosa* have been reported as antioxidant, anti-inflammatory, anti-cancer, antifungal, antibacterial, and anti-diabetic activities (Nguyen et al., 2020). In addition, the ethanol leaves extract of *P. fruticosa* exhibited anti-asthmatic, and antihistaminic, and also the n-butanol leaves extract of *P. fruticosa* showed anti-pyretic, analgesic and molluscicidal activities (Asumeng Koffuor et al., 2016; Bensita et al., 1998). Nevertheless, no study on the secondary metabolites derived from the endophytic fungus of *P. fruticosa* was against plant pathogen.

In 1992, Lutomski and Luan isolated polyacetylenes, namely, falcarinol (**8**) and heptadeca-1, 8-diene-4,6-diyne-3, 10-diol (**9**) from *P. fruticosa*. These investigators found that these compounds effective against gram-positive including cocci and dermatophytes (*Candida albicans*).



In 2018, Luyen and co-worker reported that 3-*O*-[β -D-glucopyranosyl-(1 $\rightarrow$ 4)- β -D-glucuronopyranosyl] oleanolic acid 28-*O*- β -D-glucopyranosyl ester (PFS) (**10**) is a major saponin that isolated from *P. fruticosa* leaves. These results suggest that the presence of PFS showed significantly suppressed porcine pancreas α -amylase and yeast α -glucosidase, and the reduction of the postprandial blood glucose level was observed in a mouse model.



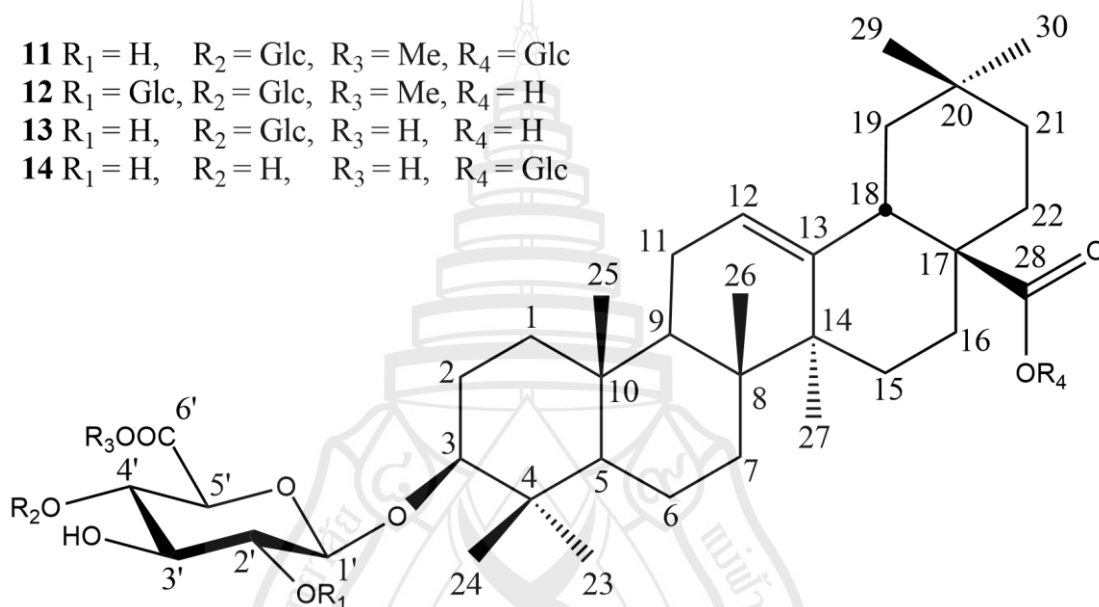
In 2019, two new oleanane-type triterpenoid saponin named polyscioside J (**11**) and polyscioside K (**12**) together with two known saponins, ladyginoside A (**13**) and chikusetsusaponin IVa (**14**) were isolated from the leaves of *P. fruticosa* (L.) harms., which reported by Do and co-worker, (2019). This is the first time of saponin from this species. Their structures have been demonstrated using IR, UV, HR-ESI-MS, NMR 1D and 2D experiments.

11 $R_1 = H$, $R_2 = \text{Glc}$, $R_3 = \text{Me}$, $R_4 = \text{Glc}$

12 $R_1 = \text{Glc}$, $R_2 = \text{Glc}$, $R_3 = \text{Me}$, $R_4 = H$

13 $R_1 = H$, $R_2 = \text{Glc}$, $R_3 = H$, $R_4 = H$

14 $R_1 = H$, $R_2 = H$, $R_3 = H$, $R_4 = \text{Glc}$



2.1.3 *Barleria prionitis*

2.1.3.1 Botanical Description of *B. prionitis*

B. prionitis Linn is belonging to Acanthaceae, and widely cultivated throughout South Africa, Sri Lanka, and tropical Asia. More than 300 species were found and famous for medicinal plants (Ata et al., 2009; Talukdar et al., 2015). *B. prionitis* plant is a small spiny shrub coming to up to 0.6-1.5 m high. The stem is erected, thick, glabrous, much branched with cylindrical tapering branchlet. The whole plant is small and considered yearly plant amid the dry season and its flowers are tubular yellow-orange long blooms and regularly single in the lower axils. Their fruits are provided ovoid and capsular while its seeds are flattened, covered with matted hairs. *B. prionitis* leaf is containing 5-20 mm long and broad with 3-10 cm and 1.5-4 cm, respectively. Generally, it is well-known perennial as “Vajradanti” in India and “Katukaradu” in Sri Lanka, and mostly used in folk medicine.



Source Naryan and Nikam (2018)

Figure 2.5 The leaves of *B. prionitis*

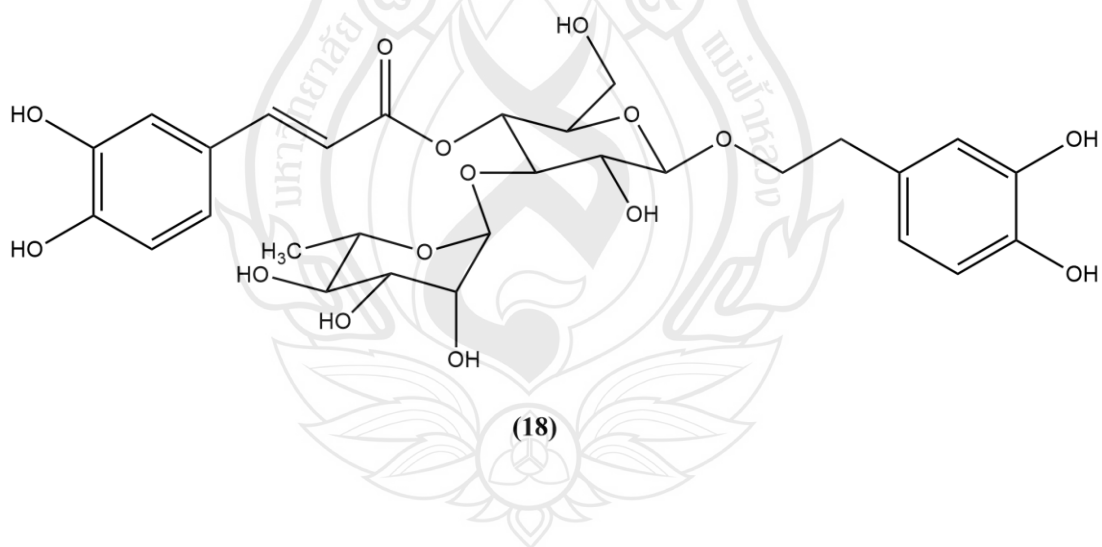
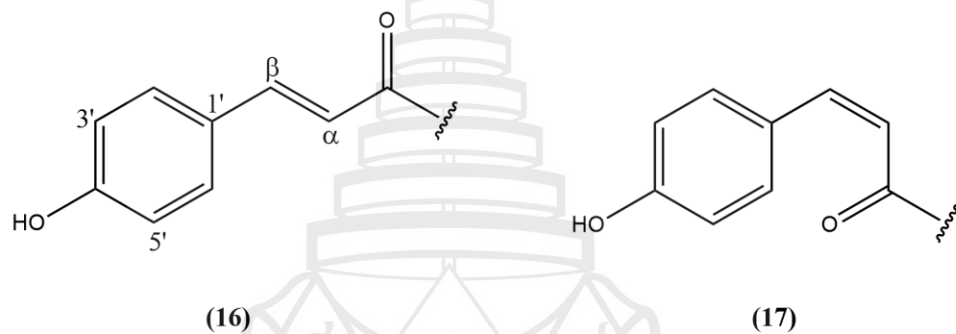
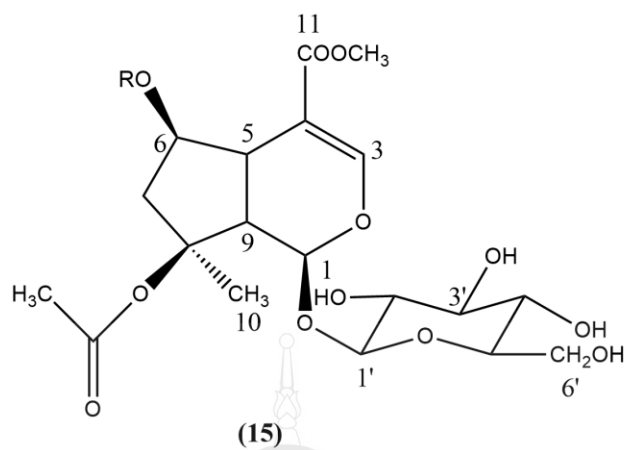
2.1.3.2 Medicinal Application

Currently, the increasing of herbal medicines is higher demand due to their property, higher safety and low cost. The entire of *B. prionitis* parts (root, leaves, and bark) were used for therapeutical treatment with numerous medicinal properties including, fever, whooping, inflammations, swellings, urinary infection, cough and cold, pain, and toothache since ancient times (Shukla & Gunjegaonkar, 2018). Several traditional uses have been reported that *B. prionitis* power is used for toothache, while root power is used for cure fever, and whooping cough can be prevent using the juice of *B. prionitis*, especially used in Uttar Pradesh and Madhya Pradesh states of India (Jadhav, 2006; Mahajan, 2007). Singh et al. (2016) have demonstrated the application for whooping cough, anti-respiratory syncytial, antiarthritic, anti-inflammatory and antifertility activities, were protected by decoction of the aerial parts of *B. prionitis*. In Southern India, the root part is taken orally for snakebite by decoction and the seed paste is taken daily once for edema. Meanwhile, the paste of the root of *B. prionitis* can be used to disperse boils and glandular swellings, and also the paste of the fresh leaf of *B. prionitis* has been used against scabies in Karnataka (Ghatapanadi et al., 2011; Gupta et al., 2000; Karuppusamy, 2007; Reddy et al., 2010). For curing wound, the crushed leaves are applied and widely used in Maharashtra (India) region (Patil et al., 2009). The other application, the leaf juice has been utilized for the treatment of catarrhal affections of children, glandular swellings, and boils (Mohammed et al., 2004). Bakshi et al. (2012) reported that the shoot paste with honey formation is uses for asthma and whooping cough. In particular, the folk medicinal healers including, anti-inflammatory and the treatment of cancer and tumor, were conducted using *B. prionitis* throughout Bangladesh (Mollik et al., 2010).

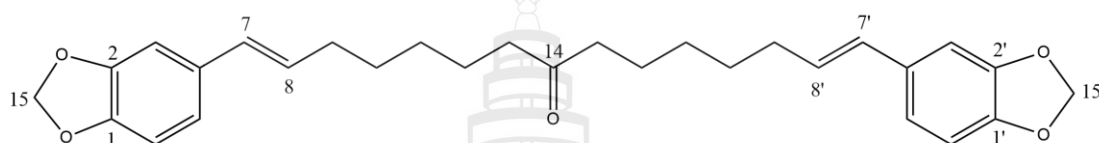
2.1.3.3 Chemical constituents and its biological activity

Consequently, the phytochemical constituents were isolated from the different parts of this plant including saponin, tannin, flavonoid, alkaloid, glycoside, steroid, sterol (stigmasterol), phenolic compound, and some essential oil (Talukdar et al., 2015). Besides, the phytochemical has been demonstrated by different workers for example, Dheer & Bhatnagar (2010) reported that the alcoholic extract derived from the leaf and root part of *B. prionitis* revealed the antidiabetic activity using alloxan monohydrate. Crude extract of leaves of *B. prionitis* was exhibited antimicrobial activity against dental decay pathogens. Surprisingly, the methanolic extract derived from bark part showed potential activity against oral pathogens including, *S. mutans*, *S. aureus*, *Pseudomonas* sp., *Bacillus* sp., *C. albicans*, and *S. cerevisiae* (Aneja et al., 2010). In addition, the ability of the extracts from the leaves, stems, and roots of *B. prionitis* showed significant antioxidant activity in the DPPH radical scavenging assay (Amoo et al., 2011). Due to their potential as a source of secondary metabolites, endophytic fungus isolated from *B. prionitis* could be useful for novel drug discovery.

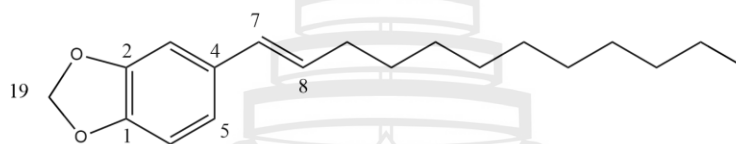
In 1998, Chen and co-worker reported two new iridoid glycosides which are 6-O-transp-coumaroyl-8-O-acetylshanzhiside methyl ester (**15**) and its cis isomer (**16**) and two known compounds which are barlerin (**17**) and verbascoside (**18**), were isolated from the whole plant of *B. prionitis*. These two new compounds were characterized with spectrometry techniques. The ratio of 3:1 of the mixture of compound (**15**) and (**16**) in the ratio of 3:1 was shown to have potent *in vitro* activity against respiratory syncytial virus.



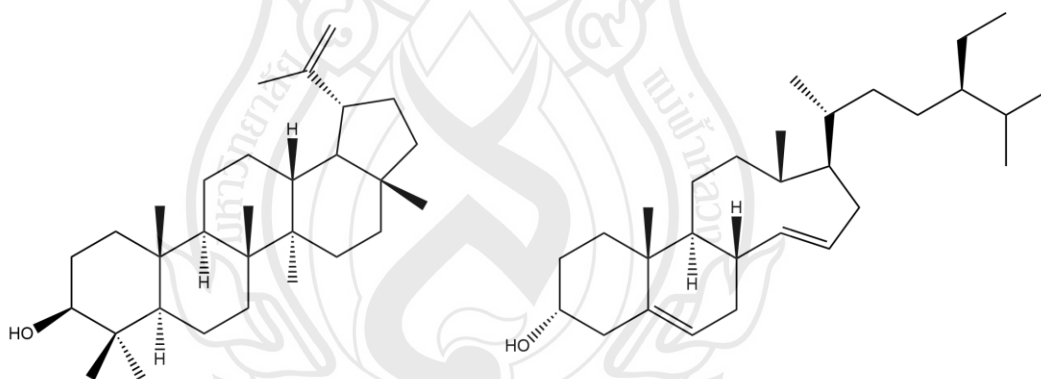
In 2007, Kosmulalage and co-worker studied the phytochemical on the ethanolic extract of *B. prionitis* in Sri Lankan origin. A new compound, balarenone (**19**), with three known compounds, namely, pipataline (**20**), lupeol (**21**) and 13,14-seco-stigmasta-5,14-diene-3- α -ol (**22**) were identified using NMR techniques. Among these compounds, it showed different levels of acetylcholinesterase (AChE) inhibitory activity. In addition, compound (**19**), (**20**), and (**22**) presented antimicrobial activity to at least one of the tested microorganisms.



(19)



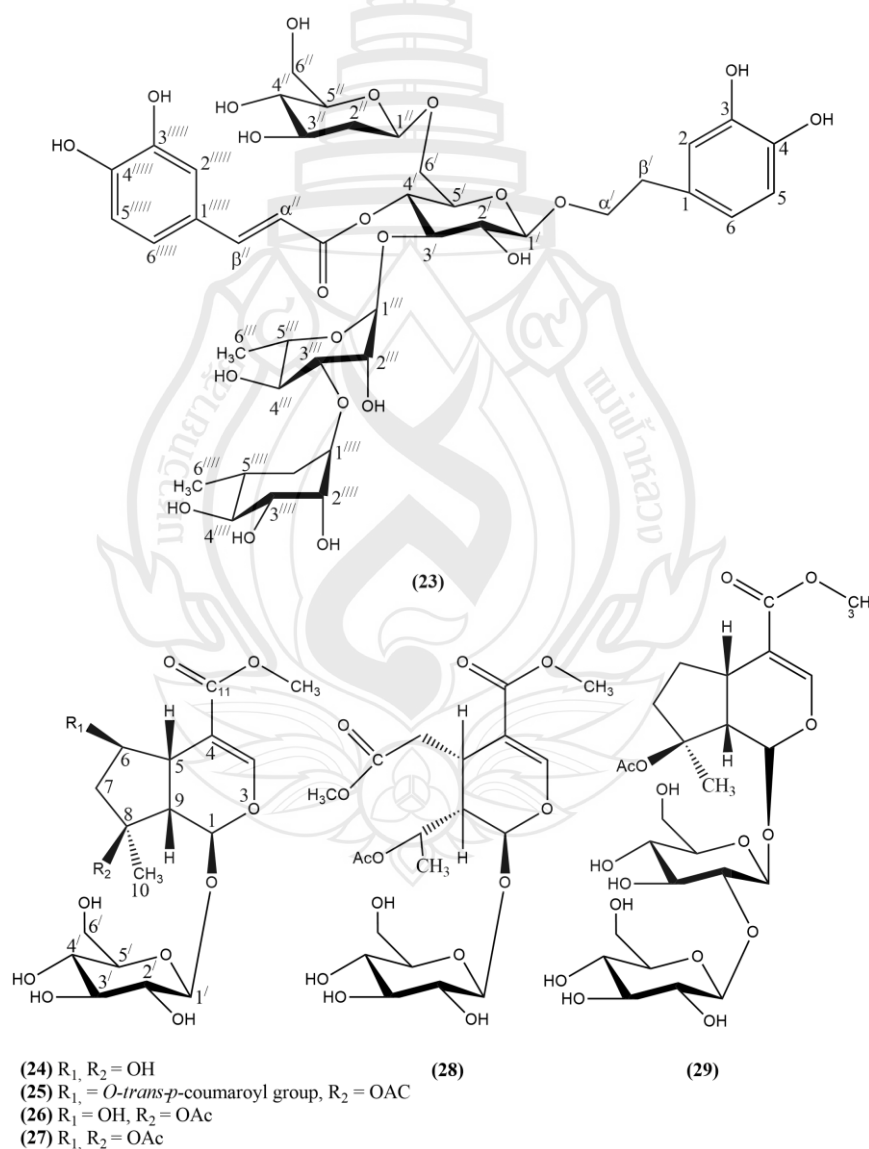
(20)



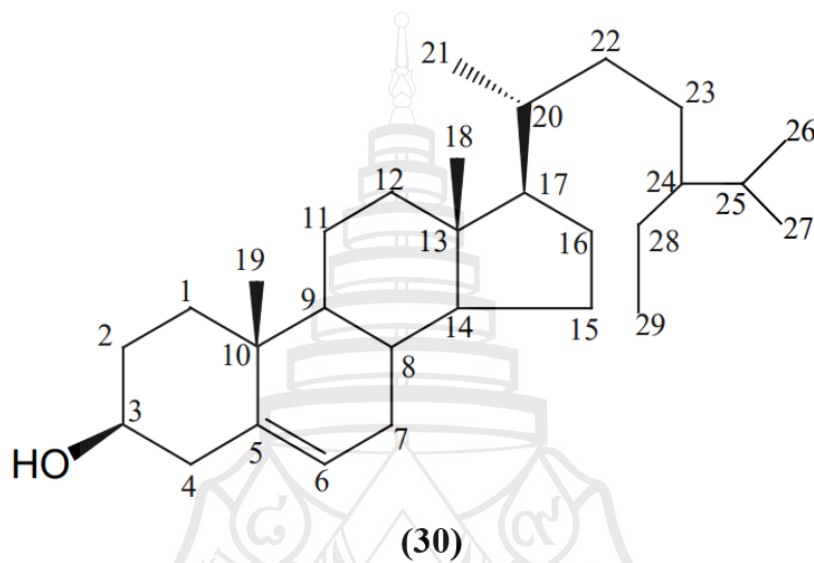
(21)

(22)

In 2009, ¹Ata and co-worker isolated seven compounds from the aerial parts of *B. prionitis*. From seven isolated compounds, it was found one new phenylethanoid glycoside, barlerinoside (**23**), confirmed using Nuclear Magnetic Resonance and chemical reactions. Furthermore, six known iridoid glycosides, namely shanzhiside methyl ester (**24**), 6-*O-trans-p*-coumaroyl-8-*O*-acetylshanzhiside methyl ester (**25**), barlerin (**26**), acetylbarlerin (**27**), 7-methoxydideroside (**28**), and lupulinoside (**29**). In glutathione *S*-transferase (GST) inhibition assay, compound (**23**) showed potential, while compounds (**24**) – (**29**) also exhibited varying levels of GST activity.



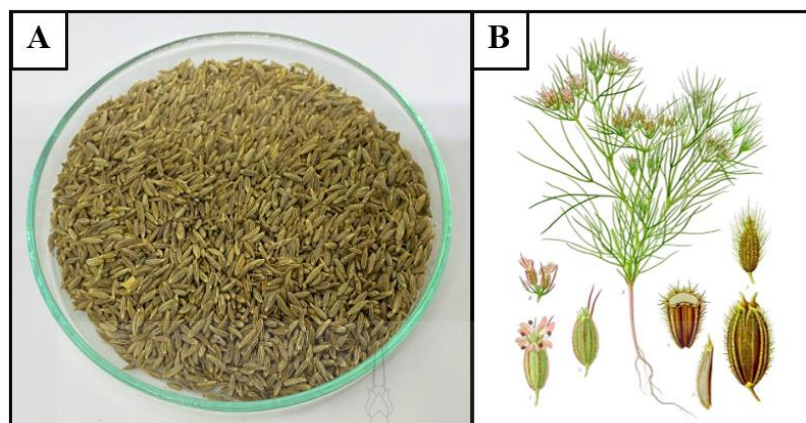
In 2016, β -sitosterol (**30**) derived from the methanolic root extract of *B. prionitis*, was reported by Singh and Gupta. These compounds were basically characterized using Infrared spectroscopy (IR) and Nuclear Magnetic Resonance (NMR). Spermatogenesis and fertility were suppressed by β -sitosterol and may lead to development of new drugs in clinical trial studies.



2.1.4 *Cuminum cuminum*

2.1.4.1 Botanical Description of *C. cuminum*

C. cyminum (cumin) is an annual herbaceous flowering plant belonging to family Apiaceae. It is native origin to the East Mediterranean to South Asia and widely cultivated all over the world (Agarwal et al., 2017). Generally, it is well known as cumin or zeera, and also called kummel, comino, zirech-e sabz, cumino, kemon, zira, and kamun in India (Peter, 2003). It is one of the oldest and economically valuable herbs that cultivated in warm regions likes Mediterranean climates (Mnif & Aifa, 2015). The physical of cumin tree is a thin, and is consisting of mericarps which may be united and separated. Bluish-green leaves are alternate, dissected, sparsely hairy, and bear thread-like leaflets. Cumin tree presents yellowish green/brown colour, elongated ovoid, and 3-6 mm in length. The surface has five primary ridges with four less distinct secondary ridges bearing numerous short hairs. The petioles have tapered crusted wings, sheathing the stem at the base. Small flower is white or pink colour with diploid allogam hermaphrodite, and contains a group of 3-5 flower (Koohsari et al., 2020). The petals present white or red colour, oblong-shaped with an intense border of a long-indented tip. Seed of cumin has a short stalk and small annual herb with long slender, and small umbels of white or rose-coloured flower which covered with tiny hairs. Cumin seed are extensively used as spice for their distinctive aroma since ancient times (Peter, 2003; Sahana et al., 2011). Multipurpose of cumin is widely use for various culinary and medicinal purposes (Mnif & Aifa, 2015).



Source adapted from Sahana et al. (2011)

Figure 2.6 The dry seeds of cumin (A) The aerial parts of cumin plant (B)

2.1.4.2 Medicinal Application

The usage of herbal obtained from natural products is interesting and commonly used in Ayurvedic medicine for prevention or treatment ailments. Scientific evidence has been reported the medicinal uses in cumin plants. For example, neck mumps is acute infectious inflammations which can prevent using cumin via external application (Bellakhdar, 1997). The powder or decoction derived from cumin seeds were used for the treatment of gastrointestinal disorders in Maghreb countries and Tunisia region. In addition, the decoction of cumin seeds is recommended as an emmenagogue which is agent to stimulate menstrual flow, and also used for stomachic, carminative, antispasmodics and anthelmintic agent. Interestingly, cumin has also been commonly used for treatment insomnia, cold, and fever which recorded by Indian herbalists. The mixture of onion juice and the paste of cumin seeds has been used for scorpion and bee stings to retard the frequency of upbeats (Mnif & Aifa, 2015). Similarly, the toothaches and epilepsy can be prevented by the fruit of cumin plants, generally in Iranian since ancient time (Janahmadi et al., 2006). In ancient Indian medicine, cumin plays a role in the body's defenses against dyspepsia, diarrhea, and jaundice (Mnif & Aifa, 2015). It has also been recorded that it is used as digestive aid and relieve coli, flatulence, and diarrhea (Bremness, 1996).

It has been illustrated that the increasing lactation and decreasing nausea during pregnancy, can be used as a safe remedy to relieve swelling of the breast or the testicles (Jalali-Heravi et al., 2007). Moreover, the integral part of their folk medicines has been reported that cumin seeds has also been used in widely region including the Northern Europe to the Mediterranean, Russia, Iran, Indonesia and North America (Johri, 2011). Among these medicinal properties, the chemical constituents and biological activities obtained from cumin should be developed in various industrial, agricultural or cosmetic applications.

2.1.4.3 Chemical constituents and its biological activity

Essential oils are a complex mixture of volatile constituents or secondary metabolites abundant in some aromatic plant family such as *Apiaceae*, *Asteraceae*, *Cupressaceae*, *Labiatae*, *Myrtaceae*, *Pinaceae*, *Piperaceae*, *Rutaceae*, *Zingiberaceae*, and *Umbelliferae* (Ishaaya et al., 2007). Although different parts of plant (flowers, leaves, seeds, roots, stem, and bark) have been produced through the internal multicellular cavities and secretory ducts with a specific essential oils and odour. For example, some flower scents (rose, jasmine or tuberose) will immediately emit the volatiles by the epidermal layers of their petals. Basically, these essential oils are obtained in various techniques including, water or steam distillation, extraction with organic solvent, supercritical carbon dioxide, which are mostly liquid at room temperature, slightly soluble in water and highly soluble in organic solvents showed in Figure 2.7 (Figueiredo et al., 2008). Generally, essential oils have been used extensively in cosmetic, therapeutic and food industries. Essential oils are consisted of terpenoids and non-terpenoid hydrocarbons. All of them are hydrocarbons and their oxygenated derivatives (nitrogen or sulphur derivatives). Various form of alcohols, acids, esters, epoxides, aldehydes, ketones, amines, sulphides, have been identified (Berger, 2007).

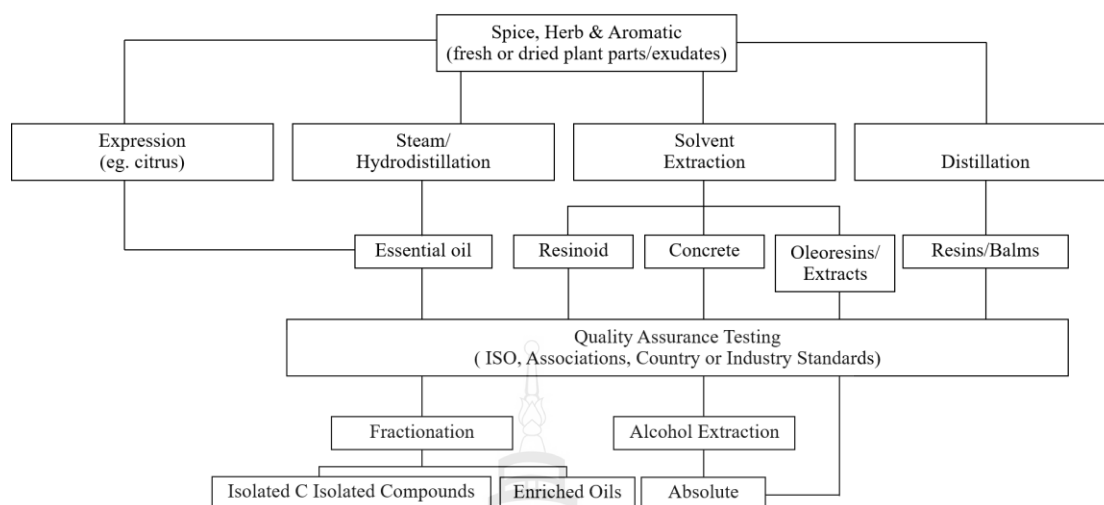


Figure 2.7 Extraction methods of chemical composition from plants

It is well-known and important phytoconstituents in Apiaceae species producing essential oils with interesting potential effects. Cumin seeds are relatively good source of fat, protein, dietary fibre, vitamin B and E, and several dietary minerals. Various classes of compounds including, terpenes, alcohols, phenols, flavonoid, and aldehydes, are present in cumin (Figure 2.8). Mnif and Aifa (2015) reported that cumin seeds contain a wide range of phenolic acids, including gallic, cinnamic, rosmarinic, coumaric, and vanillic acids, and also several flavonoid including luteolin, catechin, coumarin, quercetin, and apigenin, have been identified in cumin seeds (Figure 2.9). Cumin seeds are nutritionally rich of essential oils with several biological activity such as antiallergic, antioxidant, antiplatelet. While, cuminaldehyde, eugenol, β -pinene, are the predominant in cumin. (Mandal & Mandal, 2016).

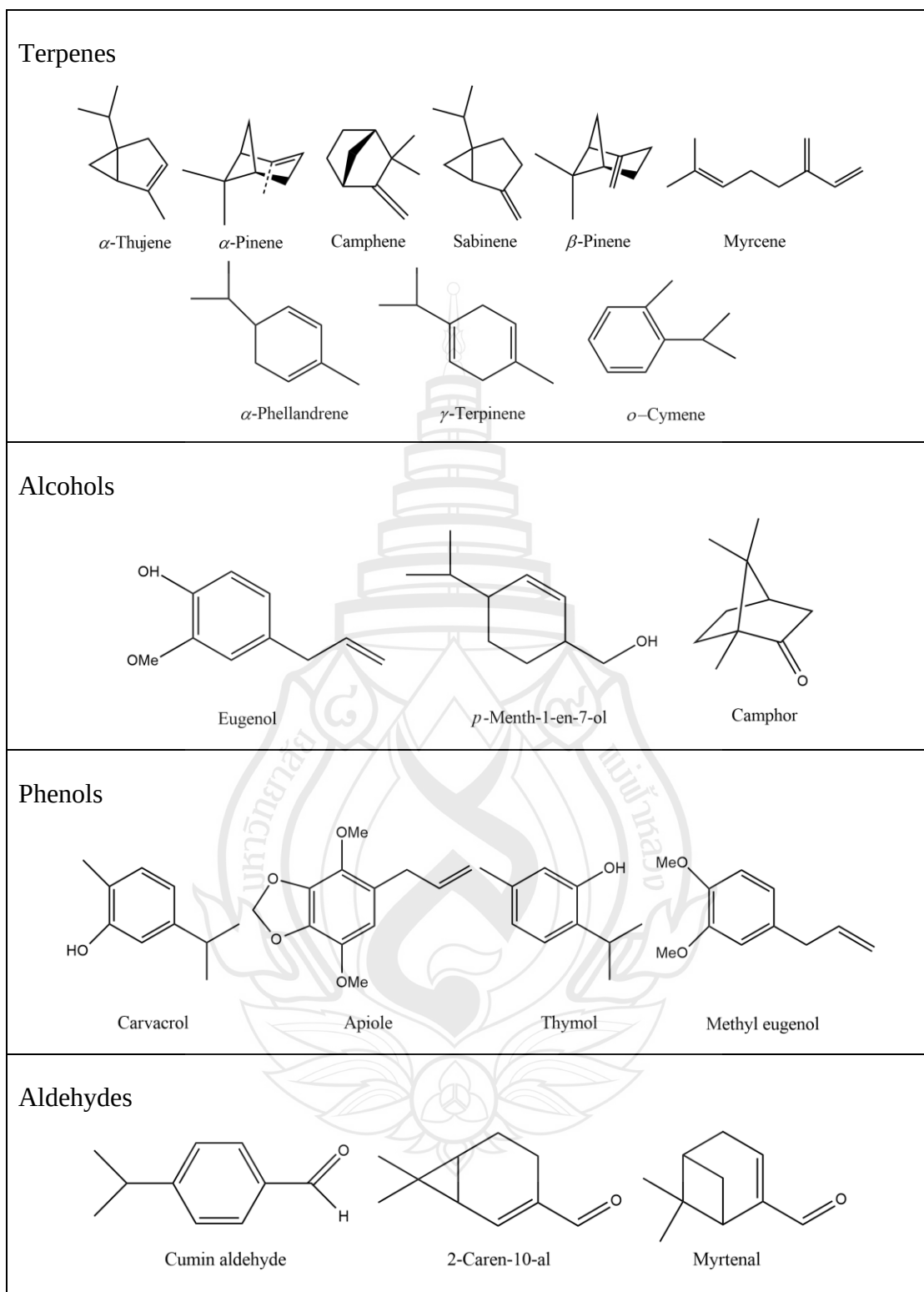
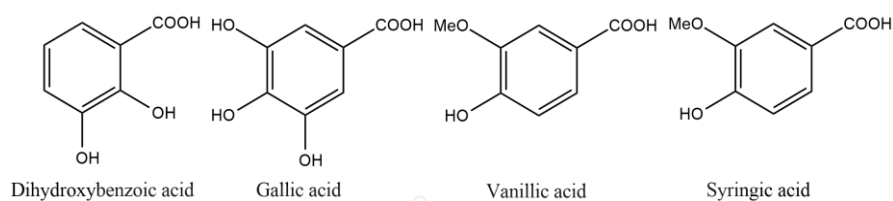
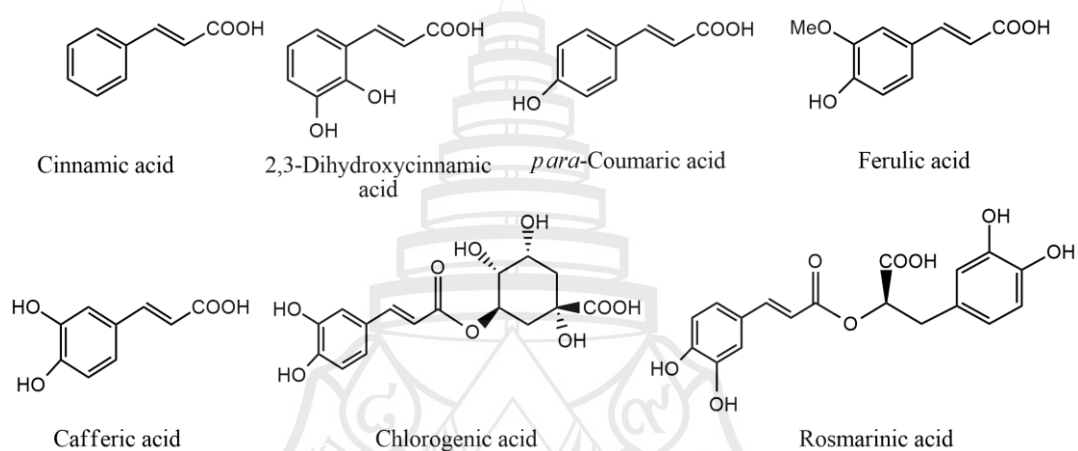


Figure 2.8 Example of some compounds found in *C. cyminum*

Phenolic Acids



Cinnamic acid and its derivatives



Flavonoids

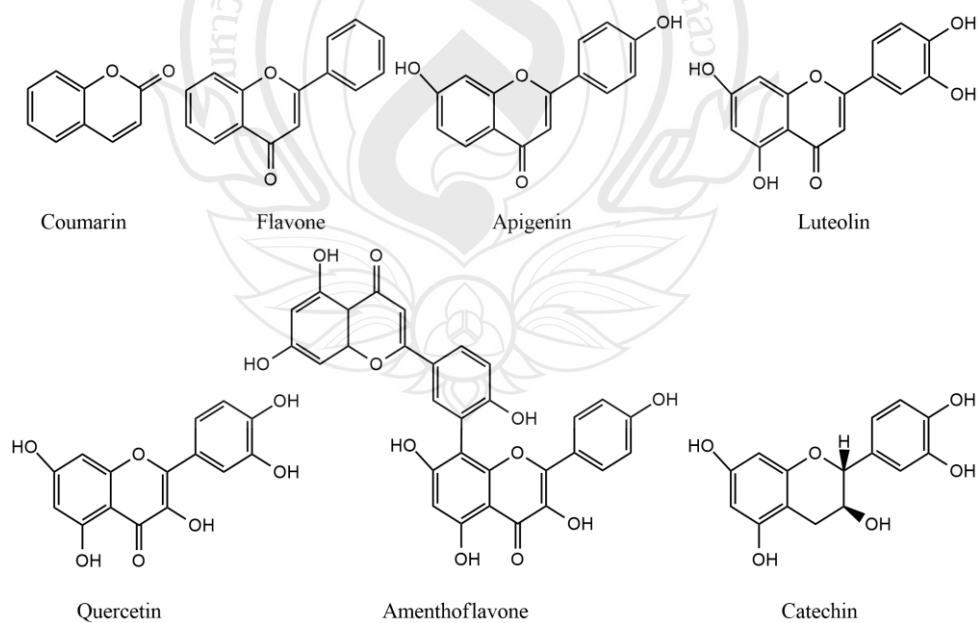


Figure 2.9 Example of some phenols and flavonoids found in *C. cyminum*

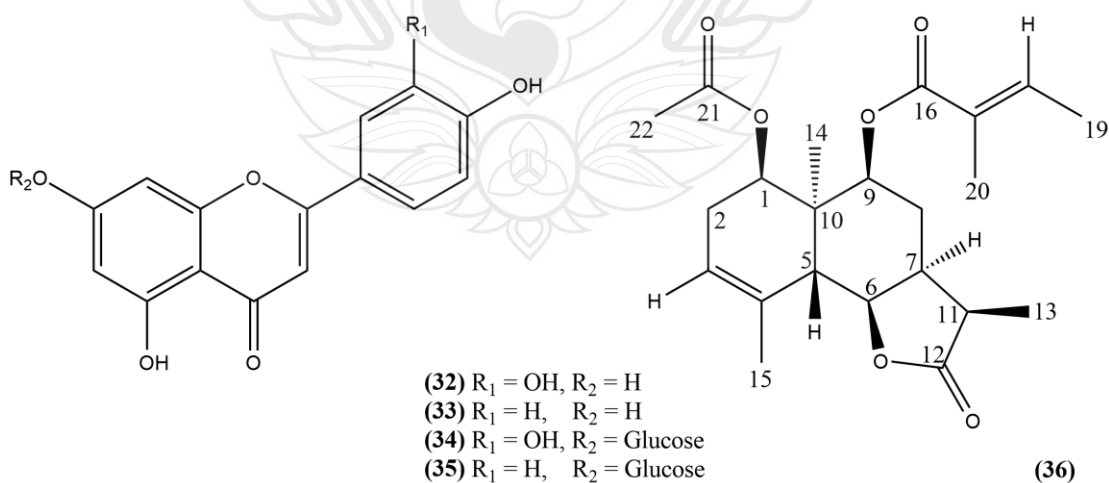
Literature review on phytochemicals of the cumin seeds revealed the presence of various bioactive compound, which are described as followed.

In 2016, Tahir and co-worker reported that α -pinene **(31)** has been found as the majority of *C. cyminum* essential oils. The antidiabetic potential was evaluate using various emulsion formulation (B1 to B5) by alpha amylase inhibition assay. B5 (essential oil + surfactant (tween 80) + co-surfactant (ethanol) + different concentrations of *C. cyminum* essential oils) showed a maximum antidiabetic activity with 83.09% inhibition of α -amylase.



(31)

In 2020, Goodarzi and co-worker isolated the four flavone compounds, namely, luteolin **(32)**, apigenin**(33)**, luteolin-7- O-glucoside **(34)** and apigenin-7-O-glucoside **(35)** which derived from the ethyl acetate fractions, and cuminoid A **(36)** derived from the hexane fractions of *C. cyminum*. Especially, Luteolin-7-O- glucoside **(34)** exhibited anticancer activity against MCF-7 cell line with IC₅₀ of 3.98 mg/mL.



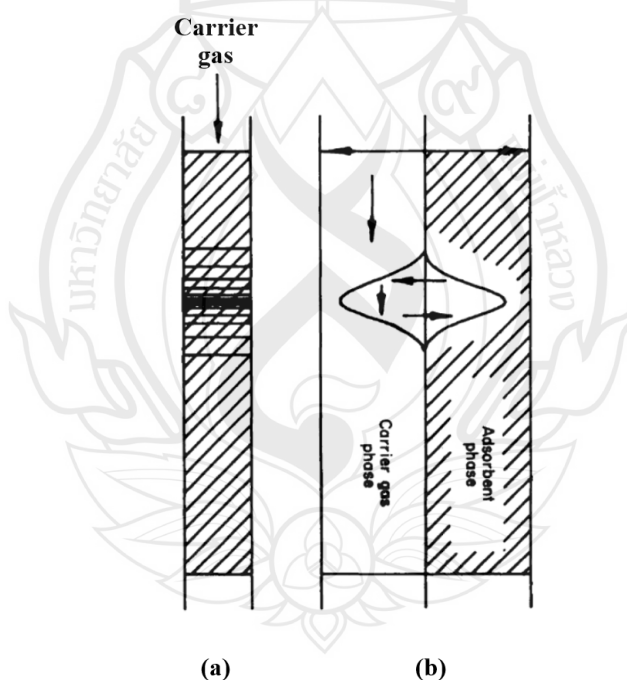
2.2 Background of Instruments

2.2.1 Gas Chromatography (GC)

GC is one of the first chromatographic and the most widely used for separating components of mixture of volatile organic compounds (VOCs). The popularity of GC is well-established method for determination the quantity of each component of the mixture, based on a favourable combination of very high selectivity and resolution, good accuracy and precision, wide dynamic concentration range and high sensitivity. Various application of GC has been analysed including volatile organic compounds (VOCs), polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins and furans (PCDD/Fs), pesticides. In addition, the other organic pollutants have also been analyzed such as PBDEs, PCAs and toxaphene (Santos & Galceran, 2002).

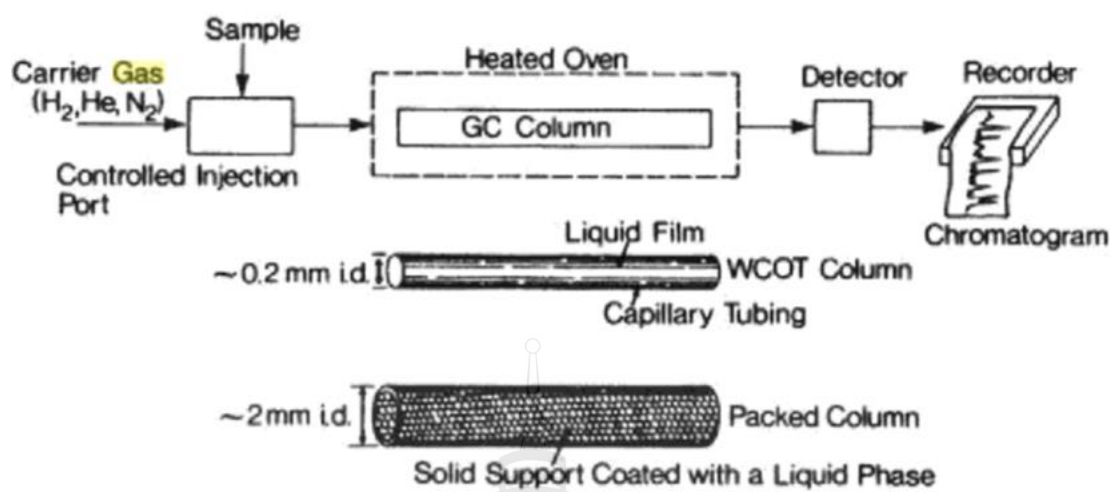
The important apparatus of GC divided into two parts: column phase and stationary phase. The column phase is a long tube packed permeably with some adsorbent, and a non-reactive gas (carrier gas) and a stationary phase (a liquid coated on to a solid surface). Generally, the elution technique is the commonest technique of GC that is a stream of inert gas, the carrier gas will pass through the column. In the beginning of the column, the mixture will be separate instantaneously as a gas or a volatile liquid, and they are detected by the measurement of some chemical or physical property. When the sample will enter the stationary phase, the form of a coiled column has been formed and kept in a thermostatic oven, and also an equilibrium is set up between the column and the gas that a proportion of the sample always remains in the gas phase. This portion moves further along the column in the carrier gas stream.

In the elution form, the sample mixture will heat and separate to vaporize into a flowing stream of carrier gas (nitrogen or an inert gas such as helium), and the vaporized mixture is carried along the stationary phase by a carrier gas, then various components elute out of the column due to the different affinity of the stationary phase, and the different rates. Temperature is an important factor that involved the influencing column performance, and usually required for sample components having a wide range of boiling points, for example, some mixture sample is necessary to elute at higher temperature, and also the appropriate temperature will assist by enclosing the column in a vapour or thermostatted block or hot-air oven. Due to the different sample components, it will give different retention times in the GC system. The retention time and peak integration values obtained from individual sample components, provide the qualitative and quantitative results (Hussain & Maqbool, 2014).



Source Hussain and Maqbool, (2014)

Figure 2.10 Diagram of a permeably packed column and its idealisation, in which gas and adsorbed phases are represented separately

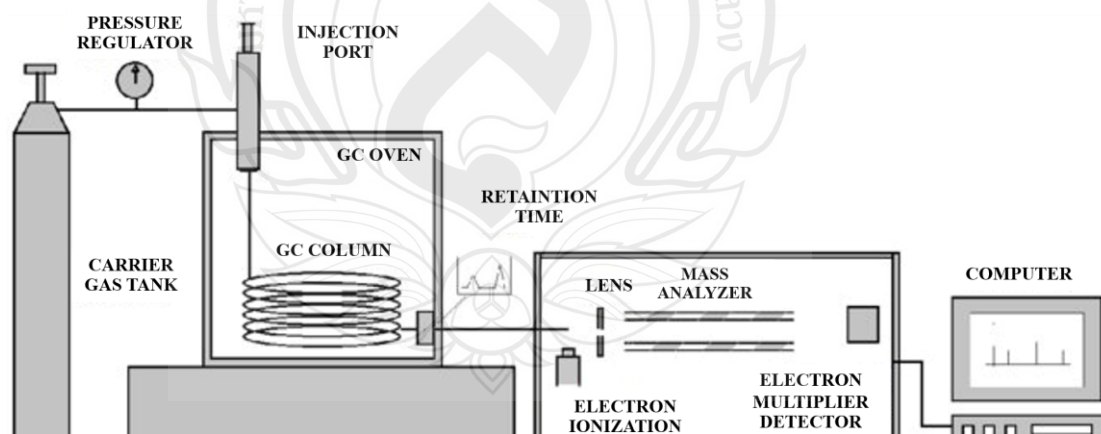


Source Karasek and Clement (2003)

Figure 2.11 Schematic Diagram of GC Instrument

2.2.2 Gas Chromatograph-Mass Spectrometry (GC-MS)

The combination between GC and MS (GC-MS) provides defensible analytical and reduces the reliance on the GC separation and lead to faster analysis times. GC-MS is the technique most commonly used for the analysis of volatile organic pollutants in environmental samples that combines the character of gas chromatography and mass spectrometry. Since 1959, GC-MS was born and the introduction of Gas Chromatography Time-Of-Flight Mass Spectrometry (GC-TOF MS) was demonstrated by Gohlke (1959). In the mid-1960s, the new technique has been reported due to the analysis of the different volatile organic mixtures, especially involved TOF and high-resolution mass spectrometers. However, the couple packed column to MS, a reduction of pressure was required into the vacuum. Until now, the coupling of capillary columns directly to the ion source is popular use. GC-MS has been established the application of high-resolution GC and bench-top MS instruments which is one of the most attractive techniques to analysis of organic compounds in complex mixtures, and as one of the most sensitivity, selectivity and accuracy in the analytical methods (Harvey, 2012). However, Amirav et al. (2020) described the limitation of GC-MS which is limited to the lower molecular weight, thermally stable compounds, and also molecular ions.



Source Kolapkar (2018)

Figure 2.12 Schematic Diagram of GC-MS System

2.2.3 High Performance Liquid Chromatography (HPLC)

HPLC is a chromatographic technique for the isolation of natural product. HPLC is widely used to separate a mixture of compounds and its phytochemical and analyze chemistry to identify, quantify and purify the individual components of the mixture (Boligon & Athayde, 2014). Recently, HPLC has become one of the most popular and the most powerful approaches to quality control of herbal plants (Fan et al., 2006). Crude extract obtained from natural products are usually isolated following the evaluation of a relatively of biological assay for characterize its properties. The primary goal of the analyst is the rapid processing of such multi-component samples on both an analytical and preparative scale (Boligon & Athayde, 2014). The characterization and quantification of secondary metabolites obtained from natural product have been described in several authors, including phenol groups, steroids, flavonoids, and alkaloids (Martin & Guiochon, 2005; Reis et al., 2014).

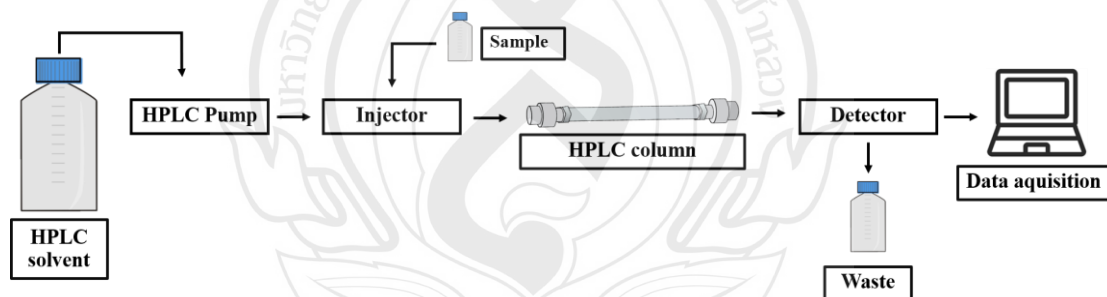
The commonly used separation technique in HPLC is reverse-phase chromatography due to its variety application range. Reversed phase HPLC has a non-polar stationary phase and watery tolerably polar portable phase. More than 65% of HPLC separation is reversed phase separation due to their properties such as the simplicity, versatility, and scope of the reversed-phase method including the separation of secondary plant metabolites (Boligon et al., 2012). Moreover, the buffer is used for the mobile phase in reversed phase chromatography in the colloquial term. The little buffering capacity are usually contained strong acids at low pH with organic solvents in the mobile phase solution. In the case of physiological conditions, the appropriate buffering capacity would help maintain the machine.

Vidushi and Meenakshi (2017) described the principle of HPLC that the sample to be analyzed is injected into the stream of the mobile phase, and the motion of analyte is associated with the specific chemical or physical interactions with stationary phases. Depending on the partition behaviour of the analyte components effect to the rates of migration through the column, and the elution at different time. The process of separation of the individual components of a mixture of chromatography is based on their relative affinities towards mobile phases and stationary phases. Normally, the greater affinity of analyte components will go to stationary, and will move slower for a shorter distance. In contrast, the less affinity of

analyte components will move faster for a longer distance. Various organic solvent has commonly been used such as methanol and acetonitrile that depend on the nature of the analyte and the stationary phase. The buffer or salts dissolved in water, improves the separation of the analyte components or compounds which acts as an ion pairing agent (Kumar et al., 2015).

The selected detector is an important to identify any compound. The identifying peak should have a reasonable retention time and should be well separated from the extraneous peaks at the detection levels. UV detectors are widely popular due to their high sensitivity. For example, UV-VIS and Photodiode Array (PDA) are identified the phenolics compounds in the range of 190-380 nm (Khoddami et al., 2013).

However, some important parameters are an important to analyze bioactive including crude source material, and the suitable solvent. The filtration is necessary part to inject into HPLC column, and also the guard columns are used for the analysis of crude extract, especially, the lifetime of the analytical columns can extend by the guard columns (Boligon & Athayde, 2014).



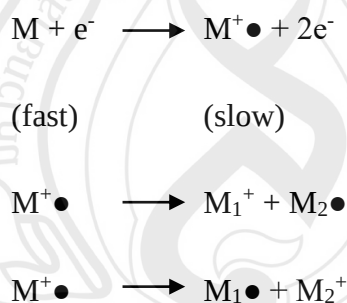
Source Boligon & Athayde (2014)

Figure 2.13 Schematic Diagram of HPLC System

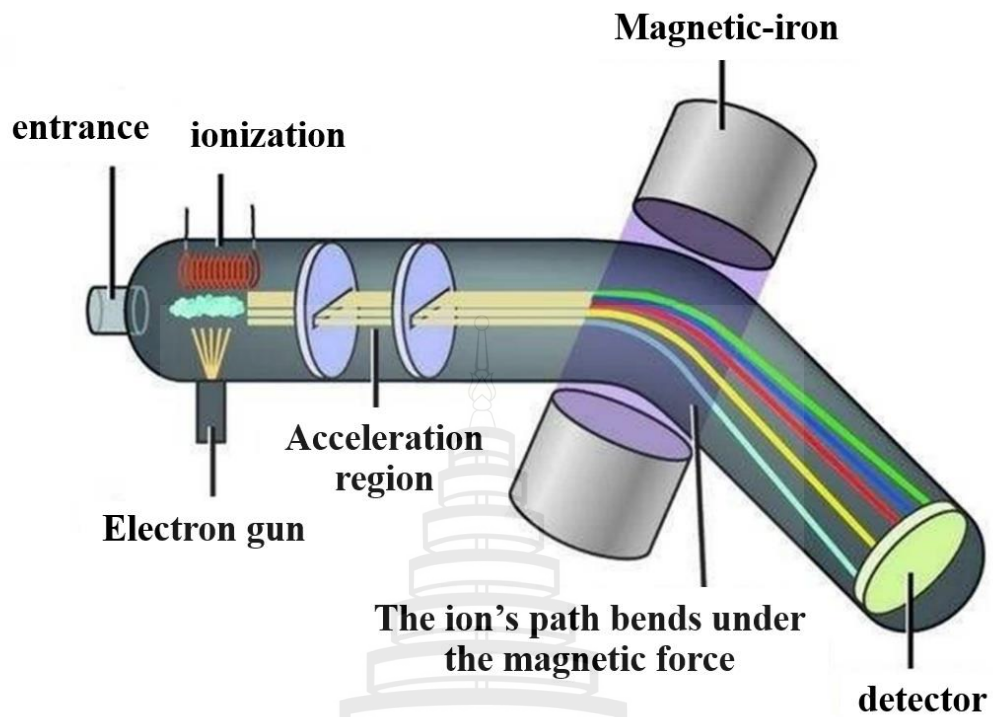
2.2.4 Mass spectrometry (MS)

MS is a technique for qualitative chemical analysis, and extensively used in various fields such as pharmaceutical, medical science, and environmental analysis (Bjarnholt et al., 2014). The potential of MS is superior to other analytical technique in the combination of several features including multi-analytic property, sensitivity, selectivity, extremely low amounts, and the identification capabilities of characterizing compounds by molecular mass or formula (Milman, 2015). Basically, this technique is studied the effect of ionizing energy on molecule, and it depend on chemical reactions in the gas phase during the formation of ionic and neutral species of sample components are formed (Agarwal & Goyal, 2017).

Paré and Yaylayan (1997) described the process of MS instruments. MS is based on the ionization of molecules so that they become more charged particles and can then be sorted according to their mass to charge ratio (m/z). Generally, electron bombardment (70 eV) was obtained under pressure between 10^{-7} to 10^{-5} mm of Hg using tungsten or rhenium filament. The breaking of molecules provides the cations and other fragments as follows:



Typically, ionization from n or π orbital are loss an electron and then molecules are formed (cations or parent ion) which can further break up into smaller ion (called ionizer). These ions are accelerated towards the detector by an electric field, and then these ions are deflected by magnetic field depending on their mass-to-charge ratio. The separated ions were measured and the output will be displayed as mass spectrum. The information consists of several bits of information including the composition of an organic compound, the position of functional groups from each mass spectrum, and the determination of molecular weight. The base peak is presented as the most intense peak in the mass spectrum (Agarwal & Goyal, 2017).



Source Chen et al. (2019)

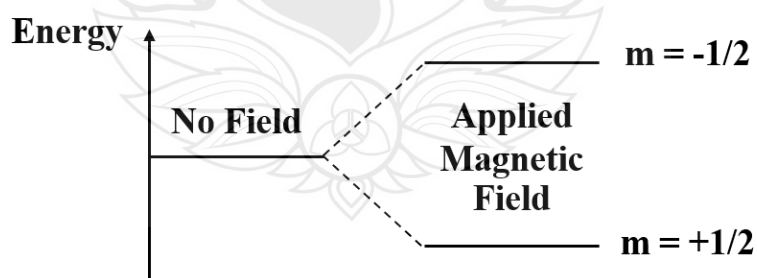
Figure 2.14 Schematic Diagram of MS System

2.2.5 Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR is an important instrumental technique for qualitative and quantitative analysis, and one of the most powerful tools in structural elucidation, including fatty acids, and their derivatives. Enhancing of the advent of high-field spectrometer (900 MHz) and cryogenic probes have been improved the sensitivity (Bonvin et al., 2005).

NMR was first discovered in 1946 by Purcell and Bloch. NMR have been developed in next 50 years into the premier organic spectroscopy to elucidate the chemical structure of the chemicals they were synthesizing. Magnetic Resonance Imager (MRI) is another well-known product of NMR that available in various utilization including medical radiology field. Currently, the production of stable permanent magnet technologies of NMR is developed high-resolution ^1H NMR spectra to become portable tools to analyze samples anywhere (Edwards, 2009).

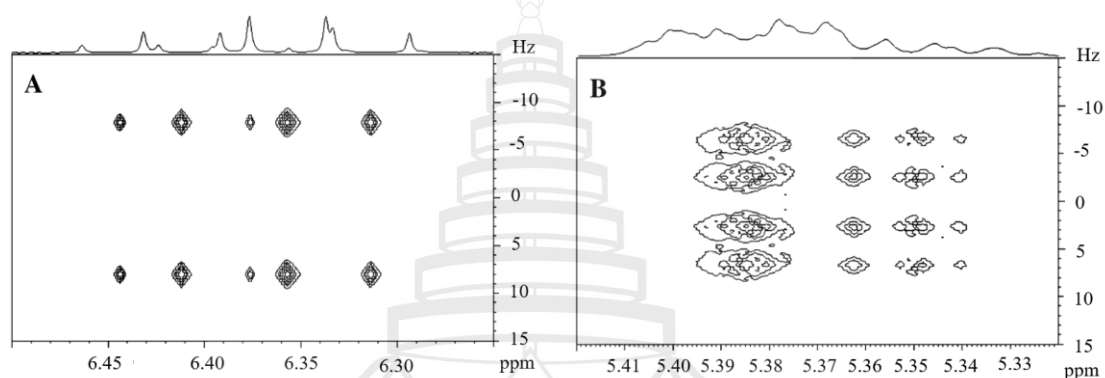
The NMR phenomenon is based on the interaction of magnetic moments of nuclei with the magnetic field. The intrinsic spin angular momentum possessed by electrons and nuclei is revealed with the magnetic moment of nuclei. The nuclear spin value is determined by a spin number. Correlation between the nuclear magnetic moment and the nuclear spin is depend on the properties of a nucleus and the spin number. No element with an even number of both protons and neutrons has been found to possess moment, whereas some element with an odd number (Edwards, 2009).



Source Edwards (2009)

Figure 2.15 Energy Levels for a Nucleus with Spin Quantum Number

Two-dimension (2D) NMR method has been developed to elucidate the chemical structure of novel compounds in a mixture as illustrated in Figure 2.16 (Verpoorte et al., 2007). In the early 1980s, Formáček and Kubeczka reported that ^{13}C NMR spectra have been used in essential oils for fingerprinting. In addition, Schripsema and Verpoorte (1991) described that ^1H NMR spectra has been used in plant cell cultures, and also used in sugars in cell cultures and media. Furthermore, the mixture of pyrrolizidine alkaloids has been analyzed (Pieters et al., 1989).



Source adapted Liang et al. (2006)

Figure 2.16 Two-dimensional *J*-resolved NMR spectra of phenylpropanoids of *B. rapa* leaves in the range of δ 6.25–6.50 (a); δ 5.32–5.42 (b). The spectra were measured in methanol- d_4

CHAPTER 3

PRODUCTION OF EUGENOL FROM FUNGAL ENDOPHYTES

Neopestalotiopsis sp. AND *Diaporthe* sp. ISOLATED FROM

Cinnamomum loureiroi Leaves¹

Abstract

Endophytic fungi, which colonize within a host plant without causing any apparent diseases, have been considered as an important source of bioactive secondary metabolites containing antimicrobial and antioxidant activities. The aim of this research was to isolate the endophytic fungi of *Cinnamomum loureiroi* and then to screen their antimicrobial and antioxidant activities. A total of 11 fungal endophytes were isolated from healthy leaves of *Cinnamomum loureiroi* belonging to six genera: *Botryosphaeria*, *Colletotrichum*, *Diaporthe*, *Fusarium*, *Neopestalotiopsis*, and *Pestalotiopsis*. All isolated strains were cultured and further extracted with ethyl acetate solvent. Antimicrobial activity of all crude endophytic fungal extracts was analyzed using disc diffusion assay against six bacterial and two fungal pathogens. Crude extracts of strains MFLUCC15-1130 and MFLUCC15-1131 showed broad-spectrum antimicrobial activity against all tested pathogens. Activity against *Bacillus cereus* and *Staphylococcus epidermidis* was notable, showing the lowest minimum inhibitory concentration at 3.91 µg/mL. Antioxidant activity of all crude endophytic fungal extracts was also evaluated based on 2,2-diphenyl-1-picrylhydrazyl assay. Significant antioxidant activity was detected in the crude extracts of fungus MFLUCC15-1130 and MFLUCC15-1131 with IC₅₀ of 22.92 ± 0.67 and 37.61 ± 0.49 µg/mL, respectively. Using molecular identification, MFLUCC15-1130 and

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MFLUCC15-1131 were identified as *Neopestalotiopsis* sp. and *Diaporthe* sp., respectively. The major chemical constituents produced by both crude extracts were identified by gas chromatography-mass spectrometry. Eugenol, myristaldehyde, lauric acid, and caprylic acid were the primary antimicrobial and antioxidant compounds in both crude extracts. This is the first report of eugenol being a biologically active compound of *Neopestalotiopsis* sp. and *Diaporthe* sp. fungal endophytes. Eugenol has been reported as antimicrobial and antioxidant agents with agronomic applications. Thus the two newly-isolated endophytes may be used for eugenol production, which in turn can be used in a variety of applications.

Keywords: Antimicrobial, Eugenol, *Neopestalotiopsis*, *Diaporthe*, *Cinnamomum loureiroi*, Antioxidant

3.1 Introduction

Endophytes, mostly identified as fungi and bacteria, are microorganisms residing inside healthy plant hosts without causing any apparent negative effects (Strobel et al., 2004). Most endophytes are classified as ascomycetes and anamorphic fungi (Arnold et al., 2003). Individual plant species may be a host to at least one endophytic fungus, thus the potential of discovering novel endophytic species is high. The relationship between fungal endophytes and plant hosts is intimate. The host plants deliver nutrients to endophytes, whereas endophytes produce secondary metabolites against various pathogenic microorganisms resulting in increasing plant growth rate (Berg, 2009; Ownley et al., 2010). Bioactive metabolites produced by fungal endophytes may be applied as sources of novel natural products for exploitation in agriculture, medicine, and other industries (Strobel et al., 2004). Given the broad range of endophytic fungi and their metabolic potential, there are good opportunities to investigate new endophytes colonizing medicinal plants in different ecosystems.

In recent years, the burden of human infectious diseases has increased tremendously worldwide. Current treatment of infections involves using chemical synthetic drugs, which have many side effects. The most notable side effect is

development of drug resistance, which has been noted in both human and plant pathogens. Consequently, the effectiveness of old antibiotics is reduced significantly. Thus, there is an urgent need to investigate newer and safer antimicrobial agents. Endophytic fungi may be a better source of antimicrobial compounds than synthetic drugs. Therefore, analysis of antimicrobial activity of natural products produced by endophytic fungi present alternative ways for drug development to control pathogens resistant to antibiotics.

Cinnamomum plants belong to the Lauraceae family and contain more than 300 evergreen aromatic plant species (Ravindran et al., 2003). They have been also employed as a health promoting agent for the treatment of inflammation, gastrointestinal, and urinary disorders (Brierley & Kelber, 2011) as well as potential antibacterial activity (Al-Jiffri et al., 2011). Four *Cinnamomum* species including *Cinnamomum zeylanicum*, *Cinnamomum loureiroi*, *Cinnamomum burmannii*, and *C. aromaticum* are important economic crops because of their numerous culinary uses as spices (Bandara et al., 2012). Of these four species, *Cinnamomum loureiroi* has been also used in Chinese traditional medicine as neuroprotective agent (Khasnavis & Pahan, 2012) and for the treatment of diabetes (Kim et al., 2006). Given the broad range of benefits of *Cinnamomum* plants, some studies have explored the metabolic potential of their endophytic fungi. For instance, *Muscodora albus*, a Xylariaceae fungus isolated from *Cinnamomum zeylanicum*, inhibited fungal and bacterial growth (Strobel et al., 2001). A total of 26 endophytic fungi were isolated from various parts of *Cinnamomum burmannii* and reported to contain antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* (Ilyas et al., 2015). Santiago et al. (2012) reported production of 5-hydroxyramulosin by *Cinnamomum mollissimum*, which inhibited growth of the pathogenic fungus *Aspergillus niger* and murine leukemia cells.

Some endophytes were reported to be able to produce the same set of or similar bioactive compounds as found in their host plants. Production of active compounds by endophytes is a result of a long period of co-evolution between endophytes and their host plants. An example of active compounds produced by endophytes is eugenol, 4-allyl-2-methoxyphenol. It is a volatile phenolic compound presenting in a variety of medicinal plants including plants in the genus *Cinnamomum* (Chericoni et al., 2005). Eugenol is known to have important pharmacological activities including anaesthetic,

antioxidant, antimicrobial, antihelmintic, anti-inflammatory, anticarcinogenic, antifumigant, and antirepellent properties (Bezerra et al., 2017). Although eugenol has been found in many plants, it was also detected in several endophytes. Cheng et al. (2014) reported that eugenol was isolated from endophytic fungus *Annulohypoxylon stygium* BCRC 34024. Eugenol was also found in bacterial endophytes isolated from *Mentha piperita* reported by Kumar and Mathur (2017). Moreover, methyl eugenol, a eugenol derivative, was also reported to be produced by endophytic fungus in *Alternaria* species from *Rosa damascena* (Kaul et al., 2008). Production of active compounds by endophytes resulted from long period of co-evolution with a friendly relationship between endophytes and their host plant. Some endophytes were reported to produce the same or similar bioactive compounds as found in their host plants.

Previous studies have found that the endophytic fungi isolated from *Cinnamomum* plants contain several phytochemical constituents inhibiting pathogenic microorganisms. Nonetheless, there has been no study on diversity and chemical profile of endophytic fungi associated with *Cinnamomum loureiroi*. Therefore the purpose of this work was to extract secondary metabolites produced by endophytic fungi isolated from *Cinnamomum loureiroi* and screen for antimicrobial and antioxidant activities. The chemical constituents of fungal endophytes with great antimicrobial and antioxidant activities were also investigated.

3.2 Materials and Methods

3.2.1 Plant Material and Chemicals

Cinnamomum loureiroi were collected from Mae Fah Luang University Botanical Garden, Mae Fah Luang University, Chiang Rai province, Thailand (20°2'42"N, 99°53'41"E) during May 2015. A voucher herbarium specimen (MFLU No. 10021) was identified and deposited at the Mae Fah Luang University Botanical Garden, Chiang Rai, Thailand. Standard C₁₁–C₁₉ *n*-alkanes were purchased from Fluka, Germany. Ethyl acetate solvent (analytical reagent grade) was purchased from Merck (Darmstadt, Germany).

3.2.2 Isolation, Culturing, and Morphology of Fungal Endophytes

The surface of healthy *Cinnamomum loureiroi* leaves was pre-sterilized by washing with deionized water. The surface was further sterilized by immersing in 70% ethanol for 60 s, 1% sodium hypochlorite (NaClO) for 60 s prior washing with sterile deionized water for 60 s. The sterilized leaves were cut into 0.5 cm² size and then immediately placed on potato dextrose agar (PDA) medium plate mixed with chloramphenicol antibiotic (100 µg/mL) to suppress the growth of bacteria. The culture medium plates were incubated at room temperature (27 °C) for three days until growth of the fungal hyphae was visible. All endophytic fungi that formed colonies were separated and cultured on new PDA plates and then incubated at room temperature (27 °C) for a week. Seven mycelial agar plugs of endophytic strain were placed into 500 mL Erlenmeyer flask containing 150 mL of potato dextrose broth. The cultures were incubated at room temperature (27 °C) for a month prior to the extracting process. The isolated fungal endophytes were deposited at the Center of Excellence in Fungal Research, Mae Fah Luang University under strain accession MFLUCC15-1112, MFLUCC15-1113, and MFLUCC15-1130 – MFLUCC15-1138. The morphological characteristics, for example, colony morphology, growth pattern, hyphae, and spore of isolated fungal endophytes were studied for preliminary identification.

3.2.3 Mycelial Culture Extraction

After incubating for 1 month, the broth culture was filtrated with WhatmanTM filter paper No. 3 under vacuum using Buchner funnel to separate the culture broth and mycelia. The medium broth and ground mycelia were portioned and macerated with ethyl acetate with ratio of 1:1 and 1:1.5 v/v, respectively, using modified method of Monggoot et al. (2017). Mycelial fraction was further sonicated using ultrasound-assisted extraction (Crest/690DAE; GuangDong GT Ultrasonic, Guangdong, China) at room temperature for 5 h. All fractions were combined and concentrated by using a rotary evaporator (Buchi Rotavapor Model R114, BUCHI) under reduced pressure. The obtained extract was subsequently stored at 4 °C until further use.

3.2.4 Screening of Antibacterial Activity

Antibacterial activity of 11 isolated endophytic fungal extracts was screened against pathogenic bacteria obtained from Department of Medical Sciences, Bangkok, Thailand. Antibacterial activity was evaluated against three Gram-positive (*Bacillus cereus* ATCC 11778, *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* ATCC 12228) and three Gram-negative (*E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella typhimurium* ATCC 13311) bacterial pathogens. Antibacterial activities were determined by using a disc diffusion assay. Initially, all crude extracts from isolated fungal endophytes were prepared with a concentration of 1,000 µg/mL using ethyl acetate as a solvent. The obtained solution was then serially diluted using the twofold dilution method until obtaining the concentration of 500, 250, 125, 62.50, 31.25, 7.81, and 3.91 µg/mL, respectively. A single colony of each pathogenic bacterium was activated by culturing in tryptic soy agar medium followed the 0.5 McFarland standard. All pathogenic strains were further cultured on Mueller Hinton agar medium plate. A total of 20 microliters of different diluted solutions were individually loaded into sterilized six mm-diameter filter paper discs (WhatmanTM, Sigma-Aldrich Corp., St. Louis, MO, USA) and placed onto agar medium plates suppressing bacterial pathogens. Chloramphenicol (20 µg/disc; Sigma-Aldrich, St. Louis, MO, USA) and ethyl acetate solvent was used as positive and negative control, respectively. Plates were then incubated at 37 °C for 24 h. All experiments were performed in triplicate. The inhibition diameter zone values of all endophytic fungal extracts at a concentration of 1,000 µg/mL and chloramphenicol were reported as mean ± standard deviation. The minimum inhibitory concentration (MIC) values were also determined. The variation of inhibition zone values was examined by one-way ANOVA (at $P < 0.05$)

3.2.5 Screening of Antifungal Activity

The antifungal activity of all crude endophytic fungal extracts was investigated against *Candida albicans* ATCC 90028 and *Aspergillus niger* ATCC 11414 using disc diffusion assay. All crude extracts were prepared as used in antibacterial activity screening. Both pathogenic fungi were cultured on Sabouraud dextrose agar media and incubated at 30 °C for a week. A plug of 1-week old fungal

culture (six mm diameter) was then placed on the center of the sterilized plates and incubated at 30 °C for 5 days. The six mm filter paper discs (no. 3; Whatman, Maidstone, UK) moistened with 20 microliters of each crude extract solution were placed onto the surface of culture plates. The plates were incubated at 30 °C for a week in while the fungal growth was also monitored. The growth inhibition of all endophytic fungal extracts against both fungal strains was measured as the percentage of inhibition of a radical growth relative to the control following formula: Percentage of inhibition (%) = $100 \times (1 - (\text{radical growth of treatment (mm)} / \text{radical growth of control (mm)}))$. All experiments were performed in triplicate. Ethyl acetate was used as negative control. In addition, MIC value of each sample were evaluated for their antifungal activity against lowest concentration of fungal extract inhibiting the visible growth of each pathogen on the agar plate. The growth inhibition and MIC are shown with the value of mean $\pm$ SD.

3.2.6 Screening of Antioxidant Activity

The electron or hydrogen atom donation or ability of endophytic fungal extracts was measured from the decolorizing of the purple colored solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) dissolving in methanol solvent. All endophytic fungal solutions were prepared at various concentrations (1,000, 500, 250, 125, 62.50, 31.25, 7.81, and 3.91 $\mu\text{g/mL}$) in methanol. One mL of various solutions of fungal extract was added to a one mL of 60 mM DPPH methanolic solution. The solution was mixed vigorously and incubated in dark place at room temperature for 30 min. After incubation time, the absorbance of all solutions was measured at 517 nm with a Perkin–Elmer–Lambda 25 UV/vis spectrophotometer. The antioxidant activity of endophytic fungal extracts was determined as percentage inhibition of DPPH (I%) using following equation: $I\% = 100 \times (A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}$ where A_{control} is the absorbance of the control solution (containing all solutions except the fungal extract), and A_{sample} is the absorbance of the crude extract solution. The antioxidant activity of all endophytic fungal extracts was expressed as IC_{50} value (mg/mL) and shown in Table 3.1. Trolox was used as standard for comparison.

Table 3.1 Antioxidant activity of endophytic fungal extracts and standard drugs, expressed as half maximal inhibitory concentration (IC₅₀) values using 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay

Crude fungal extract/standard	IC ₅₀ (µg/mL)
<i>Pestalotiopsis</i> sp. MFLUCC15-1112	77.23 ± 0.28
<i>Colletotrichum</i> sp. MFLUCC15-1113	1,067.25 ± 5.23
<i>Neopestalotiopsis</i> sp. MFLUCC15-1130	32.56 ± 0.66
<i>Diaporthe</i> sp. MFLUCC15-1131	20.78 ± 0.45
<i>Botryosphaeria</i> sp. MFLUCC15-1132	-
<i>Pestalotiopsis</i> sp. MFLUCC15-1133	88.36 ± 0.27
<i>Colletotrichum</i> sp. MFLUCC15-1134	1,224.23 ± 0.24
<i>Diaporthe</i> sp. MFLUCC15-1135	56.32 ± 1.35
<i>Diaporthe</i> sp. MFLUCC15-1136	87.14 ± 1.05
<i>Colletotrichum</i> sp. MFLUCC15-1137	-
<i>Fusarium</i> sp. MFLUCC15-1138	98.04 ± 1.11
Trolox	4.28 ± 0.23
Gallic acid	13.55 ± 0.14

Note The average IC₅₀ value and its standard deviation were calculated from a triplicate experiment. Symbol (–) indicates that the antioxidant activity was not detected.

3.2.7 Genomic DNA Extraction and Polymerase Chain Reaction

MFLUCC15-1130 and MFLUCC15-1131 were selected for genomic DNA extraction and polymerase chain reaction (PCR) due to their notable antibacterial properties. The selected fungal endophytes were grown on PDA for 1 week and the aerial mycelium of each isolate was scraped from the PDA surface. The fungal biomass was then freeze-dried and ground into a fine powder with a pestle and mortar. A modified SDS-CTAB method (Suwannarach et al., 2013) was applied for this experiment. The internal transcribed spacer (ITS) 1-5.8S-ITS2 region of ribosomal DNA was amplified by PCR using ITS4 (5'-TCCTCCGCTTATTGAT-ATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAA CAAGG-3') primers (White et

al., 1990) under the following thermal cycling conditions: initial denaturation at 95 °C for 2 min, 30 cycles of 95 °C for 30 s, 50 °C for 30 s 72 °C for 1 min, and final extension at 72 °C for 10 min on a PeqSTAR 2× thermal cycler (Peqlab, Erlangen, Germany). Negative controls lacking fungal DNA were run in parallel. PCR products were checked on 1% agarose gels stained with ethidium bromide under UV light and purified using NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel, Düren, Germany). The purified PCR products were directly sequenced at 1ST Base Company (Malaysia) using the same PCR primers mentioned above. Sequences were used to query GenBank via BLAST (<http://blast.ddbj.nig.ac.jp/top-e.html>).

3.2.8 Phylogenetic Analysis

The assembled contigs were subjected to BLAST search against the publicly available NCBI database to exclude bacterial contamination and to get a preliminary identification of the genera. Two ITS1-5.8S-ITS2 datasets were assembled. MFLUCC15-1130 and MFLUCC15-1131 were evaluated in a group of *Xylariales* and *Diaporthales*, respectively. Nucleotide sequences were aligned using MAFFT (default parameters) (Katoh & Toh, 2010). The alignment was further subjected to minor manual adjustments and then trimmed with trimAl (automated option) (Capella-Gutiérrez et al., 2009). After trimming, MFLUCC15-1130 contained 656 sites while MFLUCC15-1131 had 670 sites. A maximum likelihood phylogenetic tree was constructed using RAxML (Stamatakis, 2006) and the general time reversible model of nucleotide substitution. Bootstrap values were obtained from 1,000 replicates. A Bayesian inference (BI) (Ronquist & Huelsenbeck, 2003) analysis, available on CIPRES Science Gateway (<http://www.phylo.org/portal2/home.action>) (Miller et al., 2010), was also performed. Four Monte Carlo Markov chains were run for 1,000,000 generations. Convergence was declared when all retention indices (RI) parameters converged toward zero.

3.2.9 Analysis of Volatile Compounds by Gas Chromatography-Mass Spectrometry

The volatile constituents of crude extracts from strains MFLUCC15-1130 and MFLUCC15-1131 were identified by using a Hewlett Packard model HP6890 gas chromatograph (GC) (Agilent Technologies, Palo Alto, CA, USA), equipped with a

HP-5MS (5% phenylpolymethylsiloxane) capillary column (30 m × 0.25 mm × 0.25 µm; Agilent Technologies, Santa Clara, CA, USA). Helium was used as a carrier gas with a flow rate one mL/min, whereas the split ratio of column was operated to 1:50. The oven temperature program was started at 60 °C and increased up to 200 °C, at intervals of 2 °C/min. The GC was coupled to a mass spectrometer (MS) with HP model 5973 (Agilent Technologies, Palo Alto, CA, USA). The electronic ionization energy was set up at 70 eV, while mass range was scanned from 33 to 600 Da with scan rate at 10 spectra/s. Each crude extract was diluted 1:50 in ethyl acetate solvent. Automatically, one µL of solution was injected to the injection port of GC with split mode. The temperature of injector and detector were set at 250 and 280 °C, respectively. Data analysis was performed with ChemStation software (Agilent, Santa Clara, CA, USA). The volatile compounds of crude extract were tentatively identified by comparing the obtained fragmentation patterns of mass spectra with those of verified standards in the National Institute of Standards and Technology database. Linear RI to homologous series of *n*-alkanes, C₈-C₁₇, were calculated to those obtained by Kovat retention index method. Different RI databases of volatile compounds were obtained from Adams (2017), <http://www.pherobase.com> and http://www.flavornet.org/f_kovats.html.

3.3 Results

3.3.1 Isolation of Fungal Endophytes of *Cinnamomum Loureiroi* Leaves

A total of 11 fungal endophytes were isolated from *Cinnamomum loureiroi* leaves and then deposited at Center of Excellence in Fungal Research, Mae Fah Luang University, Thailand to obtain a strain accession number. Six genera were characterized based on morphological characteristics including conidiophore, growth rate, colony color, and texture. Colonies and morphologies of all isolated endophytic fungi are shown in the Supplemental Material (Figure 3.5). *Colletotrichum* genus was detected in stains MFLUCC15-1113, MFLUCC15-1134, and MFLUCC15-1137, *Diaporthe* genus was observed in strains MFLUCC15-1131, MFLUCC15-1135, and MFLUCC15-1136, *Pestalotiopsis* was found in strains MFLUCC15-1112 and

MFLUCC15-1133. *Neopestalotiopsis*, *Botryosphaeria*, and *Fusarium* genus was obtained in a single strain from MFLUCC15-1130, MFLUCC15-1132, and MFLUCC15-1138, respectively. The isolated endophytic fungi were cultured in PDA plates with colonies being approximately 6.0–7.0 cm in diameter after incubating at room temperature (27 °C) for a week. Colonies of *Colletotrichum* strain MFLUCC15-1113 were light gray at the border of culture, while the conidial mass of MFLUCC1115-1134 was pale gray at the center and MFLUCC1115-1137 was slightly white and their hyphae were swollen in only half the culture, the pattern of growth layer by *Diaporthe* strains MFLUCC15-1131, MFLUCC15-1135, and MFLUCC15-1136 was grown from mass center. The colony color of MFLUCC15-1131, MFLUCC15-1135 was white, whereas MFLUCC15-1136 presented pastel gray color. *Pestalotiopsis* strain MFLUCC15-1112 was pale gray color with thick-walled and smooth hyphae distribution, while the hyphae of strains MFLUCC15-1130 and MFLUCC15-1133 were septate and formed a white ring colony. Moreover, the sporulation of *Pestalotiopsis* strain was identified by its spore, which consisted of four to five conidia and appendages being dark brown. Colonies of *Fusarium* strains were grayish, but slightly yellow in the center. The growth rate of this genus was rather slow comparing to all other strains in this study. The colony of *Botryosphaeria* strains was white with abundant aerial mycelia

3.3.2 Screening of Antimicrobial and Antioxidant Activities

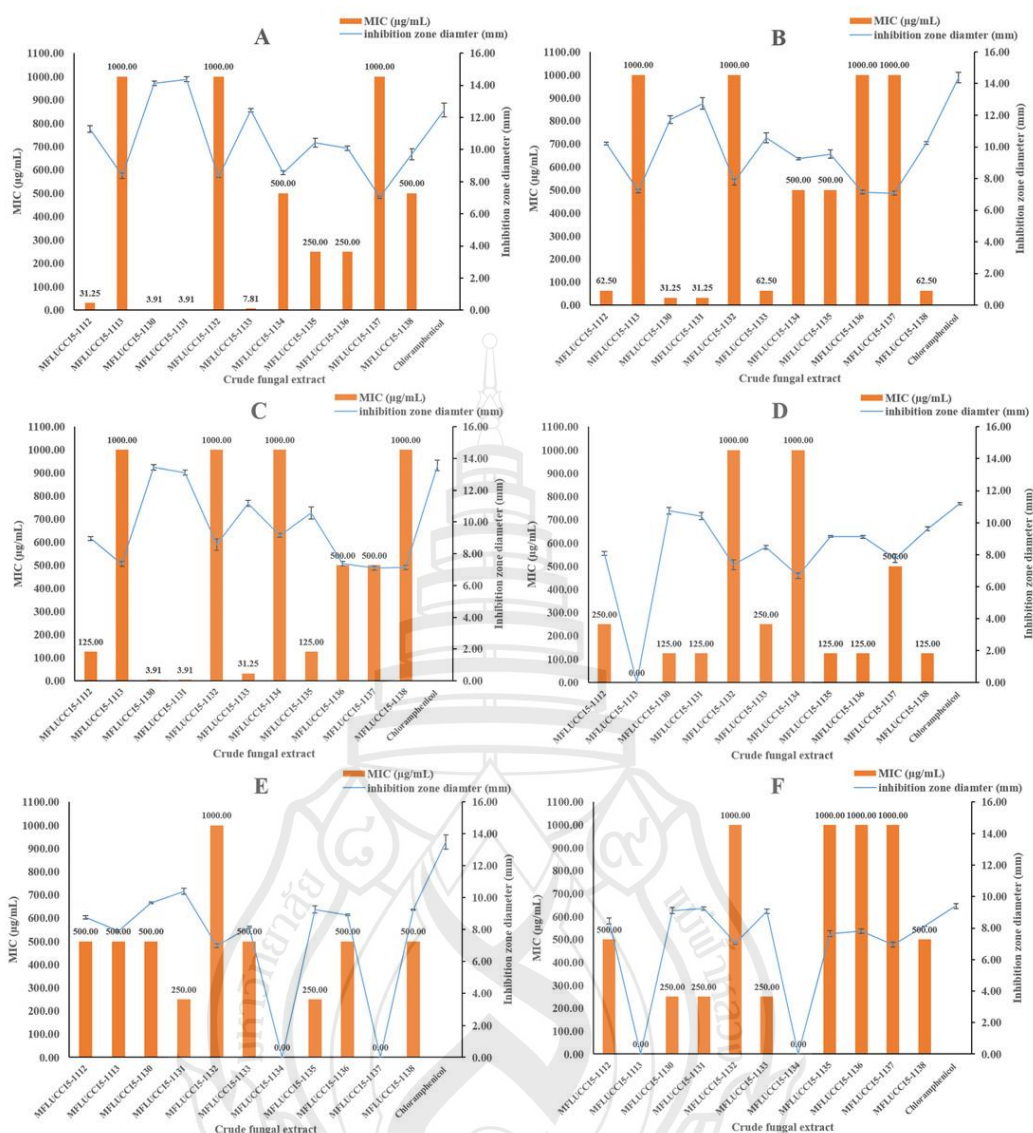
The *in vitro* antimicrobial activity of 11 endophytic fungal extracts against pathogenic strains from both Gram-positive, Gram-negative bacteria, and fungi was screened using the disc diffusion method by determining inhibition zone diameters. All crude extracts showed antibacterial activities at a concentration of 1,000 µg/mL based on these inhibition zone diameters (Figure 3.1). Most fungal extracts were effective on all tested bacteria, with inhibition zones ranging from 7.04 to 14.37 mm. Interestingly, crude extracts of strain MFLUCC15-1130 and MFLUCC15-1131 produced inhibition zone diameter against *B. cereus* much larger than those obtained of chloramphenicol, suggesting that they were more active than this antibiotic. Crude extracts of strains MFLUCC15-1113, MFLUCC15-1132, MFLUCC15-1134, MFLUCC15-1137, and MFLUCC15-1138 presented lower antibacterial activity

against test bacterial pathogens than other crude extracts, while broad spectrum antimicrobial activity with the greatest inhibition was observed by crude extracts of fungus MFLUCC15-1130 and MFLUCC15-1131 against all tested pathogens. Crude extracts of fungus MFLUCC15-1113 and MFLUCC15-1132 had no antibacterial activity against *E. coli*, while MFLUCC15-1134 and MFLUCC15-1137 fungal extracts had no effect on *P. aeruginosa*. In addition, MFLUCC15-1113 and MFLUCC15-1134 fungal crude extracts presented less antibacterial activity against *Salmonella typhimurium*. As noticed, Gram-positive bacterial strains were much more sensitive to crude extracts and chloramphenicol than Gram-negative strains. The MIC of antibacterial activity assay was also measured for these 11 endophytic fungal extracts by using the disc diffusion method. The MIC results are shown in Figure 3.1. These antibacterial activity assays indicated that crude extracts of strain MFLUCC15-1130 and MFLUCC15-1131 have a very strong activity with MIC ranging from 3.91 to 500 µg/mL and 3.91 to 250 µg/mL, respectively, while the crude extract of strain MFLUCC15-1132 had less antibacterial activity with MIC of 1,000 µg/mL against all tested bacteria. All crude extracts inhibited the growth of Gram-positive bacteria including *B. cereus*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*.

A disc diffusion assay was also used to investigate the antifungal activity of 11 endophytic fungal extracts at a concentration of 1,000 µg/mL against two fungal strains by determining the percentage of radical growth inhibition. Most crude extracts at concentrations of 1,000 µg/mL showed antifungal activity inhibiting the mycelial growth of *Candida albicans* and *Aspergillus niger* as shown in Figure 3.2 ranging from 28.09% to 77.89%. MFLUCC15-1112 and MFLUCC15-1138 crude fungal extracts exhibited a lower inhibition percentage. MFLUCC15-1137 crude extract had no fungicidal activity against *Candida albicans* while no fungicidal activity against *Aspergillus niger* was detected in the crude extracts of fungus MFLUCC15-1113 and MFLUCC15-1134, respectively. The MIC value of all crude extracts against *Candida albicans* and *Aspergillus niger* are also summarized in Figure 3.2. The greatest antifungal activity against both tested strains was presented by MFLUCC15-1130 and MFLUCC15-1131 fungal extracts which had MICs of 62.50 and 31.25–62.50 µg/mL, respectively.

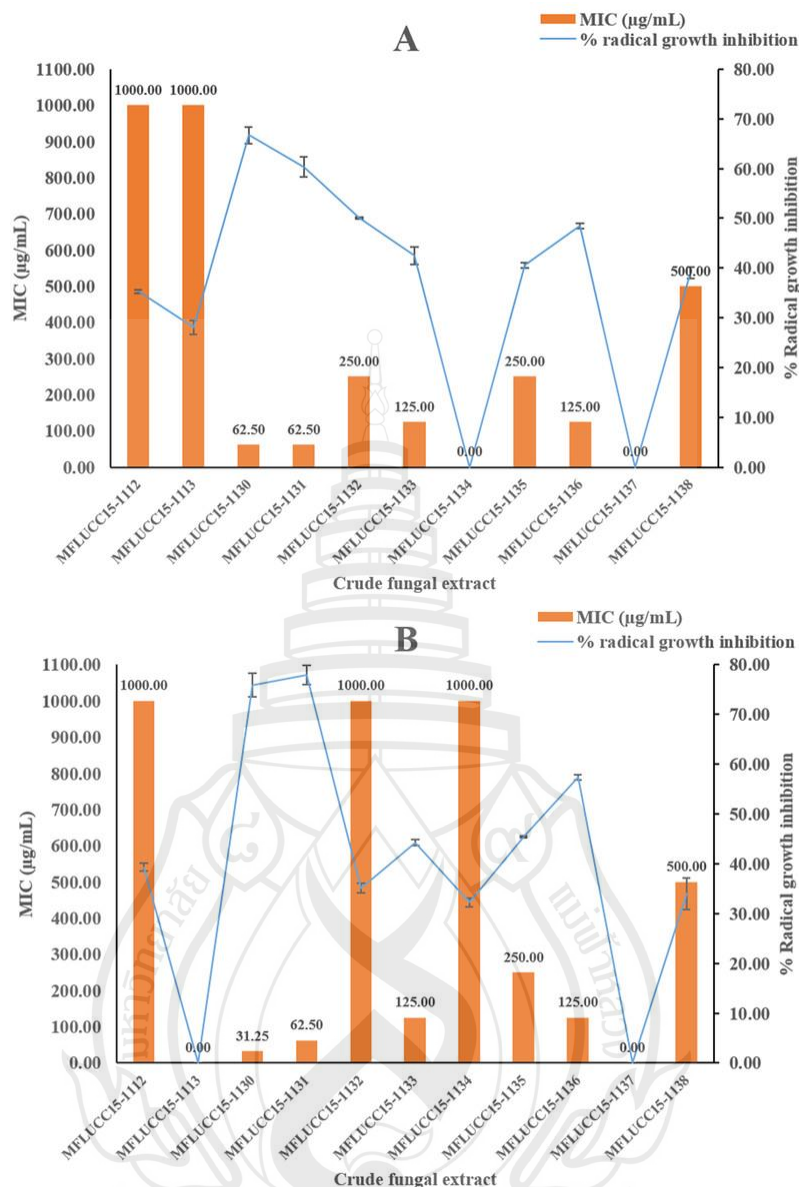
The antioxidant activity using DPPH radical scavenging assay was investigated for the 11 fungal extracts. Table 2.1 shows IC_{50} values of the fungal crude extracts ranging from 22.92 ± 0.67 to $>1,000 \mu\text{g/mL}$. The crude extracts exhibiting the greatest scavenging effects was obtained from fungus MFLUCC15-1131 with IC_{50} of $22.92 \pm 0.67 \mu\text{g/mL}$ while MFLUCC15-1131 exhibited the second best IC_{50} of $37.61 \pm 0.49 \mu\text{g/mL}$ as compared to the positive control, trolox which was $4.47 \pm 0.02 \mu\text{g/mL}$, respectively.





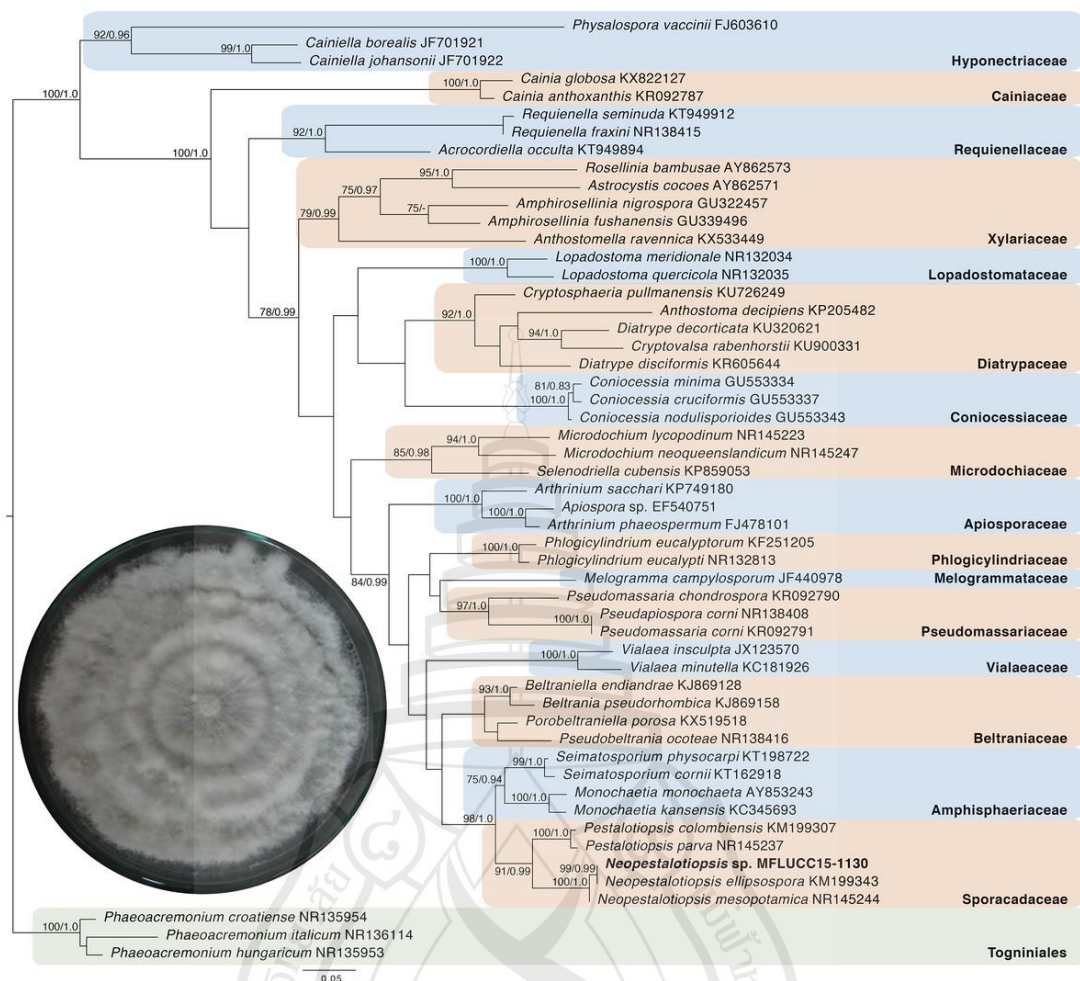
Note Each bar graph indicates the average inhibition zone diameter (mm) of crude fungal extracts and chloramphenicol at a concentration of 1,000 µg/mL and 20 µg/mL, respectively (triplicate analysis). No standard deviation value was obtained from this experiment. Each line represents the MIC (µg/mL) of crude fungal extracts against each bacterial pathogen tested. Each error bar was calculated from a standard deviation value derived from a triplicate experiment.

Figure 3.1 Antibacterial activities of crude fungal extracts and chloramphenicol against *B. cereus* (A), *S. aureus* (B), *S. epidermidis* (C), *E. coli* (D), *P. aeruginosa* (E), and *S. typhimurium* (F), expressed as an inhibition zone diameter and MIC



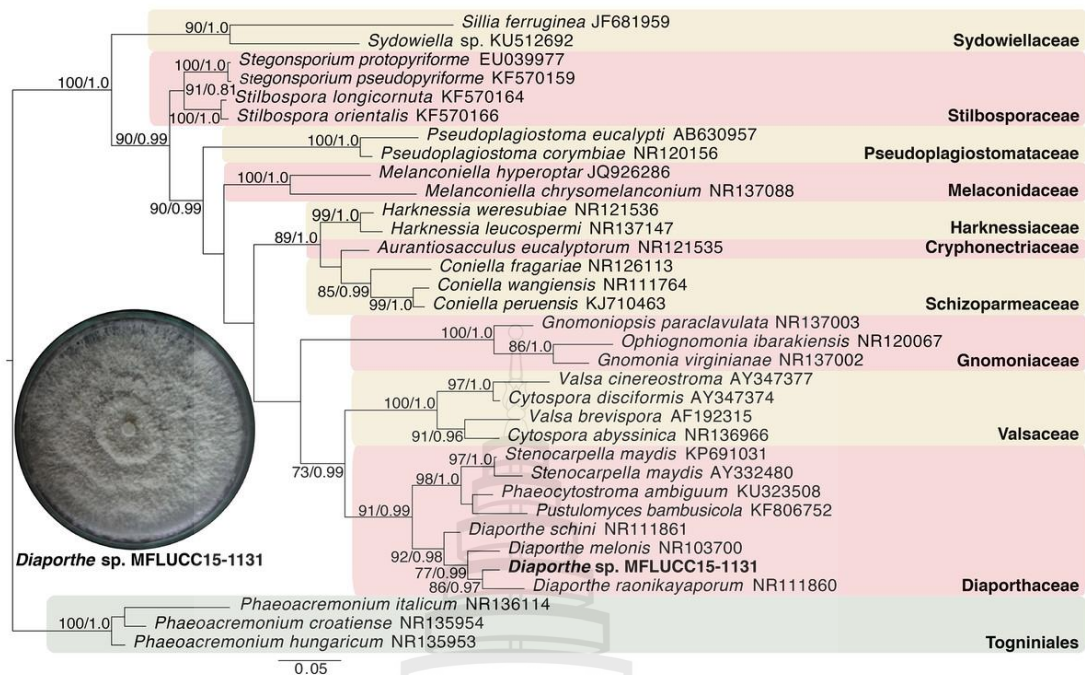
Note Each bar graph indicates the percentage of radical growth inhibition of crude fungal extracts at a concentration of 1,000 µg/mL (triplicate analysis). No standard deviation value was obtained from this experiment. Each line indicates the MIC (µg/mL) of crude fungal extracts against each fungal pathogen tested. Each error bar was calculated from a standard deviation value derived from a triplicate experiment.

Figure 3.2 Antifungal activities of crude fungal extracts against *C. albicans* (A) and *A. niger* (B), expressed as percentage of radical growth inhibition and MIC



Note This phylogenetic tree was analyzed by ITS4-5.8S-ITS5 inferred 50 taxa under the GTR model of nucleotide substitution. The newly sequenced species is shown in bold texts. Numerical values at the nodes indicate bootstrap support and posterior probabilities in this order. Only bootstrap support values over 70 are shown.

Figure 3.3 Phylogenetic tree of *Neopestalotiopsis* sp. MFLUCC15-1130



Note This phylogenetic tree was analyzed by ITS4-5.8S-ITS5 inferred 31 taxa under the GTR model of nucleotide substitution. The newly sequenced species is shown in bold texts. Numerical values at the nodes indicate bootstrap support and posterior probabilities in this order. Only bootstrap support values over 70 are shown.

Figure 3.4 Phylogenetic tree of *Diaporthe sp. MFLUCC15-1131*

3.3.3 Molecular Phylogenetic Analysis of MFLUCC15-1130 and MFLUCC15-1131

Given the great antimicrobial and antioxidant activities of crude extracts of MFLUCC15-1130 and MFLUCC15-1131, molecular phylogenetic analysis was used to complement morphology. The phylogenetic trees of the endophytic fungal strain MFLUCC15-1130 and MFLUCC15-1131 are shown in Figure 3.3 and 3.4, respectively. Strain MFLUCC15-1130 grouped in the family Sporocadaceae consisting of the genus *Neopestalotiopsis* and *Pestalotiopsis* with a supporting value of 91BS/0.99BI. The new strain sequence was also close to *Neopestalotiopsis ellipsospora* KM199343 with strong supported value of 99BS/0.99BI. Strain sequences of MFLUCC15-1131 grouped in the family Diaporthaceae and within genus *Diaporthe* with a supporting value of 92BS/0.98BI. The new isolate was sister to *Diaporthe raonikayaporum* (86BS/0.97BI). Gene sequences of the *Neopestalotiopsis* sp. MFLUCC15-1130 and strain *Diaporthe* sp. MFLUCC15-1131 were submitted to GenBank database under accession numbers KY646065 and KY646066, respectively.

3.3.4 Volatile Profiles of Fungal Crude Extract of MFLUCC15-1130 and MFLUCC15-1131

The fungal extracts obtained from *Neopestalotiopsis* sp. MFLUCC15-1130 and *Diaporthe* sp. MFLUCC15-1131 were subjected to detailed GC–MS analysis. Identified chemical constituents of crude extracts of fungus *Neopestalotiopsis* sp. MFLUCC15-1130 and *Diaporthe* sp. MFLUCC15-1131 are presented in Table 3.2. Similar profiles of chemical constituents were obtained among both crude fungal extracts. Exactly 34 constituents were identified in two samples. In *Neopestalotiopsis* sp. MFLUCC15-1130, 34 constituents were identified, representing 97.38% of the total crude extract. Eugenol, lauric acid, myristaldehyde, and caprylic acid were found as the main representatives. A total of 34 constituents were also identified in the *Diaporthe* sp. MFLUCC15-1131, representing 98.70% of the total crude extract. Similarly with *Neopestalotiopsis* sp. MFLUCC15-1130, this sample contained eugenol as the major representative with high content of 59.68%.

Table 3.2 Volatile compounds detected in crude fungal extracts of *Neopestalotiopsis* sp. MFLUCC15-1130 and *Diaporthe* sp. MFLUCC15-1131 using gas chromatography-mass spectrometry (GC–MS)

No.	Compound	RI**	RI***	Relative peak area (%) ± SD	
				<i>Neopestalotiopsis</i> sp. MFLUCC15- 1130	<i>Diaporthe</i> sp. MFLUCC15- 1130
1	Isovaleric acid	817	827	3.42 ± 0.07	-
2	Furfural	820	828	0.38 ± 0.30	1.69 ± 0.07
3	2-methyl-Butanoic acid	824	832	0.15 ± 1.18	-
4	4-Pentenoic acid	850	858	0.08 ± 0.71	-
5	2-methyl-Methyl pentanoate	861	871	0.29 ± 0.20	0.15 ± 0.37
6	Methional	888	901	-	3.20 ± 0.02
7	(2E,4E)-Hexadienal	890	907	1.11 ± 0.10	0.62 ± 0.09
8	(2E,4E)-Hexadienol	899	912	0.30 ± 0.19	0.15 ± 0.78
9	Methyl hexanoate	911	921	1.90 ± 0.03	1.12 ± 0.10
10	α-Pinene	930	939	3.19 ± 0.02	1.70 ± 0.07
11	β-Pinene	967	974	-	1.90 ± 0.03
12	3-Octanone	970	979	0.48 ± 0.12	0.20 ± 0.85
13	2-Amylfuran	974	94	0.21 ± 0.27	0.09 ± 0.65
14	Caprylaldehyde	989	998	0.20 ± 0.28	0.09 ± 0.62
15	p-Methyl anisole	1,005	1015	1.42 ± 0.08	0.71 ± 0.08
16	p-Cymene	1,010	1,020	1.06 ± 0.05	0.50 ± 0.12
17	o-Cymene	1,012	1,022	-	0.39 ± 0.15
18	trans-2-Octenal	1,035	1,049	0.89 ± 0.06	0.47 ± 0.12
19	5Z-Octenol	1,058	1,065	1.61 ± 0.04	0.73 ± 0.08
20	Caprylic alcohol	1,059	1,063	0.54 ± 0.11	0.39 ± 0.15
21	p-Cresol	1,062	1,071	4.71 ± 0.12	2.50 ± 0.02

Table 3.2 (Continued)

No.	Compound	RI**	RI***	Relative peak area (%) $\pm$ SD	
				<i>Neopetalotriopsis</i> sp. MFLUCC15- 1130	<i>Diaporthe</i> sp. MFLUCC15- 113
22	m-Cymene	1,072	1,082	1.28 $\pm$ 0.04	0.68 $\pm$ 0.09
23	o-Guaiacol	1,079	1,087	3.20 $\pm$ 0.02	1.73 $\pm$ 0.33
24	α -Pinene oxide	1,090	1,099	0.75 $\pm$ 0.15	0.40 $\pm$ 0.41
25	2-Phenylethyl alcohol	1,197	1,106	2.34 $\pm$ 0.05	1.23 $\pm$ 0.05
26	Octen-3-yl acetate	1,102	1,110	1.97 $\pm$ 0.09	1.12 $\pm$ 0.10
27	E-Tagetone	1,130	1,139	0.48 $\pm$ 0.48	0.38 $\pm$ 0.15
28	Z-Tagetone	1,139	1,148	1.09 $\pm$ 0.21	1.34 $\pm$ 0.04
29	Menthofuran	1,150	1,159	1.88 $\pm$ 0.06	1.13 $\pm$ 0.05
30	Caprylic acid	1,158	1,167	4.95 $\pm$ 0.02	2.58 $\pm$ 0.02
31	Methyl chavicol	1,189	1,195	2.57 $\pm$ 0.02	1.41 $\pm$ 0.04
32	(2E,4Z)-2,4-Decadienal	1,288	1,292	1.41 $\pm$ 0.12	0.83 $\pm$ 0.07
33	(2E,4E)-2,4-Decadienal	1,310	1,315	2.81 $\pm$ 0.04	2.24 $\pm$ 0.05
34	Methyl decanoate	1,318	1,323	4.06 $\pm$ 0.10	2.36 $\pm$ 0.02
35	Eugenol	1,350	1,356	34.12 $\pm$ 0.01	59.68 $\pm$ 0.01
36	Lauric acid	1,558	1,565	6.79 $\pm$ 0.14	2.35 $\pm$ 0.05
37	Myristaldehyde	1,604	1,611	5.72 $\pm$ 0.31	2.65 $\pm$ 0.02
Number of compounds				34	34
Total percentage of relative peak area				97.38	98.7

Notes For each compound, the average relative peak area and the standard deviation were calculated from a triplicate analysis.

Symbol (–) indicates that this compound was not detected in fungal extracts.

RI* values are the self-calculated retention indices on HP-5MS column.

RI** values are the retention indices obtained from the literature using the same column.

3.4 Discussion

In the present work, endophytic fungi of *Cinnamomum loureiroi* leaves were isolated and screened for their antimicrobial and antioxidant activities. *Cinnamomum loureiroi* is known for its antimicrobial activity and other medicinal properties. It is also widely used as food ingredient due to its attractive flavor and as a preservative. Moreover, there are no reports on endophytic fungi from this plant species. A total of 11 fungal endophytes were isolated from host plant in PDA medium plates, which grouped into six genera. Number and type of endophytic fungal genus obtained from *Cinnamomum loureiroi* differed when compared to those found in other *Cinnamomum* plant species. *Muscodor albus* was isolated from *Cinnamomum zeylanicum* and is also a key endophytic fungus of other *Cinnamomum* plants (Strobel et al., 2001). The same genus, *Muscodor cinnamomi* was also found from *Cinnamomum bejolghota* (Suwannarach et al., 2011). This finding implies that a variety of endophytic fungi inhabit different host plant species. The diversity of isolated endophytic fungi may be also largely dependent on the host plant and environment. Exploitation of fungal endophytes in plant tissues provides new possibilities in the search for active compounds. Endophytes are capable of producing novel active compounds that can be used as antimicrobials and antioxidants (Strobel et al., 2004). However, many fungal endophytes and their active compounds remain unknown. Therefore, the endophytic fungi from *Cinnamomum loureiroi* were studied and screened for their antimicrobial and antioxidant potential.

The antimicrobial activity of crude fungal extracts varied possibly due to the mechanism of antimicrobial action of their chemical constituents (Cowan, 1999). In this study, all microbial pathogens showed different sensitivity to the inhibitory action of crude fungal extracts. It was noted that all fungal extracts in this study demonstrated antimicrobial activity on some microbial pathogens, Gram-positive bacteria, Gram-negative bacteria, fungi, or yeast (Gouda et al., 2016). The bacteria and fungi used in this study were selected to cover both human and plant pathogens (Monggoot et al., 2017). The inhibitory activity of all crude fungal extracts indicated their effectiveness against the bacteria and fungi used herein. The crude extracts of

Neopestalotiopsis sp. MFLUCC15-1130 and *Diaporthe* sp. MFLUCC15-1131 had broad spectrum antimicrobial activity with the strongest antimicrobial activity against six bacterial and two fungal species compared to the other crude fungal extracts. In general, Gram-positive bacterial strains were more sensitive to crude extracts and chloramphenicol than Gram-negative. Because ethyl acetate was chosen as an organic solvent to obtain crude extracts, only organic compounds that have lipophilic/hydrophobic property were able to partition in it. Arulmozhi et al. (2018) reported that the crude extracts derived from extraction with ethyl acetate showed significant antimicrobial activity. Bacteria that have cell walls comprising with peptidoglycan could easily uptake these compounds into their cells. Diffusion of these organic compounds soluble in ethyl acetate through the microbial membranes results in alterations of membrane fluidity and permeability, disruption of membrane-embedded proteins, inhibition of respiration, and alteration of ion transport processes (Trombetta et al., 2005). The antimicrobial activities of both crude extracts may be attributed to the presence of high concentration of eugenol as reported previously (Devi et al., 2010; Shah, Davidson & Zhong, 2013; Hassan et al., 2018). In addition, the high concentration of other constituents, such as myristaldehyde (Xiangwei et al., 2006), lauric acid (Nakatsuji et al., 2009), caprylic acid (Kim & Rhee, 2016), may be also correlated to the observed antimicrobial activity. Differentiation of chemical constituents may be affected by specific enzyme action on natural biosynthesis pathway (Winter, Behnken & Hertweck, 2011) and different fungal endophyte species. The similarities in the chemical profiles between host and endophyte could stem from the long evolutionary history of symbiosis between host and fungi (Kusari, Hertweck & Hertweck, 2012) and in plant growth association. In addition, some secondary metabolites of the host and its fungal endophytes could have been enhanced with the various nutrients in the media (Dighton, 2003).

The DPPH assay results revealed high antioxidant activity in crude extracts of *Neopestalotiopsis* sp. MFLUCC15-1130 and *Diaporthe* sp. MFLUCC15-1131. High antioxidant activity of both extracts can be explained by the content of eugenol, which was much higher in the case of *Diaporthe* sp. MFLUCC15-1131 in comparison to crude extract of fungus *Neopestalotiopsis* sp. MFLUCC15-1130. It was noted that eugenol was the major antioxidant compounds between the two crude extracts. The

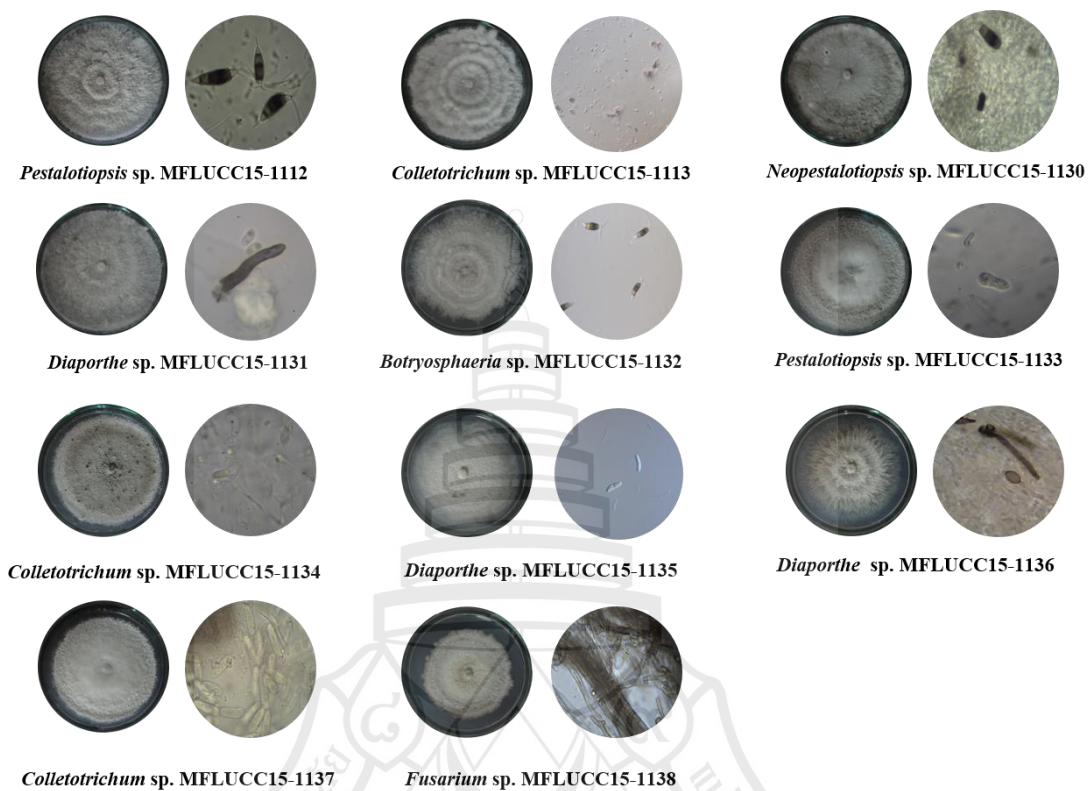
antioxidant activity of eugenol, as one of most abundant constituents in investigated crude fungal extracts, has been evaluated using both *in vitro* and *in vivo* methods (Ogata et al., 2000; Nagababu et al., 2010; Bezerra et al., 2017). Besides eugenol, synergistic activity of fatty acids, including lauric acid and caprylic acid could also account for the observed differences in antioxidant activity (Mahayothee et al., 2016). High antioxidant activity could also be due to the high amount of non-lipid compounds, such as methyl chavicol (Pripdeevech et al., 2010), p-cymene (De Oliveira et al., 2015) and methional (Sims & Fioriti, 1977), all of which were detected only in *Diaporthe* sp. MFLUCC15-1131.

Recently, eugenol, a major antimicrobial and antioxidant compound detected in *Neopestalotiopsis* sp. MFLUCC15-1130 and *Diaporthe* sp. MFLUCC15-1131, was also detected in various endophytes. Studies by Cheng et al. (2014) and Kumar and Mathur (2017) found that the endophytic fungus *Annulohyphoxylon stygium* BCRC 34024, and some bacteria isolated from *Mentha piperita* produced eugenol. Moreover, methyl eugenol, a derivative compound of eugenol was also produced by *Alternaria* species isolated from *Rosa damascaena* as reported by Kaul et al. (2008). This compound has been found as a major constituent in essential oils and extracts of *Cinamomum* host plants (Chericoni et al., 2005; Patel et al., 2007). Both endophytic fungi may have developed the ability to produce similar bioactive compounds as those originating from their host plants. Endophytes from *Neopestalotiopsis* and *Diaporthe* genus were commercially produced bioactive compounds and highly efficient in controlling microbial diseases (Bungihan et al., 2011; Xu, Ebada & Proksch, 2010). Thus, isolation of *Neopestalotiopsis* and *Diaporthe* sp. as endophyte from *Cinnamomum loureiroi* with activity against various pathogens is significant and should be further investigated.

3.5 Conclusion

This study presents a broad spectrum of antimicrobial activities of crude fungal extracts from *Cinnamomum loureiroi* leaves. Antimicrobial efficiency of 11 crude endophytic fungal extracts against six bacterial and two fungal strains was examined. Of the 11 crude fungal extracts studied the ones from *Neopestalotiopsis* sp. MFLUCC15-1130 and *Diaporthe* sp. MFLUCC15-1131 exhibited notable bactericidal and fungicidal activities against all tested bacterial and fungal pathogens in this study. The antioxidant potential of all crude extracts was also assessed for the first time in this plant species. *Neopestalotiopsis* sp. MFLUCC15-1130 and *Diaporthe* sp. MFLUCC15-1131 showed great antioxidant activity. The major constituents found in both endophytic fungi that could responsible for the observed antimicrobial potential were eugenol, lauric acid, myristaldehyde, and caprylic acid. The data reported in this study show that *Neopestalotiopsis* sp. MFLUCC15- 1130 and *Diaporthe* sp. MFLUCC15-1131 might provide an alternative safe way to fight microbes and reduce free radical contamination.

3.6 Supporting Information



Note Each colony was 15-day old when the picture was taken. All were cultured at room temperature (27 °C). Their morphology was captured under a microscope (100×).

Figure 3.5 Colonies and morphologies of endophytic fungi isolated from *C. loureiroi*

CHAPTER 4

ANTIFUNGAL ACTIVITY OF 8-METHOXYNAPHTHALEN-1-OL ISOLATED FROM THE ENDOPHYTIC FUNGUS *Diatrype* *Palmicola* MFLUCC 17-0313 AGAINST THE PLANT PATHOGENIC FUNGUS *Athelia rolfsii* ON TOMATOES²

Abstract

Thirty-four endophytic fungal isolates were obtained from the leaves of the medicinal plant *Polyscias fruticosa*, and their antagonistic activities against the growth of the common tomatoes plant pathogenic fungus *Athelia rolfsii* were initially screened using a dual culture assay. The endophytic fungus MFLUCC 17-0313, which was later molecularly identified as *Diatrype palmicola*, displayed the highest inhibition percentage (49.98%) in comparison to the others. This fungus was then chosen for further evaluation. Its culture broth and mycelia from a 10 L scale were separated and extracted using ethyl acetate, methanol, and hexane. Each extract was tested for antifungal activity against the same pathogen using a disc diffusion assay. Only the crude hexane extract of fungal mycelium showed antifungal activity. The hexane extract was fractioned using sephadex gel filtration chromatography and each fraction was tested for antifungal activity until the one with the highest inhibition percentage was obtained. The bioactive compound was identified as 8-methoxynaphthalen-1-ol using nuclear magnetic resonance spectroscopy and mass spectrometry. The minimum inhibition concentration of 8-methoxynaphthalen-1-ol

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<https://doi.org/10.3390/biom10050799>

was demonstrated at 250 µg/mL against the selected pathogen. Using the leaf assay, the solution of 8-methoxynaphthalen-1-ol was tested for phytotoxic activity against *A. rolfsii* and was found to have no phytotoxic effects. These results showed that 8-methoxynaphthalen-1-ol has the potential for controlling the growth of *A. rolfsii*, the cause of Southern blight disease on tomatoes. This study may provide the foundation for future use of this compound as a biofungicide.

Keywords: Antifungal activity, 8-methoxynaphthalen-1-ol, Endophytic fungi, Plant pathogen, Tomato, Southern blight, *Diatrype*, *Athelia rolfsii*

4.1 Introduction

Tomato (*Solanum lycopersicum* L.) is among the most popular and extensively consumed economic vegetable crop in the world. Each year, more than 100 million MT of tomatoes are produced by the major producers: China, USA, India, and Turkey (Singh et al., 2017). Production of tomatoes requires a delicate system. The quantity and quality of tomatoes are affected by various diseases across the pre- and post-harvest periods (Singh et al., 2017). Presently, there are more than 200 known diseases reported during the production of tomatoes, resulting in varying losses in overall product quantity and market values (Nowicki et al., 2013).

One of the most destructive tomato diseases is Southern blight, which is caused by infection with the basidiomycete fungus *A. rolfsii* (syn. *Sclerotium rolfsii*) and typically results in significant economic loss (Banyal et al., 2008). This disease is widespread in all tomato-growing areas throughout the world and appears at any stage of plant development during all seasons. The *A. rolfsii* fungus infects tomatoes directly and indirectly via wounds caused by nematodes or insects. Fruits, leaves, or branches that touch the soil may also be infected by the germinating sclerotia. Yield loss of tomato production caused by *A. rolfsii* infection has been reported to vary between 35% and 90% (Sikiroul et al., 2010).

Management of *A. rolfsii* infection has been performed using chemical

fungicides such as benomyl, sancozeb, thiovit, mancozeb, and carbendazim (Yaqub & Shahzad, 2006). These fungicides are able to inhibit the mycelial growth of *A. rolfsii* (Yaqub & Shahzad, 2006; Dwivedi & Prasad, 2016). One of the most effective fungicides used for controlling *A. rolfsii* infection is mancozeb (Dwivedi & Prasad, 2016). However, use of mancozeb has resulted in residue contamination in soil and crops, which may impact the environment and the health of consumers (Kaye et al., 2015; Paro et al., 2012). Moreover, the metabolite or the degradation product of mancozeb, ethylenethiourea (ETU), is classified as possibly carcinogenic to humans (Group 2B) (IARC, 1987). Exposure to mancozeb and ETU is a serious health concern. Therefore, a new fungicide that can control the growth of *A. rolfsii*, is environmentally friendly without impact on the environment and human health is highly needed.

Microorganisms are capable of producing bioactive compounds that display antimicrobial activities. A large number of microorganisms, especially endophytic fungi, have been studied for the production of their metabolic products that can be used as biocontrol agents to replace the use of synthetic fungicides (Gouda et al., 2016). For instance, (3R,4R,6R,7S)-7-hydroxyl-3,7-dimethyl-oxabicyclo[3.3.1]nonan-2-one and (3R,4R)-3-(7-methylcyclohexenyl)-propanoic acid were isolated from the endophytic fungus *Pestalotiopsis foedan* and showed significant antifungal activities against *Botrytis cinerea* and *Phytophthora nicotianae*. In addition, cladosporin, isolated from the endophytic fungus *Cladosporium cladosporioides*, was shown to inhibit the growth of *Colletotrichum acutatum*, *C. fragariae*, *C. gloeosporioides*, and *Phomopsis viticola* (Deshmukh et al., 2018).

Polyscias fruticosa (L.) Harms belongs to the family Araliaceae and is widely used in many countries of Southeastern Asia for medicinal and food purposes (Vo et al., 1998). This plant was reported to contain bioactive compounds such as saponins, oleanolic acid, and polyacetylenes that have antibacterial, antifungal, anti-inflammatory, antitoxin, antipyretic, molluscicidal, and anti-diabetic activities (Boye et al., 2018; Hanh et al., 2016). As such for the first time, the endophytic fungi that colonize within this plant were isolated and tested for their antifungal activities.

Because endophytic fungi have been important resources for antifungal compounds against phytopathogens, the present study aimed to isolate endophytes from the leaves of *Polyscias fruticosa*, screen for their antimicrobial activity against

A. rolfsii, and isolate the bioactive compound(s) from the crude extract(s) that showed significant antimicrobial activity. The obtained antifungal compound may be used as an alternative agent to control infection of *A. rolfsii* on tomatoes.

4.2 Materials and Methods

4.2.1 Plant Material

Healthy leaves of *P. fruticosa* were collected in January 2017 from Mae Chan District, Chiang Rai Province, Thailand (99°70'56"E, 20°10'36"N) for endophytic fungi isolation. The botanical specimens of this plant (MFL10011) were deposited at the Mae Fah Luang Botanical Garden of Mae Fah Luang University, Thailand.

4.2.2 Isolation of Endophytic Fungi

The isolation procedures were performed following the method described by Atiphasaworn et al. (2017). Healthy leaves of *P. fruticosa* were washed with running water for 2 min followed by 70% ethanol for 10 s and then sterilized with 1% sodium hypochlorite for 30 s and washed twice with sterile water. Sterilized leaf tissues were sectioned into 0.5 cm² pieces. Approximately 100 pieces were randomly selected and evenly distributed between 30 Petri dishes containing potato dextrose agar (PDA) mixed with chloramphenicol (30 µg/mL) to prevent bacterial growth. Petri dishes were sealed with sterile Parafilm prior to incubation at room temperature (27 °C) for 5 days. Petri dishes were observed daily, and the individual hypha tips of the emerging fungal colonies were sub-cultured on fresh PDA plates at room temperature (27 °C). All isolates were deposited at the Center of Excellence in Fungal Research, Mae Fah Luang University, Thailand (Voucher specimen no. MFLUCC 17-0280-MFLUCC 17-0313).

4.2.3 Isolation and Molecular Identification of The Phytopathogen *A. rolfsii*

For antifungal activity testing, the pathogen *A. rolfsii* was isolated from infected *S. lycopersicum* leaves following a slightly modified method described by Adesegun et al. (2013). The infected *S. lycopersicum* leaves were collected in July

2016 from a tomato farm in Sansai district, Chiang Mai province, Thailand (98°95' 58"E, 19°10'13"N). The infected leaves were washed with sterile distilled water and cut into five mm² segments. Their surface was sterilized using 1% sodium hypochlorite for 1 min and rinsed with sterilized distilled water three times. The segments were placed on Petri dishes to dry at room temperature (27 °C) and further placed on 10 Petri dishes containing PDA mixed with chloramphenicol (30 µg/mL) to prevent bacterial growth. The dishes were incubated at room temperature (27 °C) and observed for 5 days. The isolate was sub-cultured to obtain a pure culture of *A. rolfsii*, which was stored at room temperature. The pathogen was identified based on its morphological characteristics (Adesegun et al., 2013). The pathogenicity of this *A. rolfsii* isolate was confirmed by Koch's postulates. The aerial mycelium of *A. rolfsii* isolate was scraped from the PDA surface and pulverized with a mortar and pestle to obtain a mycelium pulp. The genomic DNA was extracted following the cetyltrimethyl ammonium bromide method (Elias et al., 2018). Two universal primers, ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5' - GGAAGTAAA-AGTCGTAACAAGG-3'), were used. Polymerase chain reactions (PCR) were performed at 95 °C for 5 min, followed by 40 cycles of 95 °C for 50 s, 52 °C for 50 s, 72 °C for 50 s, and 72 °C for 10 min on a PeqSTAR 2× thermal cycler (Peqlab, Germany). The PCR-amplified product was analyzed by gel electrophoresis on 1% agarose gels stained with ethidium bromide under UV light and purified using NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel, Germany). DNA sequencing was performed on an automated sequencing system ¹ST Base (Malaysia). The obtained sequence was used as a query to search for sequences in GenBank using the BLAST program (Basic Local Alignment Search Tool, <https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

4.2.4 *In vitro* Antifungal Screening by Dual Culture Assay

Endophytic fungal isolates were screened for their antifungal activity against the growth of *A. rolfsii* using the *in vitro* dual culture assay. PDA was used as a medium. For each combination of the pathogen and endophytic fungus, a mycelial plug (five mm in diameter) of *A. rolfsii* and fungal isolate were placed on Petri dishes containing PDA. The plates were incubated at 30 °C. Petri dishes inoculated only with

A. rolfsii were used as negative controls. All plates were incubated for seven days until the negative control plates were completely covered with *A. rolfsii* mycelia. The growth inhibition of each endophytic fungus against the tested pathogen was expressed as the inhibition percentage of a radical growth relative to the controls, using the following formula: Inhibition percentage (%) = $100 \times [1 - (\text{growth area of the treatment} / \text{growth area of the control})]$. All experiments were conducted in triplicate.

4.2.5 Fungal Cultivation and Extraction

From the results of the dual culture assay, the endophytic fungus MFLUCC 17-0313, which showed the strongest *in vitro* antifungal activity against *A. rolfsii*, was selected for further cultivation, extraction, isolation, and characterization of the active compound. This isolate was cultured in potato dextrose broth (PDB) using a method described by Atiphasaworn et al. (2017). Five mycelial agar plugs (five mm diameter, seven days old) of the endophytic fungus MFLUCC 17-0313 were placed in each 1,000 mL Erlenmeyer flask (40 flasks total) containing 250 mL of PDB. The flasks were incubated at room temperature (27 °C) for 28 days without shaking. The culture broth was separated from fungal mycelia by filtration through filter paper. The culture broth was extracted with an equal volume of ethyl acetate three times. The extracts were concentrated under vacuum using a rotary evaporator to produce dried broth extract. The mycelial fraction was macerated with methanol for two days and further concentrated using a rotary evaporator. They were dissolved in 200 mL of distilled water before being partitioned with an equal volume of hexane and ethyl acetate three times, respectively. The obtained hexane and ethyl acetate extracts were collected, and combined. Residue water was eliminated using anhydrous sodium sulfate. The extracts were evaporated using a rotary evaporator to obtain dried extracts from the hexane and ethyl acetate fraction, respectively. In addition, the same mycelia was macerated in dichloromethane for 2 days. The solution was filtered to obtain the dichloromethane extract. Residue water was eliminated using anhydrous sodium sulfate. The extract was evaporated using a rotary evaporator to obtain the dried dichloromethane extract. All extracts were stored at 4 °C until use.

4.2.6 *In vitro* Antifungal Activity by Disc Diffusion Assay

The antifungal activity of each crude extract obtained from the endophytic fungus MFLUCC 17-0313 was screened against *A. rolfsii* using the disc diffusion assay, as described by Tanapichatsakul et al. (2019). The dried crude extracts were dissolved using the individual extraction solvent to yield a concentration of 5 mg/mL. A plug of pathogenic fungal culture (five mm diameter, 5 days old) was placed on the center of the sterilized PDA plates and incubated at 30 °C for 2 days. A 6-mm filter paper disc (no. 3; Whatman, Maidstone, UK) moistened with 30 µL of crude extract solution was placed onto the surface of culture plates. Each crude extract solution was tested individually. The plates were incubated at 30 °C for 7 days and observed daily. Ethyl acetate, hexane, and dichloromethane were used as negative controls, while an amphotericin B solution (dissolved in distilled water to yield a concentration of 5 mg/mL) was used as a positive control (Sigma-Aldrich, USA). The growth inhibition of all endophytic fungal extracts against the tested fungal pathogen was expressed as the inhibition percentage of a radical growth relative to the control, using the following formula: inhibition percentage (%) = $100 \times [1 - (\text{radical growth of the treatment (mm)} / \text{radical growth of the control (mm)})]$. All experiments were conducted in triplicate.

4.2.7 Isolation of Antifungal Compound from The Endophytic Fungus MFLUCC 17-0313

According to the results of the disc diffusion assay, only the crude hexane extract of fungal mycelia displayed antifungal activity against *A. rolfsii*. Therefore, it was fractioned using Sephadex LH-20 (GE Health Care Bio-Sciences AB, USA) gel filtration chromatography (3× 84 cm column) and eluted with a mixture of methanol and dichloromethane (1:1 ratio) to yield a total of 24 fractions (F1–F24). All fractions were evaporated to dryness. The dried residue was dissolved with hexane to yield a solution with a concentration of 5 mg/mL. Each solution was tested for antifungal activity using the disc diffusion method. The F19 solution exhibited the strongest antifungal activity. Thin layer chromatography (TLC) was used to determine the purity of this solution. The TLC result indicated that the F19 solution contains only one compound. Therefore, the F19 fraction was subject to structural identification

using ^1H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy. ^1H - and ^{13}C -NMR spectra were recorded at 300 and 75 MHz, respectively, using a Bruker AVANCE 300 spectrometer with CDCl_3 as an internal standard. The obtained NMR data was compared to those found in the report of Emre (2018). A mass spectrum of the isolated compound was obtained using a Bruker MicroTOF_{LC} mass spectrometer (Bruker, United Kingdom).

4.2.8 Determination of the Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) value of the isolated antifungal compound against *A. rolfsii* was determined. Serial dilution was used to prepare seven concentrations (1,000, 500, 250, 125, 62.5, and 31.25 $\mu\text{g/mL}$) of the active compound in hexane. MIC was defined as the lowest concentration of the active compound that displayed visible growth inhibition of the pathogen on the agar plate. All experiments were performed in triplicate and the results were compared with the amphotericin B solution prepared at a concentration of 1,000 $\mu\text{g/mL}$.

4.2.9 Phytotoxic Assay

The phytotoxicity of the active compound was assayed using a leaf disk and absorption assay according to a method by Reveglia et al. (2019) with modifications.

The leaf disk assays were performed using fourteen-day old *S. lycopersicum* leaves. Disks of the lamina of plant leaves (six mm diameter) were prepared using a sharp corkborer. The compound was dissolved in a mixture of 0.25% tween 20 with 4% methanol into final concentrations of 1,000, 500, 250, and 125 $\mu\text{g/mL}$. The leaf disks were immersed in 1.0 mL of different compound solutions. After 18 h, the disks were washed with distilled water. The symptoms were visually observed using a 0 to four scale, where a 0 was no effect, 1 was area of necrotic spots <50%, 2 was area of necrotic spots 50-70%, 3 was area of necrotic spots 70-90%, and 4 was area of necrotic spots >90%. Distilled water and a mixture of 0.25% tween 20 with 4% methanol were used as negative controls whereas glyphosate dissolved in distilled water was used as a positive control. The experiment was carried out in triplicate.

In the leaf absorption assays, 14-day old *S. lycopersicum* leaves were cut and then placed in a tube containing 1.0 mL of compound solutions. The compound solutions were prepared by dissolving in a mixture of 0.25% tween 20 with 4%

methanol and tested at four different concentrations: 1,000, 500, 250, and 125 µg/mL. After 48 h, the leaves were moved to a new tube containing distilled water. The symptoms were visually observed after 48 h using a 0 to four scale, where a 0 was no effect, 1 was area of necrotic spots <50%, 2 was area of necrotic spots 50-70%, 3 was area of necrotic spots 70-90%, and 4 was area of necrotic spots >90%. Distilled water and a mixture of 0.25% tween 20 with 4% methanol were used as negative control whereas glyphosate dissolved in distilled water were used as positive control. The experiment was carried out in triplicate.

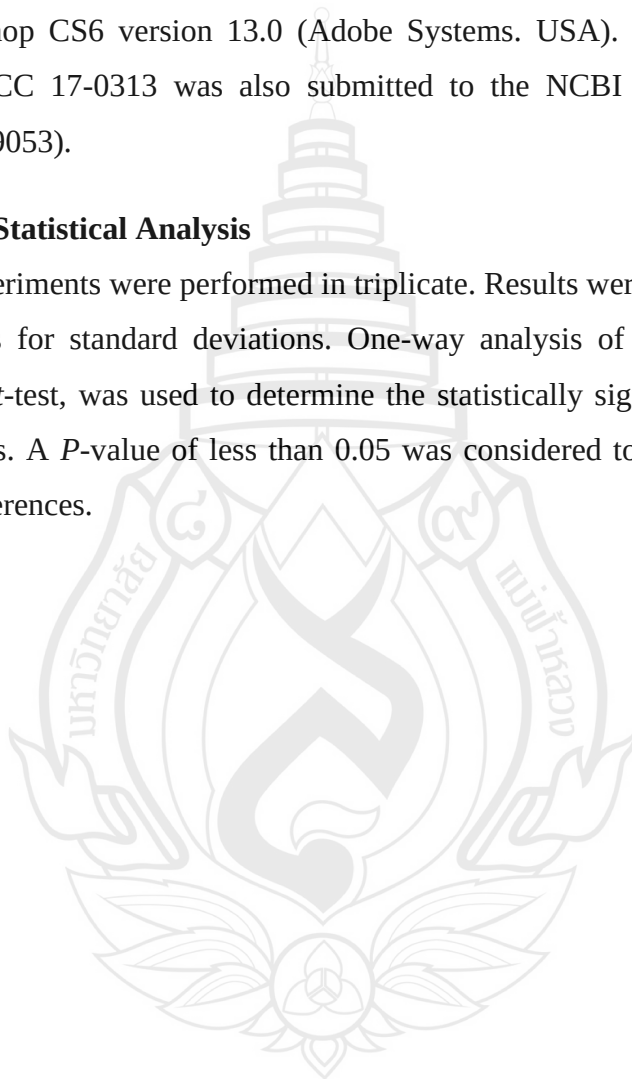
4.2.10 Identification of The Endophytic Fungus MFLUCC 17-0313

The molecular identification of endophytic fungus MFLUCC 17-0313 was preliminarily performed using the method as described in section of molecular identification of the phytopathogen *A. rolfsii*. The sequences of representative taxa in Diatrypaceae used in our phylogenetic analyses were selected from GenBank based on the BLASTn searches and recently published data (Konta et al., 2020; Senwanna et al., 2017). ITS and β -tubulin sequence datasets were selected to construct the phylogenetic tree. The combined ITS and β -tubulin sequence data were initially aligned by using MAFFT version 7 (Katoh et al., 2017; <http://mafft.cbrc.jp/alignment/server/>). The alignment was checked and improved in BioEdit v. 7.0.5.3 (Hall, 1999). The final alignment of the combined ITS and β -tubulin sequence datasets was analyzed and inferred the phylogenetic tree based on maximum likelihood (ML) and Bayesian inference analyses (BI). The estimated evolutionary model of Bayesian inference and maximum likelihood were performed independently for each locus using MrModeltest v. 2.3 (Nylander, 2004). The best-fit model is resulted as GTR+I+G model for each locus under the Akaike Information Criterion (AIC). Maximum likelihood (ML) analyses was performed by using the RAxML-HPC2 on XSEDE (v. 8.2.12) (Stamatakis, 2014) via the CIPRES Science Gateway platform (Miller et al., 2010). Bayesian inference (BI) analysis was performed by MrBayes on XSEDE, MrBayes 3.2.6 (Huelsenbeck & Ronquist, 2001) via the CIPRES Science Gateway platform (Miller et al., 2010). Bayesian posterior probabilities (BYPP) (Rannala & Yang, 1996; Zhaxybayeva & Gogarten, 2002) were evaluated by Markov Chain Monte Carlo sampling (BMCMC). Two parallel runs were conducted using the

default settings, but with the following adjustments: Six simultaneous Markov chains were set up at 5,000,000 generations. Trees were sampled every 100th generation. The first 10% of generated trees representing the burn-in phase were discarded and the remaining trees were used to calculate posterior probabilities of the majority rule consensus tree. The phylogenetic trees were figured in FigTree v.1.4.3 (Rambaut, 2016) and edited using Microsoft PowerPoint 2013 and converted to jpeg file in Adobe Photoshop CS6 version 13.0 (Adobe Systems. USA). The sequence of the fungus MFLUCC 17-0313 was also submitted to the NCBI GenBank (accession number MK329053).

4.2.11 Statistical Analysis

All experiments were performed in triplicate. Results were expressed as means with error bars for standard deviations. One-way analysis of variance (ANOVA), followed by a *t*-test, was used to determine the statistically significant difference in the experiments. A *P*-value of less than 0.05 was considered to indicate statistically significant differences.



4.3 Results

4.3.1 Isolation of Endophytic Fungi

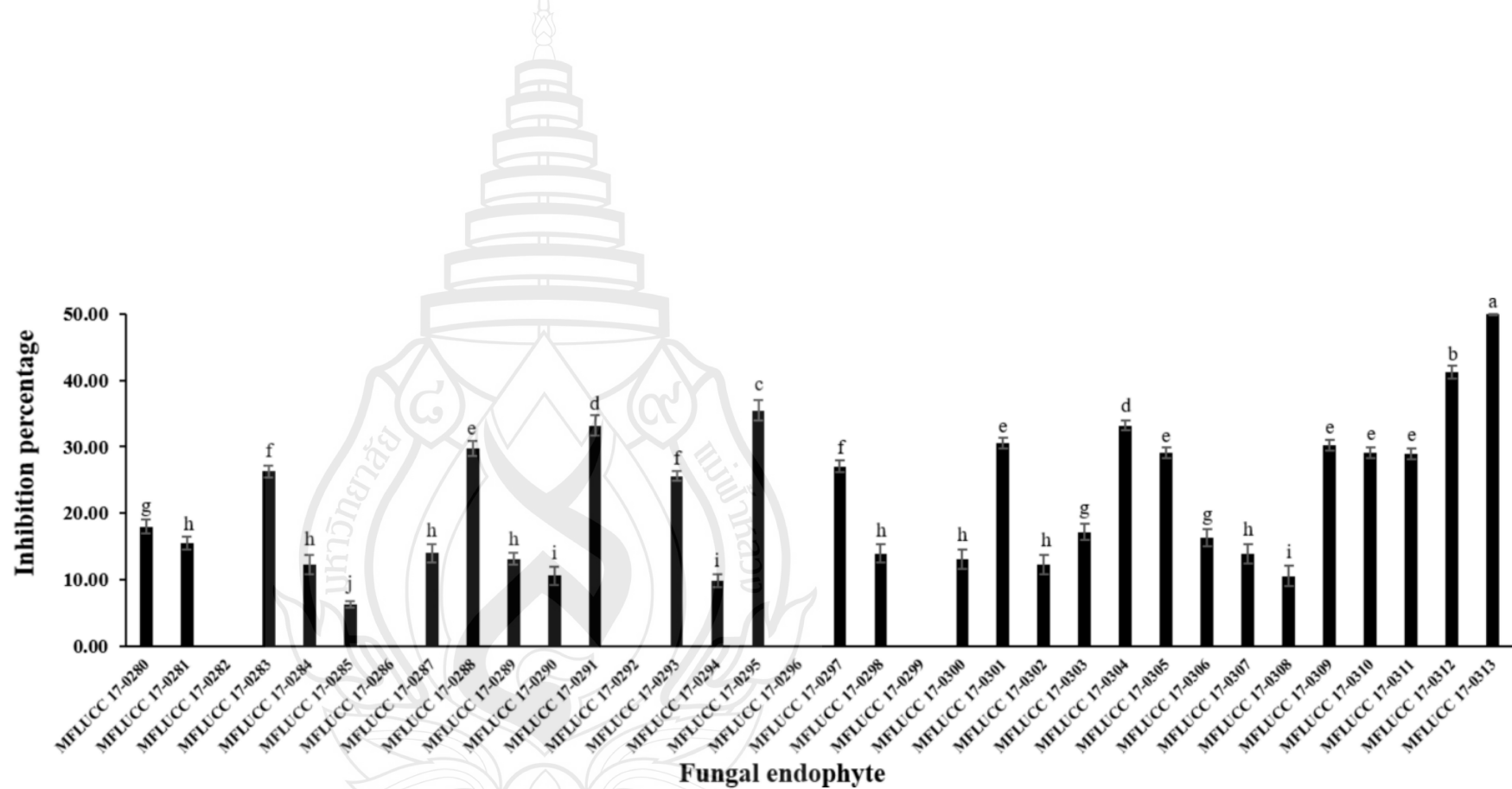
Thirty-four fungal isolates were obtained from *P. fruticosa* leaves. Morphology of the endophytic fungi isolates are shown in Figure 4.6.

4.3.2 Screening of Antifungal Activity

The antifungal activity of each isolated endophytic fungi against the pathogen *A. rolfsii* was observed. Figure 4.1 and Table 4.2 show the inhibition percentage of each endophytic fungus against the pathogen *A. rolfsii* from dual culture assay. Antifungal activity of some endophytic fungi against *A. rolfsii* for seven days using dual culture assay is shown in Figure 4.2. The percentage of growth inhibition ranged from 6.32% to 49.98%. The endophytic MFLUCC 17-0313 isolate possessed the highest inhibition percentage (49.98%). The mean inhibition percentage obtained from the endophytic fungus MFLUCC 17-0313 was significantly different from the other 33 isolates.

4.3.3 Antifungal Activity of Crude Extracts of The Endophytic Fungus MFLUCC 17-0313

The endophytic fungus MFLUCC 17-0313 was further cultured in PDB before its bioactive compound was extracted using different organic solvents. The crude extracts were tested for their antifungal activity against the pathogen *A. rolfsii*. Only the crude hexane extract of the fungal mycelium was found to exhibit a significant antifungal activity with an inhibition of 64.71%, while amphotericin B (prepared at 5 mg/mL) showed an inhibition percentage of 67.53% (Figure 4.7). No antifungal activity against *A. rolfsii* was found in the other tested broth or mycelium extracts.

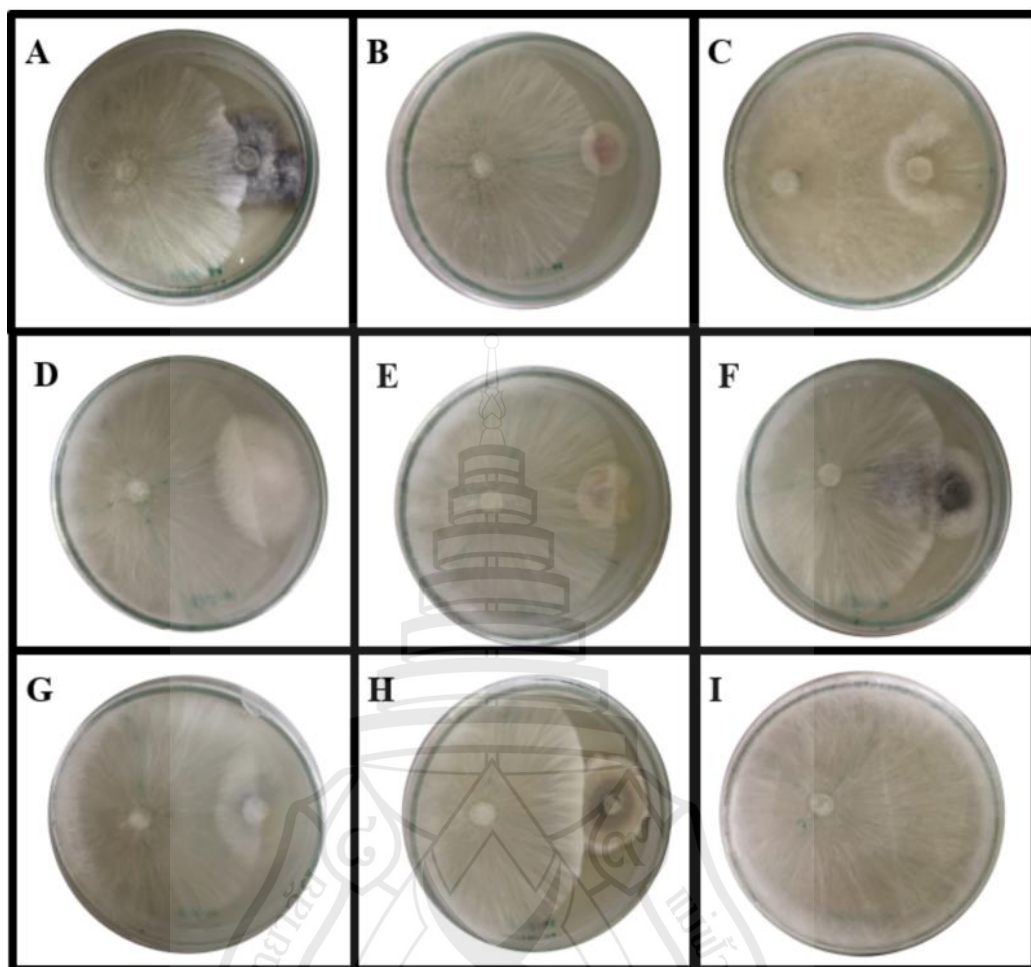


Note Each data indicates the average percentage of inhibition of endophytic fungus and a standard deviation value derived from a triplicate experiment. One-way analysis of variance (ANOVA), followed by a *t*-test, was used to determine the statistically significant difference.

Figure 4.1 Inhibition percentage of the all endophytic fungi against *A. rolfsii* from dual culture assay

4.3.4 Isolation and Identification of The Active Compounds

The crude hexane extract of fungal mycelium was fractioned to obtain the one containing the bioactive compound. Results from the disc diffusion method indicated that F19 fraction contains a bioactive compound with an inhibition percentage of $76.42 \pm 0.04\%$. The results of antifungal activity of some fractions are shown in Figure 4.8. The bioactive compound was concentrated and subjected to structural identification. The ^1H NMR (400 MHz, CDCl_3) results are as follows: δ 9.32 (1H, s), 7.42 (1H, dd, $J = 0.5, 8.3$ Hz), 7.35 (1H, t, $J = 8.1$ Hz), 7.33–7.28 (2H, m), 6.88 (1H, dd, $J = 1.3, 7.4$ Hz), 6.78 (1H, d, $J = 7.6$ Hz), and 4.06 (3H, s), while the ^{13}C NMR (CDCl_3 , 100 MHz) results are also follows: δ 156.13, 154.45, 136.71, 127.70, 125.58, 121.84, 118.84, 115.04, 110.40, 103.86, and 56.08. These NMR data are in good agreement with those reported in Emre (2018). In addition, the mass spectrum of this compound showed an m/z of 175.0759, with a predicted chemical formula of $\text{C}_{11}\text{H}_{11}\text{O}_2$. This compound was identified as 8-methoxynaphthalen-1-ol (Figure 4.3). All NMR and mass spectral data are depicted in supplement information.



Note *A. rolfsii* was grown in PDA plates together with (A) MFLUCC 17-0281, (B) MFLUCC 17-0288, (C) MFLUCC 17-0296, (D) MFLUCC 17-0297, (E) MFLUCC 17-0298, (F) MFLUCC 17-0310, (G) MFLUCC 17-0312 and (H) MFLUCC 17-0313 while (I) represents *A. rolfsii* in PDA plate. The plates were cultivated for seven days at 30 °C.

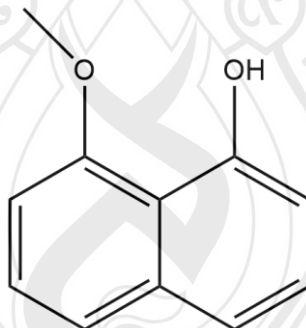
Figure 4.2 Dual culture plate assay between some endophytic fungi against the pathogen *A. rolfsii*

4.3.5 Minimum Inhibitory Concentration (MIC)

The MIC value of the isolated compound, 8-methoxynaphthalen-1-ol, was determined. The results revealed moderate antimicrobial activity of the compound against *A. rolfsii* with an MIC of 250 µg/mL. The result of antifungal activity of this compound is shown in Figure 4.9.

4.3.6 Phytotoxicity Test

In the present study, 8-methoxynaphthalen-1-ol, which showed a moderate antifungal activity against *A. rolfsii*, was further tested for its phytotoxic effect on plant leaves. Using the leaf disk and absorption assays, the development of symptoms after treatments with 8-methoxynaphthalen-1-ol at various concentrations were observed and compared to those obtained from glyphosate at the same concentrations. Distilled water and a mixture of 0.25% tween 20 with 4% methanol were used in control experiments. The effects of 8-methoxynaphthalen-1-ol on *S. lycopersicum* leaves from the leaf disk are shown in Table 4.1. Symptoms caused by compounds toward *S. lycopersicum* leaf disks at different concentrations after 18 h are shown in Figure 4.10 while leaf absorption assay is shown in Figure 4.4. From the results, 8-methoxynaphthalen-1-ol and the negative controls showed no visual effects on the leaves, while the positive control glyphosate exhibited phytotoxic effects on *S. lycopersicum* leaves at all concentrations.



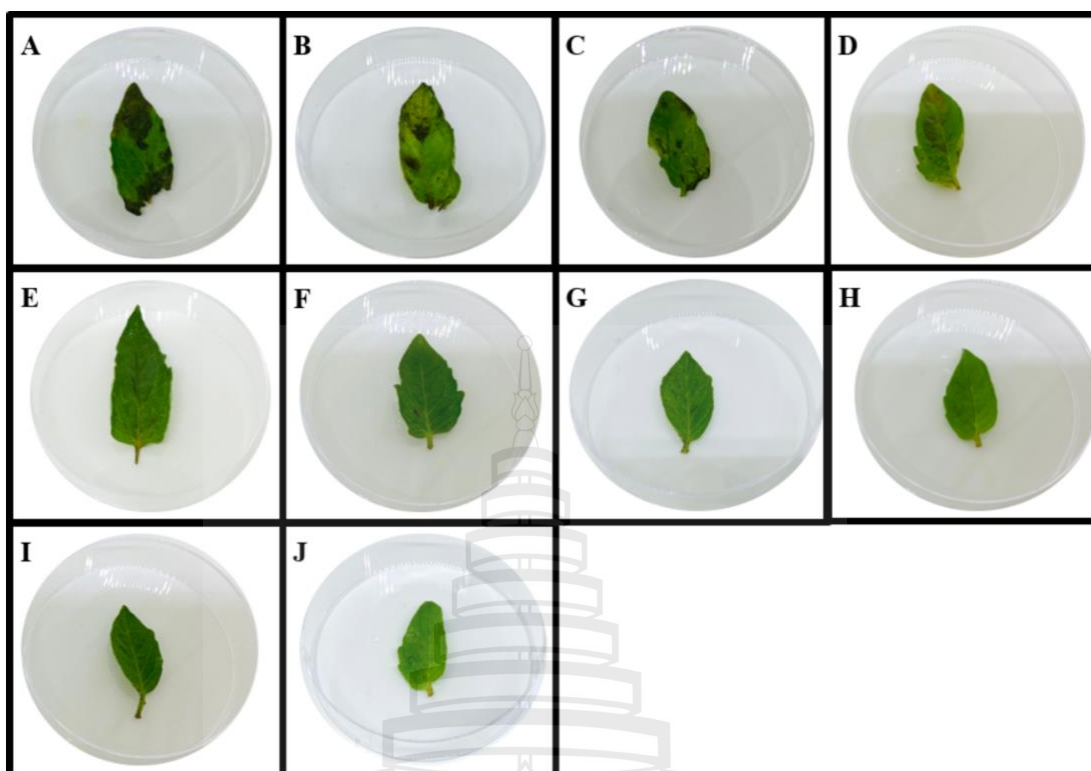
Note This compound was isolated from *Diatrype palmicola* MFLUCC 17-0313 inhibited the plant pathogenic fungus *Sclerotium rolfsii* on tomatoes.

Figure 4.3 Chemical structure of 8-methoxynaphthalen-1-ol

Table 4.1 Phytotoxicity of compounds toward *S. lycopersicum* leaf disks at different concentrations after 18 h

Compound/herbicide	Concentration (µg/mL)			
	1,000	500	250	125
8-methoxynaphthalen-1-ol	0	0	0	0
Glyphosate	4	2	1	1
Distilled water			0	
Mixture of 0.25% tween 20 with 4% methanol			0	

Note A '0' indicates no effect, '1' indicates that area of necrotic spots <50%, '2' is area of necrotic spots 50-70%, '3' indicates area of necrotic spots 70-90%, and '4' indicates that area of necrotic spots >90%.



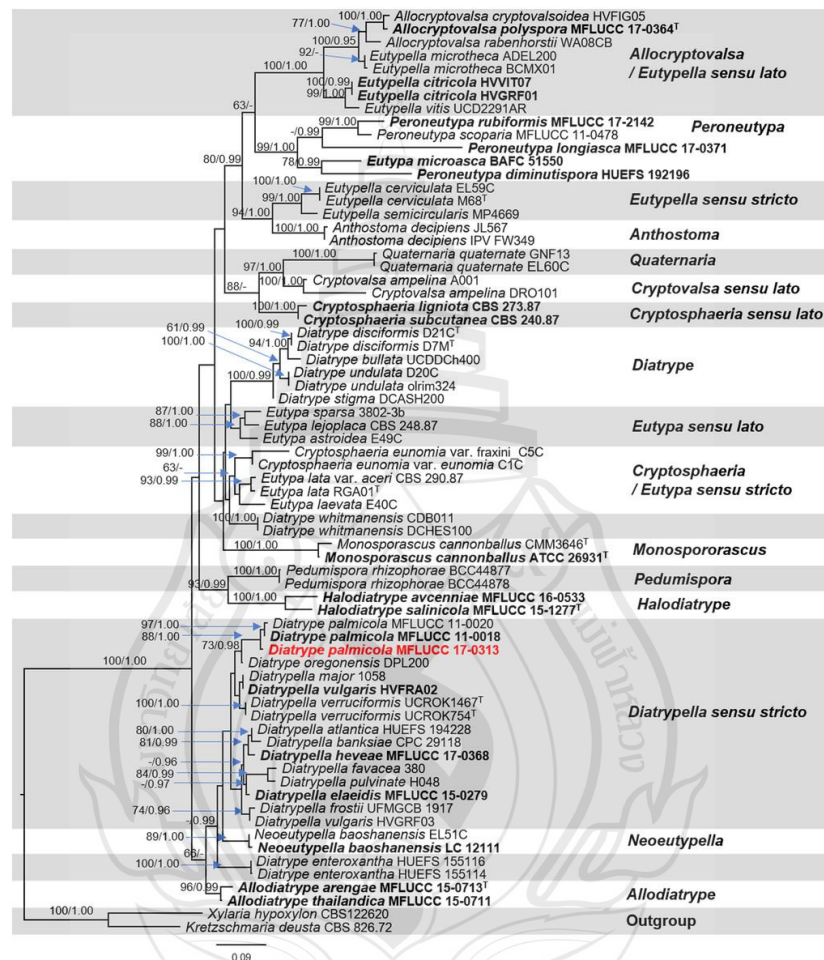
Note A-D represent symptoms caused by glyphosate at concentration of 1,000, 500, 250 and 125 $\mu\text{g/mL}$, E-H represent symptoms caused by 8-methoxy-naphthalen-1-ol at concentration of 1,000, 500, 250 and 125 $\mu\text{g/mL}$, I represents symptoms caused by a mixture of 0.25% tween 20 with 4% methanol, J represents symptoms caused by distilled water.

Figure 4.4 Symptoms caused by compounds toward *S. lycopersicum* leaf disks at different concentrations after 18 h

4.3.7 Phylogenetic Analyses

The dataset consisted of 68 taxa including our strain and the outgroup taxa. RAxML analysis of the combined ITS and β -tubulin dataset had 1,246 distinct alignment patterns and 64.54% of undetermined characters or gaps. The best scoring of RAxML analysis shown in Figure 4.5, with the final ML optimization likelihood value of -17386.379663 . Bayesian posterior probabilities (BY) from MCMC were evaluated with the final average standard deviation of split frequencies = 0.010983. Phylogenetic analysis from ML, and BI gave trees with similar overall topologies of

the generic placement and in agreement with previous studies (Konta et al., 2020; Senwanna et al., 2017). In the phylogenetic tree (Figure 4.5), our taxon was clustered with *Diatrype palmicola* (MFLUCC 11-0018 and MFLUCC 11-0020) with 90% ML and 1.00 BY bootstrap support.



Note Phylogram generated from RAXML analysis based on a combined ITS and β -tubulin sequence data. Bootstrap support values for ML equal to or greater than 70%, and Bayesian posterior probabilities (PP) equal to or greater than 0.95 are defined as ML/PP above the nodes. The tree is rooted to *Xylaria hypoxylon* (CBS 122620) and *Kretzschmaria deusta* (CBS 826.72). All sequences from ex-type strains are in bold and type species are denoted with the superscript “T” after the species name. Newly generated strains are indicated in red.

Figure 4.5 Phylogenetic tree of *Diatrype palmicola* MFLUCC17-0313

4.4 Discussion

This study presented a similar result to those found in previous studies, which revealed that the endophytic fungi capable of producing bioactive compounds could be isolated from a wide range of plants (Golinska et al., 2015a; Golinska et al., 2015b; Shen et al., 2012). According to the results, the endophytic fungus MFLUCC 17-0313 displayed significant antifungal activity against *A. rolfsii*. The BLASTn search of ITS sequence shows more than 90% similarity with Diatrypaceae taxa (i.e., *Diatrypella banksiae* (CPC 29118), *Diatrypella atlantica* (HUEFS 194228), *Eutypella parasitica* (CBS 210.39) and *Diatrypella vulgaris* (CBS 128327)). In our pre-analyses of ITS sequence dataset, our strain was grouped within Diatrypaceae, however, only the ITS sequence is not appropriate to distinguish member in Diatrypaceae. Phylogram generated from RAxML analysis based on ITS sequence data (pre-analyses) is depicted in supplement information.

We, therefore, use the combined ITS and β -tubulin sequences in our dataset to clarify phylogenetic affinities of the genera in this family. As our attempts to obtain β -tubulin for our strain was unsuccessful. Therefore, here we do not include our β -tubulin sequence in analyses. Phylogenetic analyses of a combined ITS and β -tubulin sequence dataset revealed that the endophytic fungus MFLUCC 17-0313 grouped with *Diatrype palmicola* (MFLUCC 11-0018 and MFLUCC 11-0020) with moderate support (88% ML and 1.00 BYPP). Similar results with regards to support among Diatrypaceae members were obtained from previous studies (Acero et al., 2004; De Almeida et al., 2016; Konta et al., 2020; Senwanna et al., 2017; Shang et al., 2017;). A comparison of ITS nucleotide bases showed that the endophytic fungus MFLUCC 17-0313 differed from *Diatrype palmicola* (MFLUCC 11-0018, type species) in 13/482 bp (2.7%). However, we could not obtain the reproductive structure in culture to compare the morphological characters of our strain with the type species. Despite this, for the time being our isolate will be treated as *Diatrype cf. palmicola* on the basis of the phylogenetic analysis. This taxonomic assignment is in agreement with Williams et al. (2008), who reported that fungi of the genus *Diatrype* produce secondary metabolites including lunalides A and B while treated with the

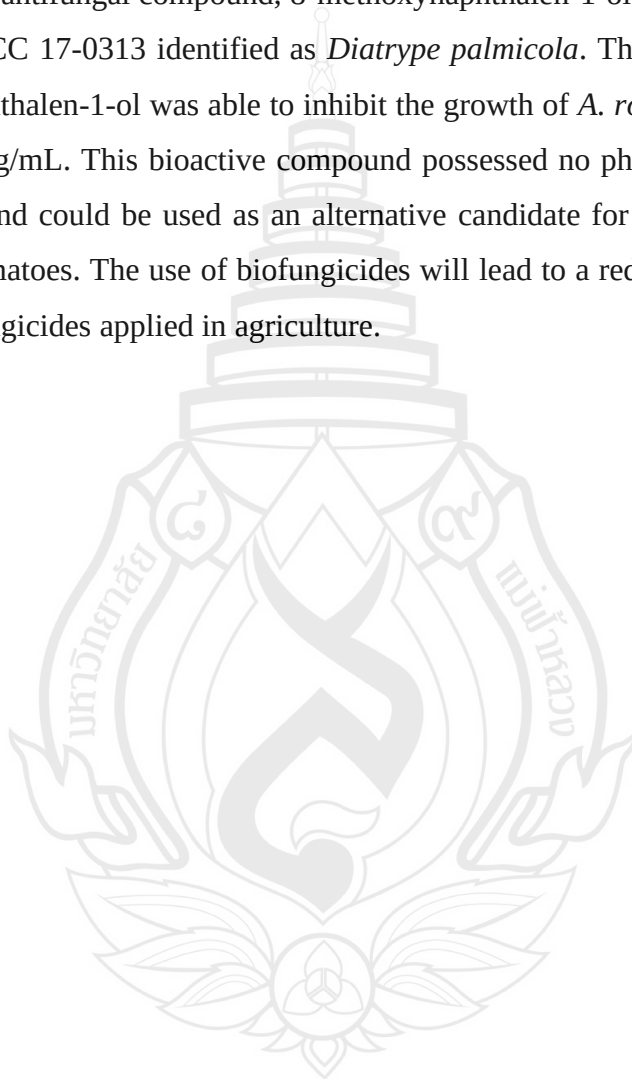
DNA methyltransferase inhibitor 5-azacytidine. Both compounds were reported to possess substantial nematocidal activities (Cichewicz, 2012). In addition, *Diatrypella frostii* endophytic fungus of Diatrypaceae family has been reported to have antimicrobial activity such as isolated from Brazilian medicinal plant *Solanum cernuum* Vell (Vieira et al., 2011). Therefore, the Diatrype fungi isolated from medicinal plants could produce antifungal compounds.

The active compound, 8-methoxynaphthalen-1-ol, isolated from the crude hexane extract of the endophytic fungus MFLUCC 17-0313 mycelium was shown to have antifungal activity against *A. rolfsii*. This compound was reported in several studies. For instance, Rukachaisirikul et al. (2007) isolated this compound from the xylariaceous fungus PSU-A80 and found that it has good radical scavenging properties. Moreover, isomers and dimers of this compound were produced by several endophytes including the endophytic fungus *Nodulisporium* sp. from *Juniperus cedre* (Dai et al., 2006), endophytic fungal strain PR35 from *Paeonia delavayi* (Hu et al., 2012), and endophytic fungus *Daldinia* cf. *concentrica* (Liarzi et al., 2016). The literature reported the use of 8-methoxynaphthalen-1-ol as an antioxidative agent but no data have been published regarding the fungicidal activity of 8-methoxynaphthalen-1-ol.

In this study, the phytotoxic effect of 8-methoxynaphthalen-1-ol was effectively determined to evaluate its potential as biocontrol agent. The similar pattern of phytotoxicity of 8-methoxynaphthalen-1-ol was observed for all concentrations when the leaf disk and absorption assays were conducted. Toxicity data obtained from phytotoxicity tests are important for ecological risk assessment. These phytotoxicity tests are useful to inform regulators on the inherent toxicity of a given active compound. However, for 8-methoxynaphthalen-1-ol, its effects on species diversity, biodiversity, ecosystem services, and functions may be assessed in further studies.

4.5 Conclusion

In summary, 34 endophytic fungal isolates were obtained from *P. fruticosa* leaves which is the first report of endophytic fungi from this plant. The antifungal activity against *A. rolfsii* of the endophytic isolates was investigated, leading to the isolation of the antifungal compound, 8-methoxynaphthalen-1-ol, from the endophytic fungus MFLUCC 17-0313 identified as *Diatrype palmicola*. The results showed that 8-methoxynaphthalen-1-ol was able to inhibit the growth of *A. rolfsii* and had an MIC value of 250 µg/mL. This bioactive compound possessed no phytotoxicity against *S. lycopersicum* and could be used as an alternative candidate for controlling *A. rolfsii* infection in tomatoes. The use of biofungicides will lead to a reduction in the amount of synthetic fungicides applied in agriculture.



4.6 Supporting Information

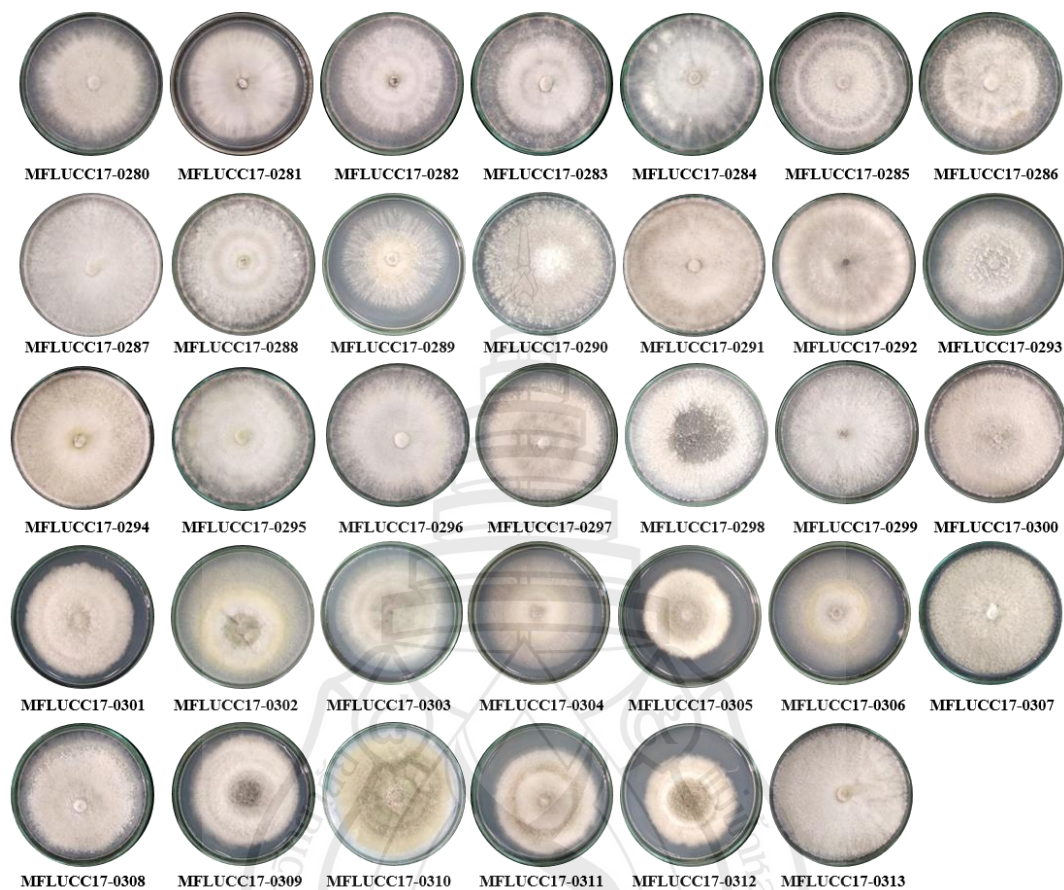


Figure 4.6 Endophytic fungi were isolated from *P. fruticosa* leaves. All endophytic fungi were cultured in PDA at room temperature for two weeks

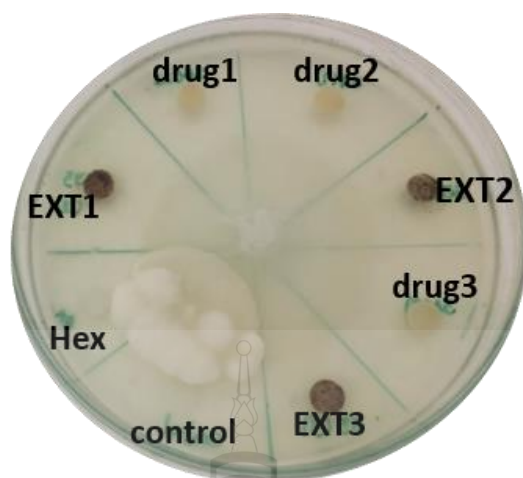


Figure 4.7 Antifungal activity of crude hexane extract (EXT), amphotericin B (drug), hexane solvent (Hex) and control against *A. rolfsii* for seven days using disk diffusion assay

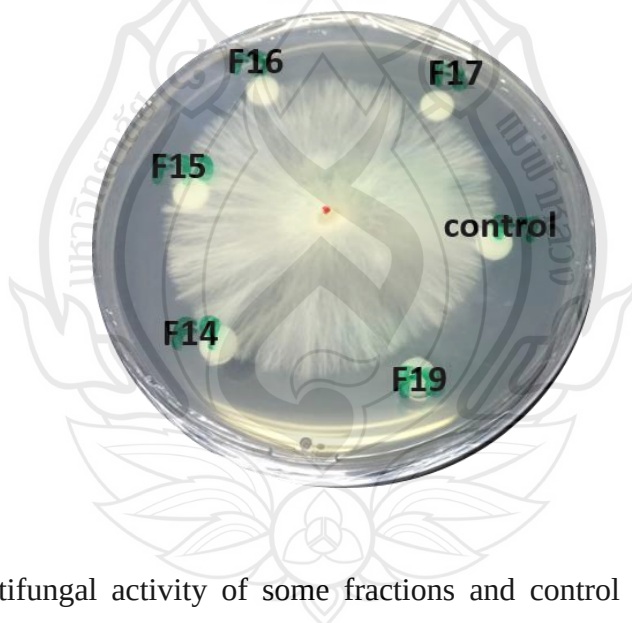


Figure 4.8 Antifungal activity of some fractions and control against *A. rolfsii* for seven days using disk diffusion assay

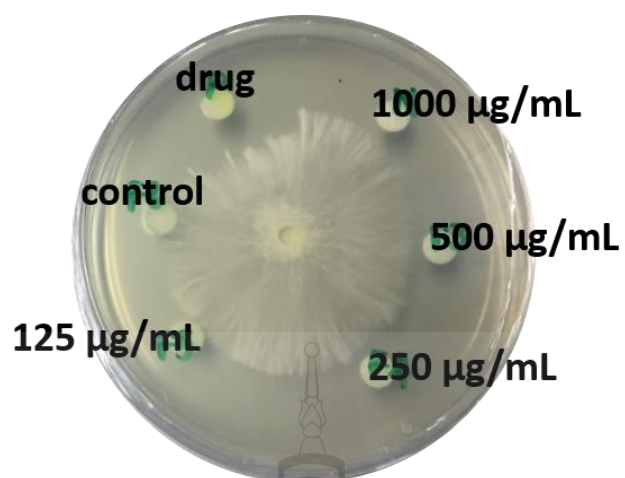
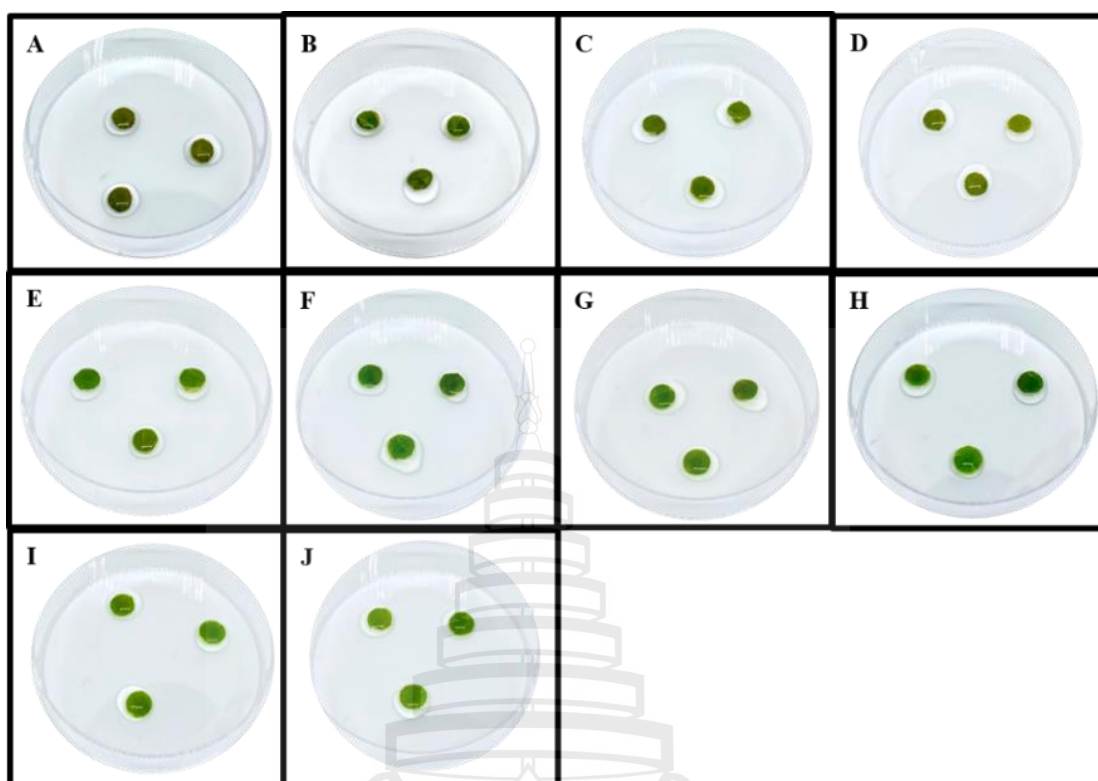


Figure 4.9 Antifungal activity of pure compounds at different concentrations and amphotericin B (drug) against *A. rolfsii* for seven days using disk diffusion assay



Note A-D represent symptom caused by glyphosate at concentration of 1,000, 500, 250 and 125 $\mu\text{g/mL}$, E-H represent symptoms caused by 8-methoxy-naphthalen-1-ol at concentration of 1,000, 500, 250 and 125 $\mu\text{g/mL}$, I represent symptoms caused by 4% methanol with 0.25% tween 20, J represents symptoms caused by distilled water

Figure 4.10 Symptoms caused by compounds toward *S. lycopersicum* leaf disks at different concentrations after 18 h

Table 4.2 Inhibition percentage of the all endophytic fungi against *A. rolfsii* from dual culture assay

Endophytic fungi	% inhibition	Endophytic fungi	% inhibition
MFLUCC17-0280	18.01±1.11 ^e	MFLUCC17-0297	27.04±0.91 ^d
MFLUCC17-0281	41.21±1.02 ^b	MFLUCC17-0298	13.97±1.39 ^f
MFLUCC17-0282	-	MFLUCC17-0300	13.15±1.43 ^f
MFLUCC17-0283	26.31±0.93 ^d	MFLUCC17-0301	30.52±0.80 ^c
MFLUCC17-0284	12.33±1.47 ^g	MFLUCC17-0302	12.31±1.44 ^g
MFLUCC17-0285	6.32±0.45 ⁱ	MFLUCC17-0303	17.21±1.27 ^e
MFLUCC17-0286	-	MFLUCC17-0304	33.22±0.68 ^c
MFLUCC17-0287	13.99±1.38 ^f	MFLUCC17-0305	29.11±0.82 ^c
MFLUCC17-0288	29.74±1.11 ^c	MFLUCC17-0306	16.39±1.30 ^e
MFLUCC17-0289	13.17±0.87 ^f	MFLUCC17-0307	13.89±1.41 ^f
MFLUCC17-0290	10.63±1.41 ^h	MFLUCC17-0308	10.57±1.51 ^h
MFLUCC17-0291	33.22±1.53 ^c	MFLUCC17-0309	30.21±0.80 ^c
MFLUCC17-0292	-	MFLUCC17-0310	29.09±0.84 ^c
MFLUCC17-0293	25.61±0.68 ^d	MFLUCC17-0311	28.94±0.84 ^c
MFLUCC17-0294	9.79±0.97 ^h	MFLUCC17-0312	-
MFLUCC17-0295	35.46±1.54 ^c	MFLUCC17-0313	49.98±0.15 ^a
MFLUCC17-0296	-		

Note Each data indicates the average percentage of inhibition of endophytic fungus and a standard deviation value derived from a triplicate experiment. One-way analysis of variance (ANOVA), followed by a *t*-test, was used to determine the statistically significant difference in the experiments.

CHAPTER 5

ANTIBACTERIAL AND CYTOTOXIC ACTIVITIES OF
ENDOPHYTIC FUNGUS *Diatrypella* sp.
MFLUCC 19-0492³

Abstract

Endophytic fungi are microorganisms living inside plant tissues without causing any disease symptoms in the plant. Various bioactive compounds have been produced and may be used as alternative drugs for controlling human pathogens. The aim of this work was to investigate antibacterial and cytotoxic activity potential of fungal crude extracts isolated from *Barleria prionitis* leaves. A total of 34 endophytic fungi were isolated. The crude ethyl acetate extracts of all fungi were preliminarily screened for their antibacterial activity. The acetate extract of isolate ANG4 showed significant antibacterial activity.

This isolate was further cultured to determine its minimum inhibition concentration (MIC). The crude ethyl acetate extracts of mycelia and culture broth from isolate ANG4 were active against all tested pathogens with low MIC values ranging from 0.39 mg/mL to 25.00 mg/mL when compared to the crude hexane extract of its mycelia. The cytotoxic effects of all crude extracts of isolate ANG4 were also determined using tetrazolium-based colorimetric (MTT and XTT) assays against T-lymphoblast, cholangiocarcinoma, liver hepatocellular and lung carcinoma cell lines. The crude ethyl acetate extract of mycelia showed the most significant activity inhibiting T-lymphoblast and lung carcinoma cell lines with 100 and 71%

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respectively whereas the crude hexane extract of mycelia showed good cytotoxic activity against the T-lymphoblast cell line with 74%. The fungus ANG4 was also identified by molecular and phylogenetic methods as *Diatrypella* sp. MFLUCC 19-0492. Therefore, this study indicates that crude extracts of *Diatrypella* sp. MFLUCC 19-0492, isolated from *B. prionitis* leaves, are potential sources of novel antibacterial compounds with good cytotoxic activity.

Keywords: Antibacterial activity, *Barleria prionitis*, cytotoxic activity, *Diatrypella*, endophytic fungi.

5.1 Introduction

Medicinal plants have played an important role as the principal natural product sources of bioactive compounds. They have been widely used in traditional treatments and studied extensively for their antimicrobial properties. Medicinal plants are also reported to host various bioactive compound-producing endophytic fungi (Bhardwaj, 2015; Nguyen et al., 2019). Endophytic fungi are microorganisms that live inside host plants without causing any visible manifestation of diseases (Ilic et al., 2017). They reside in the host plant tissues by producing secondary metabolites to protect and resist external biotic and abiotic stress to the host plant by producing secondary metabolites with antagonistic activity (Strobel et al., 2004).

New and novel bioactive compounds with various antimicrobial, anticancer, antimalarial and antioxidant activities, isolated from endophytic fungi of medicinal plants, have been reported such as alkaloids, terpenoids, steroids, quinones, flavonoids, phenols, phenolic acids and peptides (Kusari et al., 2012; Pavithra et al., 2012). Therefore, endophytic fungi are evaluated as alternative important sources of bioactive compounds that can be applied as new or novel drugs (Rajamanikyam et al., 2017).

Antimicrobial resistance has been a major health problem and still poses a concern to health care systems globally. Many studies have shown that microbial pathogens have developed resistance to antibiotics via various molecular mechanisms (Munita & Arias, 2016). Therefore, this global problem has led to the search for a new

or novel drug from endophytic fungi, particularly those isolated from medicinal plants for their antimicrobial properties (Christina et al., 2013).

Barleria prionitis belongs to the Acanthaceae family and is a well-known medicinal plant and Ayurvedic herb. It is distributed in tropical Africa and Asia including India, Malaysia, the Philippines, Sri Lanka and Thailand (Banerjee et al., 2012). This medicinal plant has been used for the treatment of fevers, toothaches, bronchial asthma, rheumatic affections, inflammation, glandular swelling, dysrhythmia and gastrointestinal disorders (Joshi et al., 2011; Khadse & Kakde, 2011). Several phytochemicals such as balarenone, pipataline, lupeol, prioniside A, prioniside B and prioniside C have been reported to possess the following biological activities: antibacterial, antiviral, antioxidant, antifertility, anthelmintic, cytoprotective, antidiabetic, antidiarrheal, hepatoprotective and antifungal activity (Gambhire et al., 2009; Kumari et al., 2015; Maji et al., 2011).

Due to the reported antimicrobial potential of various endophytic fungi, this research focused on the antibacterial and cytotoxic potential of crude extracts of endophytic fungi isolated from *B. prionitis* leaves where the most potent endophytic fungus could be used as an antibacterial agent against human pathogenic bacteria. These fungi, which are suggested to be associated with the production of compounds with medical value, could be used as alternative antibiotics.

5.2 Materials and Methods

5.2.1 Plant Material

Fresh plant materials of *B. prionitis* were collected from the flower market located in Chiang Rai Province, Thailand in October 2018. The plant material was brought to the laboratory in sterile plastic bags and processed within a few hours of sampling. Fresh, healthy leaves of *B. prionitis* were used for the isolation of endophytic fungi to reduce the chance of contamination.

5.2.2 Isolation of Endophytic Fungi

The healthy *B. prionitis* leaves were washed under running tap water several times to remove debris and soil. The leaf sterilization and the isolation of endophytic

fungi utilized the methods described by Atiphasaworn et al. (2017). The explant was immersed in 70% ethanol for 10 s followed by 1% sodium hypochlorite (NaOCl) for 30 s and washed with sterile water four times. The surface-sterilized leaves were then allowed to dry under aseptic conditions. The sterile blades were sectioned into 0.5 cm² pieces of leaf and placed on potato dextrose agar (PDA) medium plates containing chloramphenicol antibiotic (100 mg/mL) to inhibit bacterial growth.

The plates were sealed with parafilm and incubated at room temperature (25 °C), protected from light, for five days until the growth of the hypha tip was visible. Only fungal hyphae emerging from the leaf pieces were transferred to PDA plates through hyphal tipping. A culture plug was further transferred to new PDA plates and cultured at room temperature (25 °C) as soon as they outcrop from the medium, until pure colonies were formed.

5.2.3 Extraction of Chemical Compounds

All endophytic fungi were cultured in a liquid surface fermentation system followed by an ethyl acetate extraction using the experiment described previously (Atiphasaworn et al., 2017; Wang et al., 2019). Five pieces of mycelial agar plugs, 6 mm in diameter were inoculated into 150 mL potato dextrose broth (PDB). All fungi were individually incubated at room temperature (25 °C) for 28 days. After the incubation period, the broth culture was filtered with Whatmann filter paper no. 3 under vacuum using a Buchner funnel to separate the culture broth from the mycelia. The formed mycelial mat was ground using a mortar and pestle and macerated with 500 mL of ethyl acetate and further sonicated using ultrasound-assisted extraction (Crest/690DAE; GuangDong GT Ultrasonic, Guangdong, China) at room temperature (25 °C) for 5 h.

In addition, 500 mL of ethyl acetate was added to the broth culture. After 24 h, the ethyl acetate extract was separated from the culture broth using a separatory funnel and Whatmann no. 1 filter paper. The extraction process was repeated in triplicate. All extracts were combined and concentrated by using a rotary evaporator (Buchi Rotavapor Model R114, BUCHI). The obtained extracts were subsequently stored at 4 °C until use.

5.2.4 Antibacterial Activity Screening

The ethyl acetate extracts were screened for their antibacterial activity against *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922, which were representatives of gram-positive and -negative bacteria respectively using the disk diffusion method described by Razmavar et al. (2014). Both bacteria were obtained from the Department of Medical Science, Ministry of Health, Bangkok, Thailand. The bacterial suspensions were prepared from 24 h old culture of the test bacteria. They were standardized to contain approximately 1.5×10^8 CFU/mL based on the 0.5 McFarland Standard. All bacteria were inoculated on the dried surface of a Mueller-Hinton agar. The inoculum was incubated at 37 °C for 5 min to ensure the confluent lawn of bacterial growth before applying the test discs.

The dried crude extracts were dissolved in ethyl acetate to a concentration of 50 mg/mL. Each crude fungal extract (30 µL) was dropped on a sterilized 6 mm diameter paper disc (Whatmann Paper No. 1, USA) and allowed to dry prior to incubating at 37 °C for 24 h. The clear zone diameter of inhibition was determined. Paper disks eluted with 30 µL of ethyl acetate and chloramphenicol (5 mg/mL) were used as negative and positive controls respectively. Each experiment was carried out in triplicate.

5.2.5 Extraction of Various Crude Extracts of Fungus ANG4

Due to the highest antibacterial activity of endophytic fungus ANG4 against selected bacterial pathogens, a large-scale (20 L) fermentation of isolate ANG4 was performed using PDB culture medium at room temperature (25 °C) for 28 days. For the isolation of ANG4, the mycelia of the fungus were separated from the culture broth by filtration and macerated sequentially with methanol (400 mL) and dichloromethane (400 mL) for two days before concentrating and partitioning with hexane and ethyl acetate respectively.

Both hexane and ethyl acetate extracts were collected and dried over anhydrous sodium sulfate before solvent evaporation using a rotary evaporator to give crude extracts of 0.444 and 0.147 g from the hexane and ethyl acetate extracts respectively. The culture broth was partitioned with ethyl acetate three times

(400 mL) and further concentrated using a rotary evaporator to obtain the crude ethyl acetate extract of 1.086 g.

5.2.6 Antibacterial Activity Assay of Various Crude Extracts of ANG4

Eight human pathogenic bacteria were selected for this study. Four gram-positive bacteria, including *S. aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Streptococcus pyogenes* ATCC 12384 and *Enterococcus faecium* ATCC 29212 and four Gram-negative bacteria including *E. coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853 and *Salmonella typhimurium* ATCC 13311 were used as test pathogens.

All strains were obtained from the Department of Medical Science, Ministry of Health, Bangkok, Thailand. The antibacterial activity assay was performed as described above. The dried crude extracts were dissolved in hexane or ethyl acetate to concentrations of 50.00, 25.00, 12.50, 6.25, 3.12, 1.56, 0.78 and 0.39 mg/mL.

In addition, hexane and ethyl acetate were used as negative controls. Chloramphenicol was used as a positive control and prepared at concentrations of 500, 250, 125, 62.50, 31.25, 15.62, 07.81 and 3.91 µg/mL. The clear zone diameter of inhibition and minimum inhibition concentration (MIC) were measured and determined.

5.2.7 Cytotoxicity Assay

Cytotoxic activity of all crude extracts of endophytic fungus ANG4 was investigated against non-adhesive T-lymphoblast (Molt-3) and adhesive cell lines including human cholangiocarcinoma (HuCCA-1), liver hepatocellular carcinoma (HepG2) and lung carcinoma (A549) based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 2,3-bis-2-methoxy-4-nitro-5-sulfonylphenyl]-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) assays using a modified method by Thongnest et al. (2019). Cells (5×10^3 cells/well) were seeded into 96-well plates in complete medium and incubated at 37 °C. After 24 h of cell seeding, the cell lines were treated with crude extracts at a concentration of 30 µg/mL and incubated at 37 °C for 48 h.

After the incubation period, 20 µL of a stock MTT solution were added to each well and incubated at 37 °C under 5% CO₂ for 4 h before processing. The

supernatant was removed and dimethyl sulfoxide was added to the cell pellet. The absorbance of solutions was measured at 540 nm using a microtiter plate reader (VersaMax tunable multi-well plate reader). Doxorubicin was used as the positive control for the HuCCA-1, A549, Molt-3 and HepG2 cell lines whereas etoposide was used as the positive control for the Molt-3 and HepG2 cell lines. All experiments were carried out three times. The percentages of cytotoxicity were calculated spectrophotometrically as: $[100 - ((A_{540} \text{ of treated cells}) - (A_{540} \text{ of blank cells}) / (A_{540} \text{ of controlled cells}) - (A_{540} \text{ of blank cells}) \times 100)]$.

5.2.8 Identification of Endophytic Fungus ANG4

A pure culture of isolate ANG4 was selected to confirm the fungal genus according to its antibacterial and cytotoxic activities compared to other fungal extracts. Pure fungus strain mycelium was scraped from the PDA surface and pulverized with a mortar and pestle to obtain a fine powder. Analysis of genomic DNA was conducted using the cetyltrimethyl ammonium bromide method (Kato & Toh, 2010). The fungus was amplified with ITS barcoding using universal primers, ITS1 (5'- TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'- TCCTCCGCTTATTGATAT GC-3'). Polymerase chain reaction (PCR) was performed at 95 °C for 5 min followed by 40 cycles of 95 °C for 50 s, 52 °C for 50 s, 72 °C for 50 s and finally 72 °C for 10 min on a PeqSTAR 2x thermal cycler (Peqlab, Germany). The PCR-amplified products were analyzed by gel electrophoresis on 1% agarose gels stained with ethidium bromide under UV light and purified using NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel, Germany).

The DNA sequencing was performed on an automated sequencing system at 1st BASE Laboratories (Malaysia). The obtained sequences were used as a query to search for similar sequences in GenBank using the BLAST program (Basic Local Alignment Search Tool, <https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to confirm the classification of unidentified species. The phylogenetic analysis was performed using MEGA v.6.0. This isolate was stored at the Institute of Excellence in Fungal Research, Mae Fah Luang University as code MFLUCC19-0492 and further submitted to the NCBI Genbank (accession number MN715786).

5.2.9 Statistical Analysis

Experiments were performed in triplicate. All data were statistically analyzed using an Analysis of Variance. Statistical significance was analyzed by Student's *t*-test and confidence limits were set to $P < 0.05$. Results were expressed as means with an error bar of standard deviations.



5.3 Results

5.3.1 Isolation of Endophytic Fungi

Thirty-four endophytes were isolated from healthy leaves of *B. prionitis* collected from Chiang Rai Province, Thailand. Colony of isolated cultures is depicted in figure 5.1.

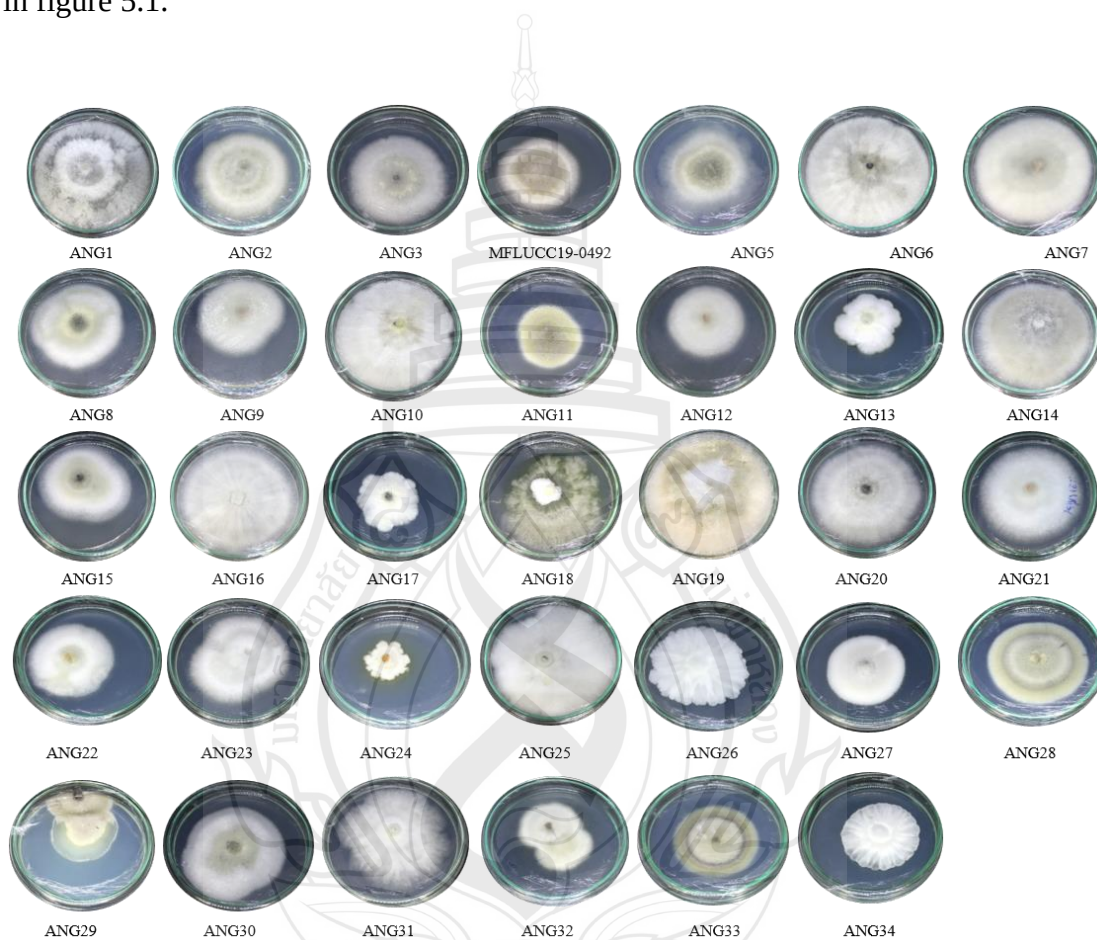


Figure 5.1 Colony of endophytic fungi isolated from *B. prionitis* leaves

Table 5.1 Antibacterial activity of crude extracts of endophytic fungi isolated from *B. prionitis* leaves (50 mg/mL) and chloramphenicol (5 mg/mL) against *S. aureus* and *E. coli*

Isolate	<i>S. aureus</i>	<i>E. coli</i>	Isolate	<i>S. aureus</i>	<i>E. coli</i>
ANG1	++++	++	ANG19	+	-
ANG2	+++	+	ANG20	-	+
ANG3	++	-	ANG21	-	-
ANG4	+++++	+++++	ANG22	+	++
ANG5	++	++	ANG23	++	++
ANG6	++	++	ANG24	++	-
ANG7	+	+++	ANG25	++	+
ANG8	++	-	ANG26	++	++
ANG9	++	-	ANG27	++	+
ANG10	-	-	ANG28	+	+
ANG11	+++	+	ANG29	-	++
ANG12	-	++	ANG30	+	-
ANG13	-	+	ANG31	++	-
ANG14	-	-	ANG32	-	-
ANG15	-	+	ANG33	-	-
ANG16	++	+	ANG34	+	+++
ANG17	+	-	Chloramphenicol	+++++	+++++
ANG18	+	++			

Note – not detected.

5.3.2 Antibacterial Activity Assay

The crude extracts were preliminarily screened for their antibacterial activities against the growth of *S. aureus* and *E. coli* which are representative of gram-negative and gram-positive bacterial pathogens respectively. The antibacterial activity of all crude extracts (50 mg/mL) and chloramphenicol (5 mg/mL) is demonstrated in table 5.1. In the present study, 34 fungal isolates were isolated from *B. prionitis*, of which 94% (32 isolates) demonstrated antibacterial activity in preliminary screening against *S. aureus* and *E. coli*. One of the isolates, ANG4, was the isolate with the highest antibacterial activity. It was selected for further investigation to determine its antibacterial and cytotoxic activities.

5.3.3 Antibacterial Activity Assay for Various Crude Extracts of ANG4

The fungus ANG4 was further cultured and the bioactive compounds were extracted using different solvents including ethyl acetate and hexane. The antibacterial activity assay was performed using eight concentrations of crude ethyl acetate extract of culture broth, crude ethyl acetate extract of mycelia and crude hexane extract of mycelia. MIC values of all crude extracts against tested human pathogens are presented in table 5.2. The antibacterial activities were observed in various values with inhibition zone diameters ranging from 6.27 ± 0.09 mm to 11.22 ± 0.14 mm. The MIC values of all crude extracts of fungus ANG4 were investigated in the range of 0.39 mg/mL to 50.00 mg/mL. Among these concentrations, MIC value of 1.56 μ g/mL was the most active against various tested pathogens. The crude ethyl acetate extracts of mycelia and culture broth of isolate ANG4 were active against all tested pathogens with low MIC values ranging from 0.39 mg/mL to 25.00 mg/mL when compared to the crude hexane extract of mycelia. However, the lowest MIC (0.39 mg/mL) was detected in the crude ethyl acetate extract of mycelia against *K. pneumoniae*. Conversely, the crude hexane extract of mycelia showed weak antibacterial activity in the range of 12.50 mg/mL to 50.00 mg/mL. Among the tested pathogens, the crude hexane extract of mycelia showed no activity against *S. typhimurium* as shown in table 5.1. Chloramphenicol, which was used as a positive control, had MIC values ranging from 62.50 to 500.00 μ g/mL.

Table 5.2 Antibacterial activity of crude extracts of ANG4 and chloramphenicol against tested human pathogens

Extract/Drug	MIC (mg/mL)							
	Zone inhibition diameter [mm] (mean $\pm$ SD)							
	Gram-negative				Gram-positive			
	<i>K. pneumoniae</i>	<i>S. typhimurium</i>	<i>P. aeruginosa</i>	<i>E. coli</i>	<i>E. faecium</i>	<i>S. pyogenes</i>	<i>S. epidermidis</i>	<i>S. aureus</i>
Ethyl acetate-	3.12	12.5	6.25	12.5	1.56	25	12.5	3.12
broth	6.41 $\pm$ 0.28	8.88 $\pm$ 0.07	10.48 $\pm$ 0.18	7.45 $\pm$ 0.28	6.37 $\pm$ 0.29	6.36 $\pm$ 0.15	6.80 $\pm$ 0.06	6.65 $\pm$ 0.07
Ethyl acetate-	0.39	1.56	1.56	0.78	1.56	0.78	1.56	0.78
mycelia	7.95 $\pm$ 1.25	10.49 $\pm$ 0.30	6.57 $\pm$ 0.26	8.55 $\pm$ 0.31	6.37 $\pm$ 0.29	7.48 $\pm$ 0.39	6.62 $\pm$ 0.37	6.77 $\pm$ 0.47
Hexane-mycelia	25.055	-	25	50	50	50	25	12.5
	7.34 $\pm$ 0.16		8.60 $\pm$ 0.25	11.22 $\pm$ 0.14	6.51 $\pm$ 0.27	6.27 $\pm$ 0.09	6.59 $\pm$ 0.18	7.22 $\pm$ 0.15
Chloramphenicol*	62.5	500	125	125	62.5	125	500	500
	9.42 $\pm$ 0.27	12.47 $\pm$ 0.37	10.54 $\pm$ 0.35	10.50 $\pm$ 0.35	9.44 $\pm$ 0.27	10.57 $\pm$ 0.35	9.52 $\pm$ 0.19	9.52 $\pm$ 0.19

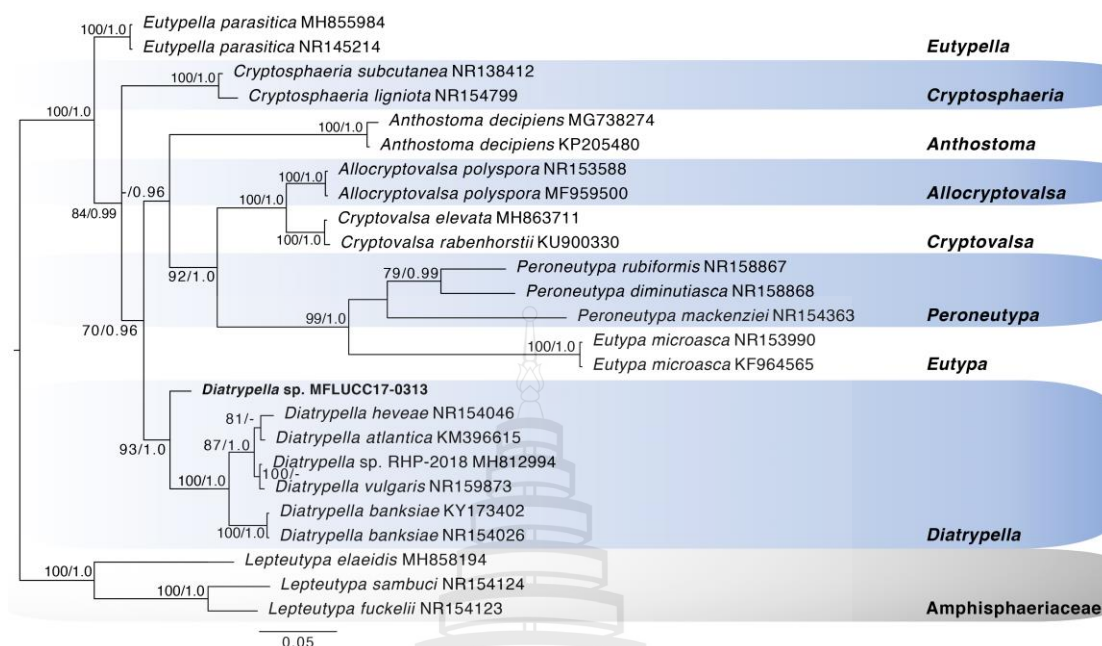
Note *MIC unit is μ g/mL.

- not detected.

Table 5.3 Percentage of cytotoxicity of crude extracts of ANG4 (30 µg/mL), doxorubicin and etoposide against four human cancer cell lines

Extract/Drug	Molt-3	HuCCA-1	A549	HepG2
Ethyl acetate-broth	-	-	-	-
Ethyl acetate-mycelia	100	66	71	54.17
Hexane-mycelia	74	-	-	-
Doxorubicin (IC ₅₀ , µg/mL)	0.013±0.006	0.5±0.028	0.32±0.035	0.32±0.01
Doxorubicin (IC ₅₀ , µg/mL)	0.024±0.006	-	-	29.35±0.63

Note Molt-3 (acute lymphoblastic leukemia), HuCCA-1 (lung cholangiocarcinoma), A549 (lung carcinoma) and HepG2 (hepatocellular liver carcinoma), the percentage of cytotoxicity of crude extracts that less than 50% is reported as no activity (-).



Note The values at each node represent the percentage of bootstrap values and the Bayesian posterior probability values per nucleotide.

Figure 5.2 Phylogenetic tree of endophytic fungus *Diatrypea* sp. MFLUCC19-0492

5.3.4 Cytotoxic Activity Assay

The cytotoxic effects of crude ethyl acetate extract of culture broth, crude ethyl acetate extract of mycelia and crude hexane extract of mycelia were evaluated by MTT and XTT assays against four selected cell lines including Molt-3, HuCCA-1, A549 and HepG2 against doxorubicin and etoposide as reference drugs. The results of the cytotoxicity investigations are presented in table 5.3.

The cytotoxicity percentages of the extracts less than 50% were reported as no activity. Results showed that crude ethyl acetate extract of culture broth was the least active with no cytotoxic activity against all tested human cell lines. The crude ethyl acetate extract of mycelia showed the most significant activity, inhibiting the Molt-3 and A549 cell lines with 100 and 71% respectively. In addition, the crude hexane extract of mycelia showed cytotoxic activity against the Molt-3 cell line with 74%. However, doxorubicin and etoposide exhibited IC_{50} values ranging from 0.013 to 0.5

µg/mL against all tested cell lines and 0.024 to 29.35 µg/mL against Molt-3 and HepG2 respectively.

5.3.5 Molecular Identification

Results from molecular and phylogenetic analyses were used to characterize the endophytic fungus ANG4 taxonomically. Partial sequences obtained from ITS1-5.8S-ITS2 of the strain were compared to ITS sequences of organisms represented in the database Genbank. The highest score sequences were collected from the databases and aligned with the ITS sequences of this endophytic fungus. Based on morphological characteristics, figure 5.2 shows the phylogenetic tree of the endophytic fungus ANG4 deposited at the Institute of Excellence in Fungal Research, Mae Fah Luang University as code MFLUCC19-0492.

The bootstrap percentage of the fungus ANG4 into family classification was 93% Diatrypella. The abundance distribution of fungal species was evaluated to be highly tilted with various frequent and incidental species. Based on the data, this fungus was identified in the genera of *Diatrypella* as *Diatrypella* sp. MFLUCC19-0492.

5.4 Discussion

In recent years, various human pathogens have developed antibiotic resistance due to indiscriminate use of commercial antibiotics. Therefore, natural products that possess antimicrobial properties are needed as alternatives to antibiotic drugs. It has been discovered that endophytic fungi are an alternative source of bioactive compounds with biological activities (Sharma et al., 2016; Toghueo & Boyom, 2019). Moreover, the populations of endophytic fungi may be dependent on host species and planting area. Medicinal plants are considered an important source for bioactive compound-producing endophytes (Golinska et al., 2015). Medicinal plant *B. prionitis* is an Ayurvedic herb used in herbal medicines for the treatment of toothaches, catarrhal affections, whooping cough, inflammation, glandular swelling, urinary infections, jaundice, fever, gastrointestinal disorders and as a diuretic and tonic (Kapoor et al., 2014). A wide range of bioactive compounds isolated from different

parts of *B. prionitis* has been reported such as balarenone, pipataline prionisides, barlerinoside, verbascoside, acetylbarlerin, lupulinoside and scutellarein (Rokade et al., 2017).

These isolated compounds have also been reported to possess various pharmacological properties including; antimicrobial, anthelmintic, antifertility, antioxidant, antidiabetic, anti-inflammatory and anti-arthritic activities without any toxic effects (Singh & Gupta, 2016). In addition, endophytes colonizing medicinal plants are reported to possess biological activities. Therefore, the medicinal properties of a plant may be correlated to their endophytes. Although phytochemical and pharmacological screenings of endophytic fungi of medicinal plants have progressed, there is little information about the bioactivity of endophytic fungi associated with *B. prionitis*. All isolates were cultured for further antibacterial and cytotoxic investigation.

The crude extracts were obtained from extraction with ethyl acetate solvent due to several pieces of research reported that crude extract from ethyl acetate solvent showed a broad spectrum of antimicrobial activities (Bhardwaj et al., 2015; Nguyen et al., 2019; Santas et al., 2010). However, hexane was also used to extract nonpolar bioactive compounds according to other studies that reported use of hexane to extract nonpolar bioactive compounds (Kumari et al., 2018; Musa et al., 2015). In this study, ethyl acetate crude extracts from mycelia and culture broth of fungus ANG4 showed potent antibacterial activity against all tested pathogens, while the hexane extract of mycelia of fungus ANG4 showed less inhibition against these same organisms.

The cytotoxicity of all crude extracts of ANG4 was investigated against four human cancers. The crude ethyl acetate extract of mycelia from ANG4 was found to be cytotoxic to cancer cell lines with stronger growth inhibition against Molt-3 compared to A549, HuCCA-1 and HepG2 cancer cell lines. High cytotoxic activity against tested cell lines was observed for ethyl acetate and hexane extracts of ANG4 mycelia especially against the Molt-3 cell line.

Therefore, each extract contained different bioactive compounds with various potential activities that are specific to a particular cell line. Various crude extracts of endophytic fungi isolated from medicinal plants have been reported to possess cytotoxic activity against many human cancer cell lines. However, the crude extracts of endophytic fungi isolated from *B. prionitis* have not previously been reported and

their bioactive compounds have also not been identified. This study describes the antibacterial and cytotoxic activities of the crude extracts of endophytic fungi isolated from *B. prionitis* against human pathogens and cancer cell lines. Moreover, endophytic fungus ANG4 was determined to produce bioactive compounds having cytotoxic activities. Many studies reported the potential of antimicrobial compounds isolated from endophytic fungi to be toxic to human cancers (Jouda et al., 2016).

5.5 Conclusion

This work demonstrates that *Diatrypella* sp. MFLUCC19-0492, isolated from *B. prionitis* leaves, has bioactive properties, highlighting its *pharmacological* potential as antibacterial and cytotoxic against human pathogens and cancer cell lines. The crude ethyl acetate extract of *Diatrypella* sp. MFLUCC19-0492 mycelia of this plant inhibits the growth of various human pathogenic bacteria and cancer cell lines on a highly active level.

Thus, there could be the possibility of new bioactive compounds in the crude extract which may provide new bioactive compounds to replace antibiotics from the *Diatrypella* genus. This study presents new antimicrobial fungal extracts that could be potential therapeutic candidates in the future. Further research needs to be conducted to identify the antibacterial and cytotoxic compounds produced by this fungus.

CHAPTER 6

***In vitro* AND *In vivo* ANTIFUNGAL ACTIVITY OF *Cuminum cyminum* ESSENTIAL OIL AGAINST *Aspergillus aculeatus* CAUSING BUNCH ROT OF POSTHARVEST GRAPES⁴**

Abstract

Bunch rot in grapes is an aggressive disease and needs to be controlled during the postharvest period. We investigate the antifungal potential of *Zanthoxylum bungeanum* Maxim., *Zanthoxylum rhetsa*, *Cuminum cyminum*, *Coriandrum sativum*, and *Zingiber montanum* (J. Koenig) Link ex A. Dietr. essential oils against *Aspergillus aculeatus* that cause bunch rot disease on postharvest grapes. *C. cyminum* essential oil exhibited stronger significantly inhibition percentage of 95.08% than other treatments in *in vitro* assay. Cumin aldehyde (33.94%) and α -terpinen-7-al (32.20%) were identified as major volatile compounds in *C. cyminum* oil. Antifungal potential of *C. cyminum* oil was then tested in conidia germination and *in vitro* tests compared to cumin aldehyde and α -terpinen-7-al. Their EC₅₀ values against the conidial germination were also estimated. Significant reduction of conidia germination was also detected in *C. cyminum* essential oil and cumin aldehyde at a concentration of 1,000 and 100 μ g/mL, respectively. EC₅₀ values of the *C. cyminum* essential oil, cumin aldehyde, and α -terpinen-7-al were 67.28 μ g/mL, 9.31 μ g/mL, and 13.23 μ g/mL, respectively. *In vivo* assay, the decrease of the disease severity (0.69%) and incidence (1.48%) percentage of *A. aculeatus* on grape berries treated at 1,000 μ g/mL of *C. cyminum* essential oil was significantly greater than that obtained from other treatments after 10 days incubation. In addition, grape berries treated with

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C. cyminum essential oil decreased weight loss and retained fruit firmness. The changing of total soluble solids, total phenolic content, and antioxidant activity are also delayed in treated fruits. Therefore, essential oil of *C. cyminum* may be applied as a biological antifungal agent to control *A. aculeatus* in postharvest grapes without any negative effects on its quality.

Keywords: Antifungal activity, *Aspergillus aculeatus*, *Cuminum cyminum*, Postharvest, Bunch rot.

6.1 Introduction

Grape (*Vitis vinifera* L.) is one of the world's most abundant fruit crops with various varieties (Kim et al., 2020). FAO (2019) reported that grapes are produced at least 50 million metric tons in 2018. It can be cultivated all over the world but mostly in China, which accounts for 16.8% of global production, followed by Italy and the United States at 10.9% and 9.6%, respectively (FAOSTAT, 2019). Wide consumption of grapes and its products, such as wine, juice, jam and raisin showing its importance in worldwide economics. They are also generally known as an essential source of natural antioxidants, including flavonoids, phenolic acids, stilbenes, and lignans (Rinaldi et al., 2017). These compounds possess protective effects against many human diseases such as cancer and cardiovascular diseases (Covas, 2008). In Thailand, grapes can be cultivated in many regions of the country. The Central region is the major production area of table grapes, while the Northeastern and Northern regions produce table and wine grapes. Beauty Seedless is the main grape variety cultivated in the Northern region due to its good growth, production, and quality, followed by Ruby Seedless and Early Muscat (Khalil et al., 2019). It is considered that grape production in Thailand has been successful. However, an increase in domestic consumption has made it necessary to import grapes from several countries at a value of one hundred million baht per year (Nilnond et al., 1996).

Grape quality depends on various factors such as cultivar type, climate, harvest and postharvest periods which affect their firmness, taste, flavor, and defect presence (Harindra Champa et al., 2014; Mirdehghan & Rahimi, 2016). Quality

degradation of grapes is mainly caused by infections from phytopathogens during cultivation and postharvest periods (Sivakumar et al., 2011). The major pathogen type that causes significant bunch rot disease in grapes during the postharvest period is the fungal pathogen *Aspergillus aculeatus* (Jarvis & Traquair, 1984; Leong et al., 2004). Infection by this pathogen contributes to a significant loss in quality and quantity during the transportation and storage process. Estimation of worldwide production loss is approximately 10 to 40% of total grape production in the world (Imlak et al., 2017; Sapper et al., 2019). Sulfur dioxide is typically used to control bunch rot disease in grapes during cultivation and postharvest period (Parafati et al., 2015; Salimi et al., 2013). Recently, its use in organic food has been made illegal due to environmental and health hazards, including residual toxicity, carcinogenicity, ecological pollution, and hormonal imbalance (Parafati et al., 2015). The development of safe and ecofriendly sustainable agriculture has been increased to control postharvest diseases with less impact on human, animal, and environment (Nunes, 2011; Pedrotti et al., 2019). Therefore, readily accessible alternative treatments other than sulfur dioxide are needed for controlling bunch rot disease in fresh grapes during postharvest storage, transportation, and market periods.

Over the years, various biological agents have been applied extensively to control diseases from pests and phytopathogens, which is known as biocontrol (Talibi et al., 2014). Essential oils are considered as biocontrol agents inhibiting phytopathogen growth on fruit surfaces by *in vitro* and *in vivo* assay during the storage period (Kaddes et al., 2019). The use of essential oils controlling postharvest phytopathogens is relatively inexpensive and may not require a sophisticated system. It can be performed by local farmers to extend the shelf lives of their agricultural products and is considered as an environmentally- and user-friendly technique. Although essential oils are advantageous as antimicrobial agent in controlling postharvest diseases, only a few studies have been reported on the control and inhibition of bunch rot disease in fresh grapes caused by *A. aculeatus* (Jarvis & Traquair, 1984).

Cuminum cyminum L., a member of Apiaceae family, is widely known as an aromatic plant. Fruits of this plant is known as cumin seeds containing high amount of essential oil. Cumin seeds have been used therapeutic purposes in traditional medicine

(Wan et al., 2008). The essential oil of cumin seed is used as natural antioxidants, flavoring the foods and in the treatment of toothache, diarrhea, epilepsy, dyspepsia, and jaundice (Wan et al., 2008). Cumin essential oil has been reported to possess antimicrobial activity against various human pathogens such as *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Staphylococcus haemolyticus*, *Propionibacterium acnes*, *Corynebacterium diphtheriae*, and *Candida albicans* (Wan et al., 2008). Moreover, this essential oil also exerted antifungal activity against various phytopathogens in postharvest fruits such as *Aspergillus flavus*, *Penicillium italicum*, *Penicillium expansum*, *Penicillium commune*, *Rhizopus stolonifera*, *Rhizopus lyococcus*, and *Botrytis cinerea* (Esmail Al-Snafi, 2016).

However, this study aimed to investigate inhibitory effect of essential oils produced by *C. cyminum* against the growth of *A. aculeatus* and its infection on fresh grape berries compared to those essential oils obtained from other plants; *Zanthoxylum bungeanum* Maxim., *Zanthoxylum rhetsa*, *Cuminum cyminum*, *Coriandrum sativum*, and *Zingiber montanum* (J. Koenig) Link ex A. Dietr. These essential oils from selected plants are known as Ayurvedic herbs, which is endemic to tropical Asia and have been used for centuries as herbal medicine to treat various symptoms such as astringent, asthma, bronchitis, diarrhea, and stomach aches. Previous reports have demonstrated that essential oils from these plants showing antimicrobial activities. Thus, the aim of the present study is to investigate the inhibitory effect of these essential oils against *A. aculeatus* *in vitro* and *in vivo* assay. The volatile compounds of plant species were also identified.

6.2 Materials and Methods

6.2.1 Plant Material

Healthy *Z. bungeanum* Maxim., *Z. rhetsa*, *C. cyminum*, *C. sativum*, and *Z. montanum* (J. Koenig) Link ex A. Dietr. were collected from Chiang Rai province, Thailand in May 2019. Age of *Z. rhetsa* and *Z. montanum* (J. Koenig) Link ex A. Dietr. is approximately seven and two years, respectively. Other plants aged approximately five months. They were washed and further dried at room temperature

(27 °C) for five days. Each plant species was deposited in the botanical garden of Mae Fah Luang University having a voucher specimen of accession number 10044, 10045, 10046, 10047, and 10048 for *Z. bungeanum* Maxim., *Z. rhetsa*, *C. cyminum*, *C. sativum*, and *Z. montanum* (J. Koenig) Link ex A. Dietr., respectively.

6.2.2 Extraction of Essential Oils

Each dried sample was subjected to hydrodistillation for 3 h according to procedures reported by Insawang et al. (2019). Anhydrous Na₂SO₄ was added in the obtained essential oils to remove water prior placing pure essential oils in a sealed vial.

6.2.3 Isolation and Identification of Phytopathogen *A. Aculeatus*

Isolation of fungal pathogen was performed using a modified method (Adesegun et al., 2013). Infected berries of Beauty Seedless grapes (*Vitis vinifera*) were collected from DoiFang Vineyard Farm (19°51'59.8"N 99°09'25.9"E), Fang district, Chiang Mai province, Thailand in July 2019. The collection site access has been approved by Ms Sawittree Kaewprasert, the owner of DoiFang Vineyard Farm. They were washed with sterile water before placing the fungal spores on Petri plates containing potato dextrose agar (PDA) mixed with chloramphenicol (30 µg/mL) to prevent bacterial growth. The plates were incubated at room temperature for seven days to complete fungal growth. The obtained fungus was further purified using the hyphal tips technique on PDA medium. It was identified based on micro- and macro-morphological characteristics using their cultures and spores according by Adesegun et al. (2013). In addition, the species level of isolated fungus was identified using molecular technique. The genomic DNA of isolated fungus was also extracted using a method of Tanapichatsakul et al. (2020). obtained sequences were compared to all sequences deposited in the National Center for Biotechnology Information database.

6.2.4 *In vitro* Test of Essential Oils on The Mycelial Growth of *A. Aculeatus*

The antagonistic activity of essential oils obtained from different plant species against *A. aculeatus* which caused bunch rot disease in Beauty Seedless grapes was evaluated using the contact phase method according to Aminifard and Mohammadi

(2013) and Pedrotti et al. (2017). with some modifications. The contact phase method was carried out using various concentrations of essential oils (200, 400, 600, 800, and 1,000 $\mu\text{g/mL}$) diluted in 5% v/v Tween 20. PDA (9.0 mL) was placed into 9 cm diameter Petri dish plates. A 100 μL sample of each essential oil solution was placed at the center of a Petri plate. After five minutes, an agar plug (5 mm) of pathogen *A. aculeatus* from seven days precultures was placed at the center of the PDA plate. All plates were sealed with Parafilm and further incubated at room temperature for seven days. Mycelia growth was determined after seven days. The mycelial diameter of *A. aculeatus* pathogen treated with essential oil in the PDA medium and the control group (no essential oil treatment) was measured daily with a digital Vernier caliper (MITUTOYO-ABS Digimatic Caliper CD-AX) in a mm unit. Plates containing 9.0 mL of PDA medium mixed with 5% v/v Tween 20 (100 μL) were used as control groups. The obtained results were reported as inhibition percentage of mycelial growth: $[(\text{mycelial diameter of the untreated} - \text{mycelial diameter of treated essential oil}) / \text{mycelial diameter of untreated essential oil}] \times 100$. There were five replicates for each treatment, and the experiment was repeated three times.

6.2.5 Viability Test of The Treated Pathogen

To evaluate the viability of *A. aculeatus* after essential oils treatment in *in vitro* test, an agar plug (5 mm) of *A. aculeatus* pathogen was transferred to a fresh PDA plate and further cultured at room temperature for seven days. The mycelial growth was determined by measuring their mycelial diameter. This experiment was conducted with five replicates, and repeated three times.

6.2.6 Analysis of The Volatile Compounds of *C. Cuminum* Essential Oil

The volatile compounds of *C. cuminum* essential oil was identified using gas chromatograph (HP6890, Agilent Technologies) interfaced to a mass-selective detector (HP model 5973, Agilent Technologies). The columns used was a HP-5MS (5% phenyl polymethylsiloxane) capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μm) (Agilent Technologies). Purified helium gas was used as the carrier gas. The temperature program was initially set at 60 $^{\circ}\text{C}$ and increased to 220 $^{\circ}\text{C}$ at a rate of 3 $^{\circ}\text{C}/\text{min}$. Injector temperature was set at 250 $^{\circ}\text{C}$ while detector temperature was set at 280 $^{\circ}\text{C}$, respectively. The ionization energy was 70 eV and molecular mass were

scanned ranging from 30–300 AMU. The voltage of electron multiplier was programmed at 1150 V. The temperature of ion source was set at 230°C while quadrupole temperature was set at 250 °C. The volatile compounds were identified by comparing their retention indices relatively to C₈–C₁₇ n-alkanes, with those obtained from Adams databases [Adams, 2017]. Moreover, their spectra were also compared to those found in the Wiley7N, NIST98 Mass Spectral Library. The quantitation of volatile compounds was performed using gas chromatograph (HP 6890, Agilent Technologies) equipped with a flame ionization detector. The volatile components were reported as the relative peak area percentage.

6.2.7 *In vitro* Test of Pure Compounds on The Mycelial Growth of *A. Aculeatus*

Antifungal activity of pure volatile compounds including cumin aldehyde and α -terpinen-7-al against *A. aculeatus* using the contact phase method described by Aminifard and Mohammadi (2013) and Pedrotti et al. (2017). with some modifications. These compounds were purchased from Hairui Chemical (China) and various concentrations of 20, 40, 60, 80, and 100 μ g/mL were prepared by diluting in 5% v/v Tween 20. After that, 9.0 mL of PDA medium solution was placed into 9 cm diameter Petri dish plates. A 100 μ L of each sample was placed at the center of a Petri plate. After five minutes, an agar plug (5 mm) of pathogen *A. aculeatus* from seven days precultures was placed at the center of the PDA plate. All plates were sealed with Parafilm and further incubated at room temperature for seven days. Mycelia growth was determined after seven days. The mycelial diameter of *A. aculeatus* pathogen treated with pure compounds in the PDA medium and the control group (no pure compound treatment) was measured daily with a digital Vernier caliper (MITUTOYO-ABS Digimatic Caliper CD-AX) in a mm unit. Plates containing 9.0 mL of PDA medium mixed with 5% v/v Tween 20 (100 μ L) were used as control groups. The obtained results were reported as inhibition percentage of mycelial growth as explained previously. There were five replicates for each treatment, and the experiment was repeated three times.

6.2.8 Antifungal Activity of *C. Cyminum* Essential Oil and Pure Volatile Compounds on Conidia Germination

C. cyminum essential oil showing strong inhibition percentage in *in vitro* experiments were selected for conidia germination experiments in accordance with the procedure described by Pedrotti et al. (2017). Pure volatile compounds including cumin aldehyde and α -terpinen-7-al were also tested to determine their effect against conidia germination of *A. aculeatus*. Conidia of 14-day old *A. aculeatus* were used in this experiment. The conidia were slowly poured in 5 mL of sterile water before scraping its surface using a sterile glass rod. The mycelia fragments extracted from the suspensions by filtering through three layers of cheesecloth. Conidial concentration of 1×10^6 conidia/mL was prepared using a hemocytometer under a microscope. A 50 μ L conidia suspension was immersed in microtubes containing 500 μ L of potato dextrose broth (PDB). Different concentrations of *C. cyminum* essential oil (200, 400, 600, 800, and 1,000 μ g/mL) or pure volatile compounds (20, 40, 60, 80, and 100 μ g/mL) were prepared in 5% v/v Tween 20. PDB, with the addition of 5% v/v Tween 20 was used as the control treatment. All microtubes were further incubated at room temperature for 16 h before transferring samples to a hemocytometer tube. The number of conidia germination was counted under microscope at 10 $\times$ magnification. EC₅₀ values were also calculated as the effective concentration that inhibited conidial germination by 50% in comparison to the control. This experiment was conducted with five replicates, in which 100 conidia were determined in each replicate. This experiment was repeated three times.

6.2.9 *In vivo* Antifungal Assay of *C. Cyminum* Essential Oil and Pure Volatile Compounds

The *in vivo* experiment was performed using the procedure described by Pedrotti et al. (2019) with some modifications. All healthy Beauty Seedless grape berries collected from a greenhouse at DoiFang Vineyard Farm (19°51'59.8"N 99°09'25.9"E), Fang district, Chiang Mai province, Thailand located in March 2020. The collection site access has been approved by Ms Sawittree Kaewprasert, the owner of DoiFang Vineyard Farm. They were chosen based on their maturity, size, color without physical infections or injuries. The grape berries were sterilized by washing

with distilled water for 2 min followed by 1% sodium hypochlorite for 60 s, and distilled water three times. Those berries were placed on a clean, pre-aseptic bench to airdry for 30 min before inoculation with the pathogens. One location on each berry was wounded using a sterile inoculating needle. Conidia of pathogen *A. aculeatus* was collected from its 14-day old colony in PDA at room temperature with a 12-h photoperiod. The suspension was prepared in sterile water to obtain a suspension concentration of 1×10^6 conidia/mL. The wound depth was approximately 2 mm. In the postharvest curative treatment, twenty healthy grape berries were inoculated simultaneously with 10 μ L of a conidia suspension of *A. aculeatus*. They were then placed at the bottom of a 2 L plastic box that was covered with sterilized tissue paper. After 4 h, each grape berry was sprayed with *C. cyminum* essential oils at various concentrations of 200, 400, 600, 800, and 1,000 μ g/mL or pure volatile compounds (cumin aldehyde and α -terpinen-7-al) solutions at various concentrations of 20, 40, 60, 80, and 100 μ g/mL in 5%v/v Tween 20. Each box was closed with a fitted plastic lid and air-sealed using Parafilm. All boxes were separated in two groups and then kept at room temperature for 10 and 20 days, respectively. Different clusters of grape berries were prepared for each solution treatment: pathogen-uninoculated (negative control, NC), essential oil-treated (essential oil treatment, TE), cumin aldehyde-treated (cumin aldehyde TE), and α -terpinen-7-al-treated (α -terpinen-7-al TE) grape berries. Groups from NC treatments were treated with *C. cyminum* oil at a concentration of 1,000 μ g/mL or pure compounds at a concentration of 100 μ g/mL, while grape berries in the TE group were treated with *C. cyminum* oil and pure volatile compounds at various concentrations. Results from these clusters were compared to those obtained from the clusters without essential oil or pure compound treatments: pathogen-inoculated (positive control, PC) and pathogen-uninoculated (control, CC) grape berries. A digital Vernier caliper (MITUTOYO-ABS Digimatic Caliper CD-AX) was used to measure the mycelial diameter (mm) of *A. aculeatus* pathogen detected in each sample. Each treatment was conducted with five replicates. This experiment was repeated three times. *In vivo* antifungal effect was expressed in terms of disease incidence and severity percentage. Disease incidence was calculated as [(numbers of diseased fruit/total Number of fruit) $\times$ 100]. Disease severity was calculated using the following formula: [(sum of symptomatic fruits and their

corresponding score scale)/(total number of fruits $\times$ highest score scale)] $\times$ 100. The severity scale was considered from 0 to 100% according to Pedrotti et al. (2017).

6.2.10 Quality of Grape Berries Obtained from All Treatments

The quality of Beauty Seedless grape berries was evaluated after treatments with *C. cyminum* essential oil, cumin aldehyde, and α -terpinen-7-al for 10 and 20 days. In this experiment, weight loss percentage, fruit firmness, total soluble solids, and pH were measured following the study by Jahani et al. (2020). Total phenolic content and antioxidant activity of grape berries after treatment were determined following the study by Molyneux (2004) and Yao et al. (2004). Each experiment was conducted with five replicates, and repeated three times.

Weight loss percentage. Weight loss of grape berries was determined as the percentage of weight loss with the following formula: (Initial weight—terminal weight)/(terminal weight) $\times$ 100.

Fruit firmness. Fruit firmness of samples was measured using a TA-XT Plus texture analyzer (Stable Micro Systems Ltd., UK).

Total soluble solids. Total soluble solids of the grape juices were determined at room temperature using a digital refractometer (RF 10, 0–32° Brix, Extech Co., USA).

pH. The pH value of grape juices was measured at room temperature using a pH meter (Thermo Scientific Orion Star A111 Benchtop pH Meter, USA).

Total phenolic content and antioxidant activity. Grape berries from all treatments were homogenized and centrifuged at 13,000 rpm at room temperature for 10 min to extract their bioactive compounds. The obtained supernatants were used to be determined their phenolic content and antioxidant activity.

For the total phenolic content assay, extract (100 μ L) was mixed with phosphate buffer (400 μ L) and 10% v/v Folin-Ciocalteu phenol reagent (5 mL). After 1 min, 4 mL of 7.5% w/v sodium carbonate solution was added to the mixture and further kept in a dark place at room temperature for 1 h. Absorbance spectrum of the solution was determined using a UV/Vis spectrophotometer (Perkin-Elmer-Lamda 25) at 765 nm. Gallic acid was used as a standard.

Antioxidant activity of grape berries extracts was analyzed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Grape extract (1 mL) was added to 60 mM DPPH methanolic solution (1 mL). The solution was vigorously mixed and stored in a dark place at room temperature for 30 min. Absorbance of all solutions were determined using UV/Vis spectrophotometer (PerkinElmer-Lambda 25) at 517 nm. Trolox was used as a standard.

6.2.11 Statistical Analysis

The differences in all experiments were analyzed using analysis of variance (ANOVA), using the SPSS 20.0 software. Duncan's post-hoc test was used to compare the mean values. A significance level at $P < 0.05$ was set to determine the significant differences. All data were reported as mean values with standard deviations.

6.3 Results

6.3.1 *In vitro* Antifungal Activity of Essential Oils on The *A. Aculeatus* Mycelial Growth

Z. bungeanum Maxim., *C. sativum*, *Z. rhetsa*, *C. cyminum*, *C. sativum*, and *Z. montanum* (J. Koenig) Link ex A. Dietr. presented essential oil yields of 0.28%, 0.48%, 0.57%, 0.89%, and 0.93%, respectively. All essential oils were screened *in vitro* antifungal activity against *A. aculeatus* on mycelial growth using contact phase assay. The effects of different essential oil concentrations on the *A. aculeatus* mycelial growth are listed in Table 6.1. The inhibition rate of essential oils on the *A. aculeatus* mycelial growth was most prominent at day 1, while the lowest was observed at day 7. A significant inhibition was observed until the day 7 compared to the control. High inhibition rate (>80%) was observed in *C. cyminum* essential oil from a concentration of 800 and 1,000 µg/mL at all days while other essential oils showed low inhibition rate (<50%) at most concentrations and number of days. Therefore, *C. cyminum* essential oils had significantly highest inhibition rate and were chosen for further tests.

6.3.2 Viability of The Treated Pathogen

After the treatment of *A. aculeatus* with the essential oils *C. cyminum*, pathogen *A. aculeatus* was able to grow and produce its mycelia slowly again on the PDA plates. The diameters of *A. aculeatus* pathogen after treating and without treating with *C. cyminum* essential oil on medium agar plates are shown in Figure 6.1. The growth of *A. aculeatus* after treating with *C. cyminum* essential oil was significantly slower than those obtained from untreated plates. After 14 days, diameter of *A. aculeatus* pathogen after treating and without treating with *C. cyminum* was 2.51 cm and 8.63 cm, respectively.

6.3.3 Analysis of Volatile Compounds of *C. cyminum* Essential Oil

Volatile compounds of *C. cyminum* oil are listed in Table 6.2. Twenty-four components were identified in *C. cyminum* essential oil, comprising 100% of the essential oil. The major compounds in *C. cyminum* oil were cuminaldehyde (33.94%), α -terpinen-7-al (32.20%), γ -terpinen-7-al (13.74%), γ -terpinene (6.67%), β -pinene (5.34%) and p-cymene (3.58%).

Table 6.1 Antifungal activity of *C. cyminum*, *Z. bungeanum* Maxim., *C. sativum*, *Z. montanum* (J. Koenig) Link ex A. Dietr., and *Z. rhetsa* essential oils at different concentrations against *A. aculeatus* using contact phase assay

Essential oil	Concentration (µg/mL)	% inhibition						
		Day1	Day2	Day3	Day4	Day5	Day6	Day7
<i>C. cyminum</i>	200	78.27±0.28 e	76.44±1.09 e	76.37±0.33 e	72.66±0.58 e	72.64±0.98 e	70.41±0.83 e	69.69±1.68 d
	400	82.06±0.81 d	80.79±0.30 d	80.05±0.50 d	79.27±0.60 d	79.08±0.27 d	76.19±1.13 d	75.76±0.76 c
	600	85.04±0.23 c	84.13±0.53 c	83.75±0.93 c	83.43±0.28 c	82.43±0.47 c	79.87±0.90 c	76.13±1.49 b
	800	91.09±0.34 b	90.23±0.25 b	87.05±0.56 b	86.09±1.12 b	85.49±0.43 b	82.55±0.90 b	81.48±0.66 b
	1,000	95.08±0.12 a	92.25±0.38 a	89.29±0.51 a	87.64±0.52 a	87.20±0.85 a	86.04±0.57 a	82.13±1.49 a
<i>Z. bungeanum</i>	200	38.52±2.00 d	34.09±0.83 e	31.47±1.26 e	27.49±0.00 e	25.57±1.15 e	24.21±0.00 e	22.49±1.01 e
	400	39.26±0.98 d	36.94±1.29 d	33.87±1.42 d	31.03±1.13 d	29.57±1.00 d	28.91±0.96 d	26.91±0.94 d
	600	44.97±1.81 c	40.65±1.42 c	39.29±1.35 c	37.83±1.77 c	36.31±0.88 c	35.05±1.46 c	33.46±0.84 c
	800	47.72±1.79 b	44.69±1.31 b	43.03±1.05 b	41.75±1.61 b	39.10±1.69 b	37.25±0.82 b	35.05±1.42 b
	1,000	50.39±1.98 a	47.73±1.42 a	45.20±1.66 a	43.66±0.95 a	41.82±1.37 a	41.22±0.52 a	39.10±0.53 a
<i>C. sativum</i>	200	41.27±0.68 e	29.77±0.40 e	27.18±1.08 e	26.94±0.54 e	23.62±0.55 d	21.69±0.98 d	21.39±1.20 c
	400	45.85±0.54 d	34.22±0.39 d	30.62±0.91 d	30.54±1.45 d	22.79±0.92 c	21.94±0.93 c	21.11±1.64 b
	600	47.84±0.64 c	46.64±1.07 c	43.60±0.51 c	43.25±1.42 c	31.52±0.90 b	29.40±0.53 b	22.50±0.67 a
	800	50.98±0.88 b	49.06±0.74 b	47.44±0.37 b	45.13±0.38 b	31.19±1.34 a	31.22±0.80 a	28.52±1.27 a

Table 6.1 (Continued)

Essential oil	Concentration (µg/mL)	% inhibition						
		Day1	Day2	Day3	Day4	Day5	Day6	Day7
<i>Z. montanum</i>	1,000	53.38±0.30 a	51.00±0.47 a	50.06±0.37 a	48.74±0.58 a	47.65±0.98 a	36.37±0.87 a	36.00±0.75 a
	200	40.13±0.56 d	39.29±1.32 e	37.25±0.54 d	26.47±1.64 d	24.74±1.09 d	23.65±0.66 d	23.14±0.96 c
	400	43.66±0.95 d	42.00±2.06 d	41.91±1.70 c	28.79±0.92 d	25.05±0.95 c	24.03±1.32 c	23.78±0.55 b
	600	46.46±0.92 c	44.24±1.33 c	43.31±0.89 b	43.00±1.82 c	30.31±1.39 b	28.18±0.53 b	25.16±1.74 a
	800	50.97±1.54 b	49.06±0.97 b	45.56±1.31 b	44.48±2.18 b	42.71±1.03 a	40.62±0.91 a	40.55±1.13 a
<i>Z. rhetsa</i>	1,000	51.86±0.88 a	51.51±0.59 a	50.72±0.48 a	48.78±0.49 a	47.52±1.12 a	45.64±0.50 a	45.12±1.29 a
	200	46.16±0.54 d	45.56±2.16 d	33.36±0.77 e	32.56±0.54 c	14.74±0.55 e	12.50±0.56 e	12.11±1.64 e
	400	48.75±0.64 c	46.63±2.13 c	44.24±1.33 d	31.29±1.13 b	27.40±0.93 d	24.78±1.73 d	24.58±0.55 d
	600	49.19±1.27 b	48.75±1.82 b	45.79±0.38 c	45.50±0.61 b	40.16±0.64 c	26.94±0.66 c	26.29±1.72 c
	800	52.29±1.85 b	51.51±0.29 a	48.07±0.49 b	46.86±2.23 a	44.63±0.51 b	31.97±0.52 b	28.52±1.27 b
	1,000	54.16±0.98 a	53.52±0.58 a	51.10±0.72 a	50.45±0.48 a	47.36±1.31 a	44.62±1.60 a	42.42±0.89 a

Note Data are mean ± standard deviation. Different letters indicate significant difference within each essential oil concentrations determined in each day according to Duncan's post-hoc test (P<0.05).

6.3.4 *In vitro* Test of Pure Compound on The Mycelial Growth of *A. Aculeatus*

All concentrations of pure major volatile compounds of *C. cyminum* oil (cumin aldehyde and α -terpinen-7-al) were tested to determine their antifungal activity against *A. aculeatus* using contact phase assay that showed in Table 6.3. The reduction on mycelial growth of *A. aculeatus* was most prominent at day 1 and continuously reduced at day 7. High inhibition rate (>80%) was recorded significantly in cumin aldehyde at concentration 60, 80, and 100 $\mu\text{g/mL}$, respectively, at day 1. Meanwhile, at all concentrations and number of days of α -terpinen-7-al showed low inhibition rate (<70%) at most concentrations and number of days. Hence, cumin aldehyde had significant inhibition rate on the mycelial growth of *A. aculeatus*.

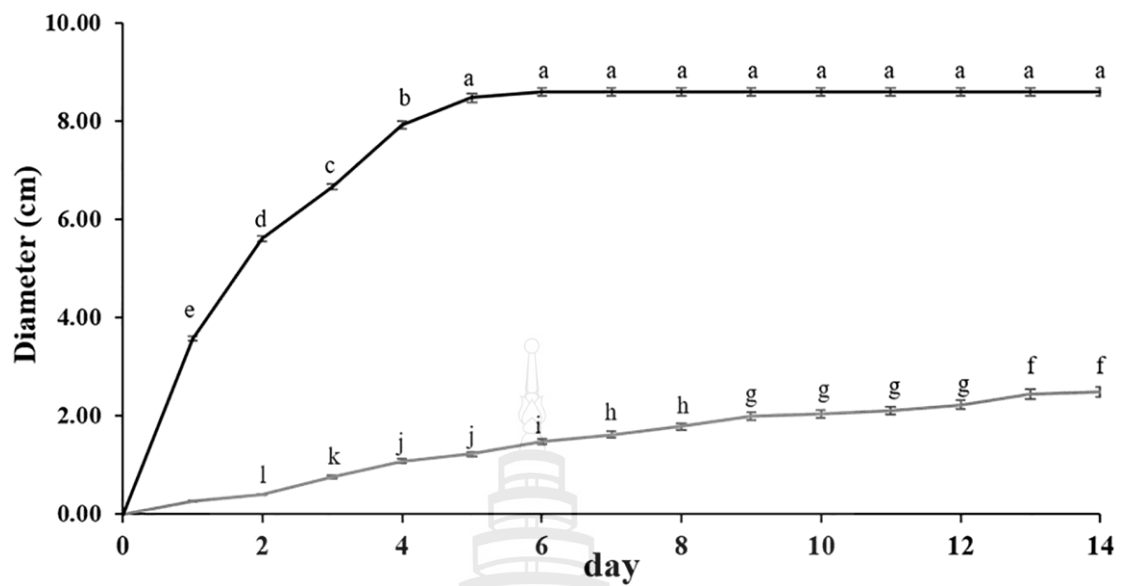
6.3.5 Antifungal Activity of *C. Cyminum* Essential Oil and Pure Volatile Compounds on Conidia Germination

The inhibitory effect of *C. cyminum* essential oil and pure volatile compounds from different concentrations on conidia germination of *A. aculeatus* is depicted in Figure 6.2. The conidia germination of *A. aculeatus* was significantly lower in the highest concentration. At all concentrations of *C. cyminum* essential oil, cumin aldehyde and α -terpinen-7-al, a significant reduction of conidia germination was observed in comparison with the control group. *C. cyminum* essential oil and cumin aldehyde inhibited conidia germination of *A. aculeatus* at the highest concentration (1000 and 100 $\mu\text{g/mL}$), remaining 1.0 and 4.8 conidia germinated, respectively, while α -terpinen-7-al showed significant reduction among concentration 60 – 100 $\mu\text{g/mL}$. EC_{50} values of the *C. cyminum* essential oil, cumin aldehyde, and α -terpinen-7-al were 67.28 $\mu\text{g/mL}$, 9.31 $\mu\text{g/mL}$, and 13.23 $\mu\text{g/mL}$, respectively.

6.3.6 *In vivo* Antifungal Assay of *C. Cyminum* Essential Oil, Cumin Aldehyde, and α -Terpinen-7-Al

Grape berries after 10- and 20-day incubation from various treatments are shown in Figure 6.3 and 6.4, respectively. No physical damage on the grape berries was detected in the CC and NC treatments among all treatments. Less physical damage was observed on grape berries inoculated with *A. aculeatus* and treated with *C. cyminum* oil (≥ 800 $\mu\text{g/mL}$) and cumin aldehyde (100 $\mu\text{g/mL}$) after 10-day

incubation. Moderate physical damage was observed on grape berries inoculated with *A. aculeatus* and treated with *C. cyminum* oils at concentrations of ≤ 600 $\mu\text{g/mL}$, cuminaldehyde at concentrations of ≤ 80 $\mu\text{g/mL}$, and α -terpinen-7-al at all concentrations after 10-day incubation. However, high physical damage was observed on grape berries inoculated with *A. aculeatus* and treated with all concentrations after 20-day incubation. Incidence and severity percentage of *A. aculeatus* in grape berries treated with *C. cyminum* oil, cuminaldehyde, and α -terpinen-7-al from various concentrations after 10- and 20-day incubation are shown in Figure 6.5. Significant reduction of the incidence and severity percentage was observed in all treatments. After 10-day incubation, *C. cyminum* oil (1,000 $\mu\text{g/mL}$) and cuminaldehyde (100 $\mu\text{g/mL}$) treatments showed the highest reduction among these concentrations with 1.48% incidence and 0.69% severity for *C. cyminum* oil and 2.04% incidence and 1.11% severity for cuminaldehyde while no significant reduction of the incidence and severity percentage was observed in α -terpinen-7-al treatment at concentration of 60 $\mu\text{g/mL}$. After 20-day incubation, the highest reduction of incidence and severity percentage was also detected from the treatment with *C. cyminum* oil followed by cuminaldehyde, and α -terpinen-7-al at the highest concentration with % incidence of 3.70, 5.56, and 25.93, and % severity of 2.59, 3.33, and 18.06, respectively.



Note Data are mean $\pm$ standard deviation. Different letters indicate significant difference within each essential oil concentrations determined in each day according to Duncan's post-hoc test ($P < 0.05$).

Figure 6.1 Diameter of *A. aculeatus* pathogen after treating (■) and without treating (■) with *C. cyminum* essential oil on medium agar plates

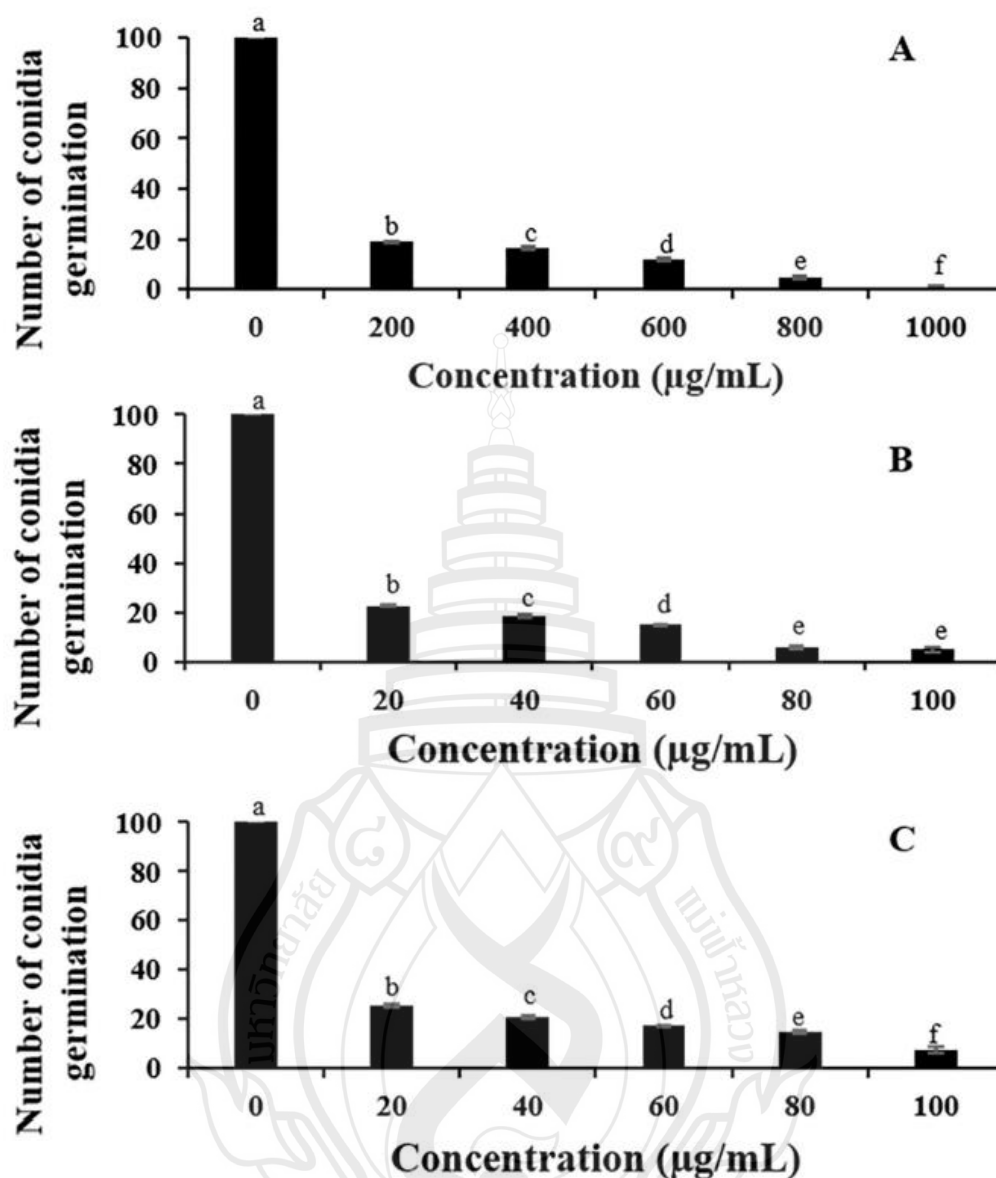
Table 6.2 Volatile compounds of *C. cyminum* essential oil

No.	Compound	RI	% Relative peak area
Monoterpene Hydrocarbons			
1	Tricyclene	921	0.11
2	α -Thujene	924	0.25
3	α -Pinene	932	0.37
4	β -Pinene	974	5.34
5	Myrcene	988	0.39
6	α -Phellandrene	1002	0.06
7	γ -carene	1008	0.02
8	α -Terpinene	1014	0.06
9	p-Cymene	1020	3.58
10	Limonene	1024	0.22
11	γ -Terpinene	1054	6.67
12	Terpinolene	1086	0.03
Oxygenated monoterpenes			
13	1,8-Cineole	1026	0.15
14	cis-Sabinene hydrate	1065	0.06
15	trans-Sabinene hydrate	1098	0.10
16	cis-p-Menth-2-en-1-ol	1118	0.04
17	trans-p-menth-2-en-1-ol	1136	0.10
18	cis- β -Terpineol	1140	0.22
19	α -Terpineol	1186	0.13
20	Pulegone	1233	1.58
21	Cumin aldehyde	1238	33.94
22	γ -Terpinen-7-al	1260	13.74
23	α -Terpinen-7-al	1290	32.20
24	p-Mentha-1,4-dien-7-ol	1325	0.65

Table 6.3 Antifungal activity of cumin aldehyde and α -terpinen-7-al at different concentrations against *A. aculeatus* using contact phase assay

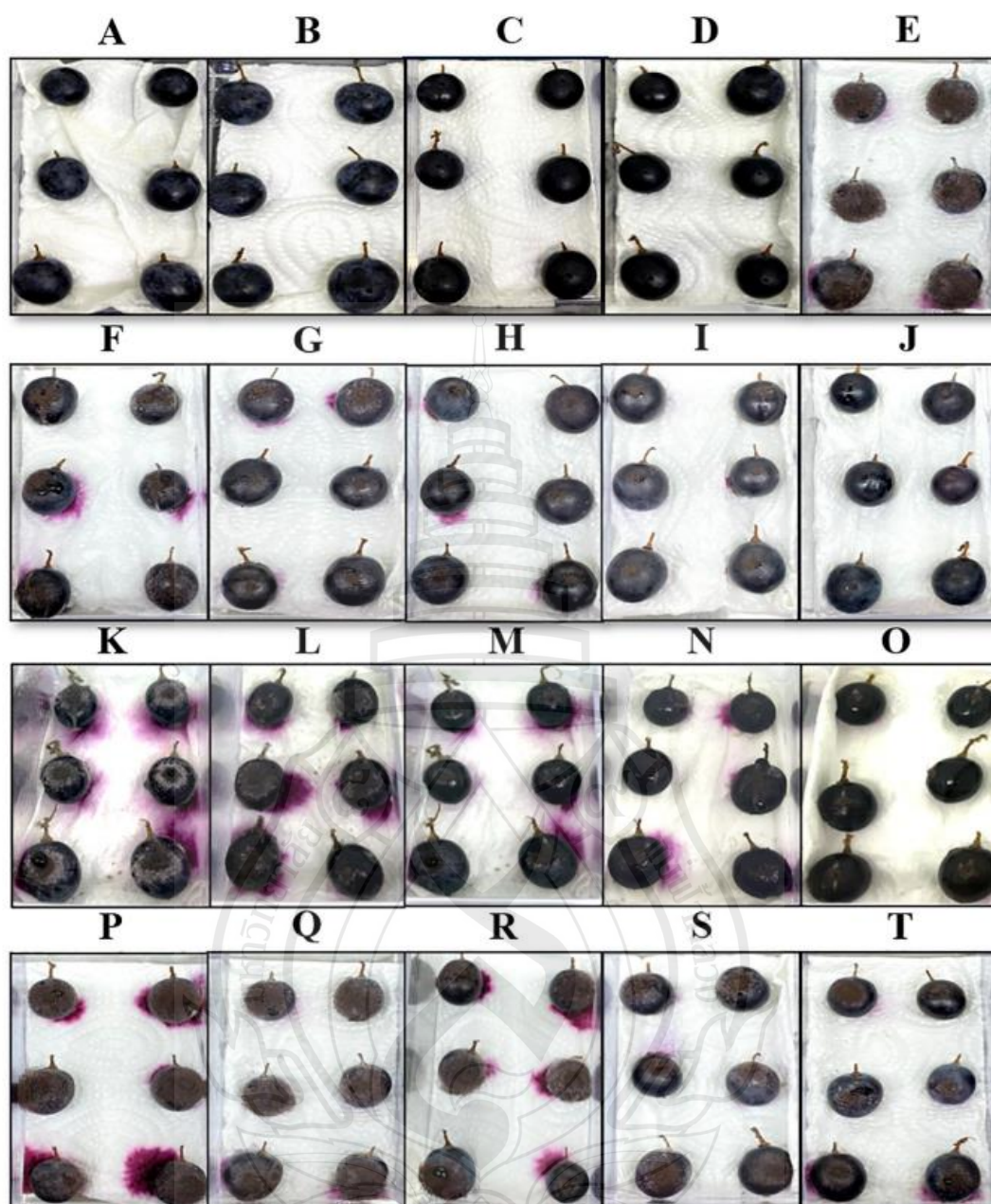
Essential oil	Concentration ($\mu\text{g/mL}$)	% Inhibition						
		Day1	Day2	Day3	Day4	Day5	Day6	Day7
Cumin aldehyde	20	72.04 $\pm$ 0.85 e	69.55 $\pm$ 1.52 d	62.61 $\pm$ 0.98 d	60.90 $\pm$ 1.00 d	58.57 $\pm$ 1.02 d	56.77 $\pm$ 1.04 d	54.94 $\pm$ 1.07 c
	40	77.13 $\pm$ 0.76 d	71.06 $\pm$ 0.00 d	70.55 $\pm$ 1.71 c	64.81 $\pm$ 1.64 c	61.46 $\pm$ 1.71 c	59.16 $\pm$ 1.02 c	56.16 $\pm$ 1.83 c
	60	80.11 $\pm$ 0.71 c	74.42 $\pm$ 0.80 c	72.04 $\pm$ 0.85 c	69.55 $\pm$ 1.52 b	66.44 $\pm$ 0.00 b	63.16 $\pm$ 1.67 b	60.90 $\pm$ 1.00 b
	80	82.10 $\pm$ 0.67 b	78.86 $\pm$ 0.74 b	74.88 $\pm$ 0.80 b	71.55 $\pm$ 0.85 b	68.53 $\pm$ 0.89 b	64.27 $\pm$ 0.96 b	62.04 $\pm$ 0.98 b
	100	85.07 $\pm$ 1.07 a	81.32 $\pm$ 0.69 a	78.43 $\pm$ 0.74 a	75.33 $\pm$ 1.37 a	72.52 $\pm$ 1.45 a	68.53 $\pm$ 0.89 a	65.36 $\pm$ 0.93 a
α -terpinen-7-al	20	54.94 $\pm$ 1.07 e	51.16 $\pm$ 1.11 e	48.55 $\pm$ 1.98 d	45.88 $\pm$ 1.18 e	41.04 $\pm$ 1.22 e	38.18 $\pm$ 2.17 d	33.01 $\pm$ 2.59 d
	40	59.16 $\pm$ 1.02 d	55.55 $\pm$ 1.07 d	51.80 $\pm$ 1.11 c	49.21 $\pm$ 1.13 d	47.23 $\pm$ 1.15 d	43.14 $\pm$ 1.20 c	39.62 $\pm$ 1.24 c
	60	62.61 $\pm$ 0.98 c	60.32 $\pm$ 1.00 c	57.37 $\pm$ 1.04 b	53.70 $\pm$ 1.09 c	50.52 $\pm$ 0.00 c	47.23 $\pm$ 1.15 b	43.82 $\pm$ 2.37 b
	80	66.97 $\pm$ 0.91 b	63.72 $\pm$ 0.96 b	59.16 $\pm$ 1.02 b	56.16 $\pm$ 1.83 b	53.07 $\pm$ 1.09 b	49.21 $\pm$ 1.13 b	45.20 $\pm$ 1.18 ab
	100	71.55 $\pm$ 0.85 a	67.49 $\pm$ 0.91a	63.72 $\pm$ 0.96 a	59.16 $\pm$ 1.02 a	55.55 $\pm$ 1.07 a	51.80 $\pm$ 1.11 a	47.89 $\pm$ 1.15 a

Note Data are mean $\pm$ standard deviation. Different letters indicate significant difference within each essential oil concentrations determined in each day according to Duncan's post-hoc test ($P < 0.05$).



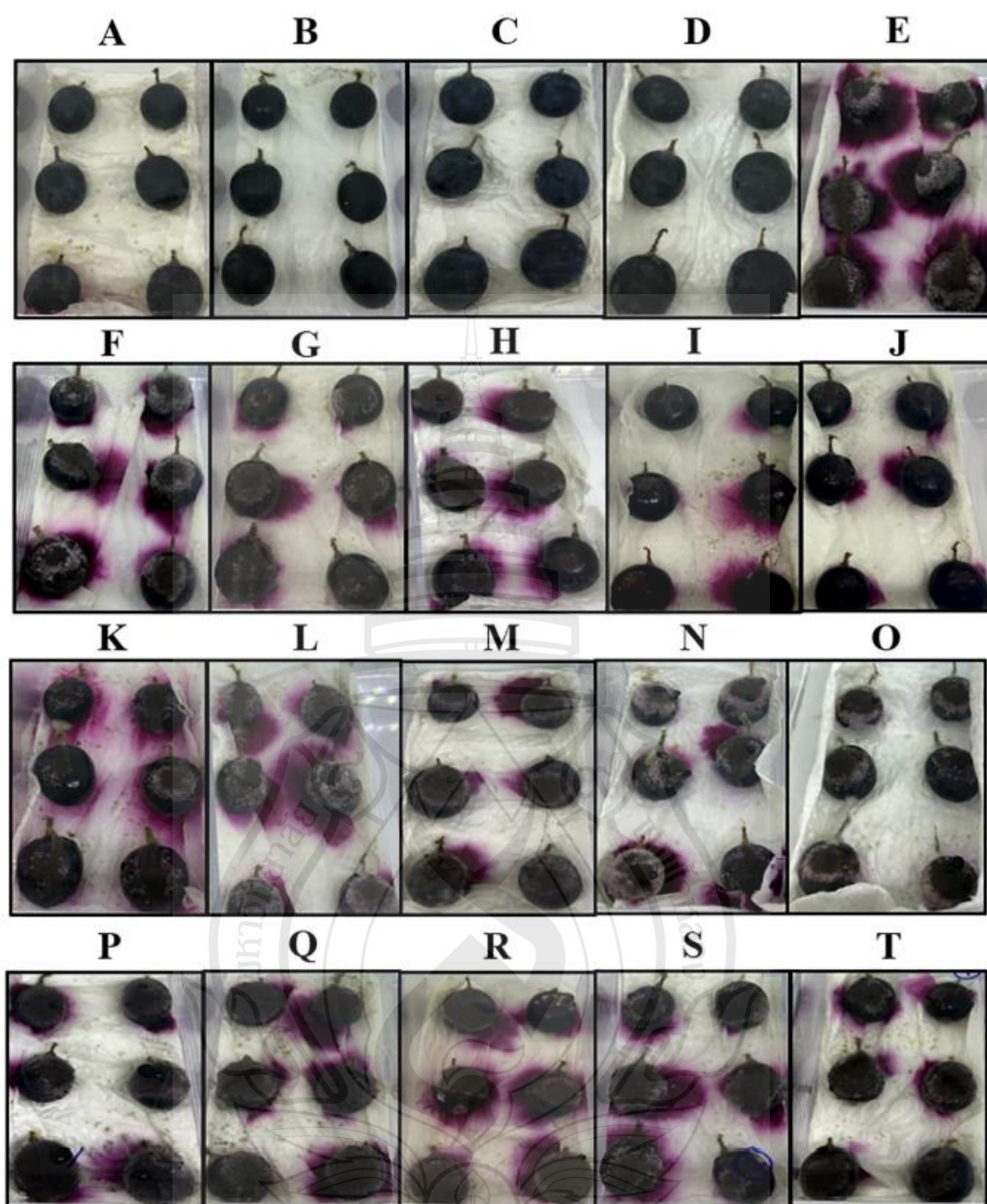
Note Data are mean $\pm$ standard deviation. Different letters indicate significant difference within each essential oil concentrations determined in each day according to Duncan's post-hoc test ($P < 0.05$).

Figure 6.2 Effect of different concentrations of *C. cyminum* essential oil (A), cumin aldehyde (B), and α -terpinen-7-al (C) on conidia germination of *A. aculeatus*



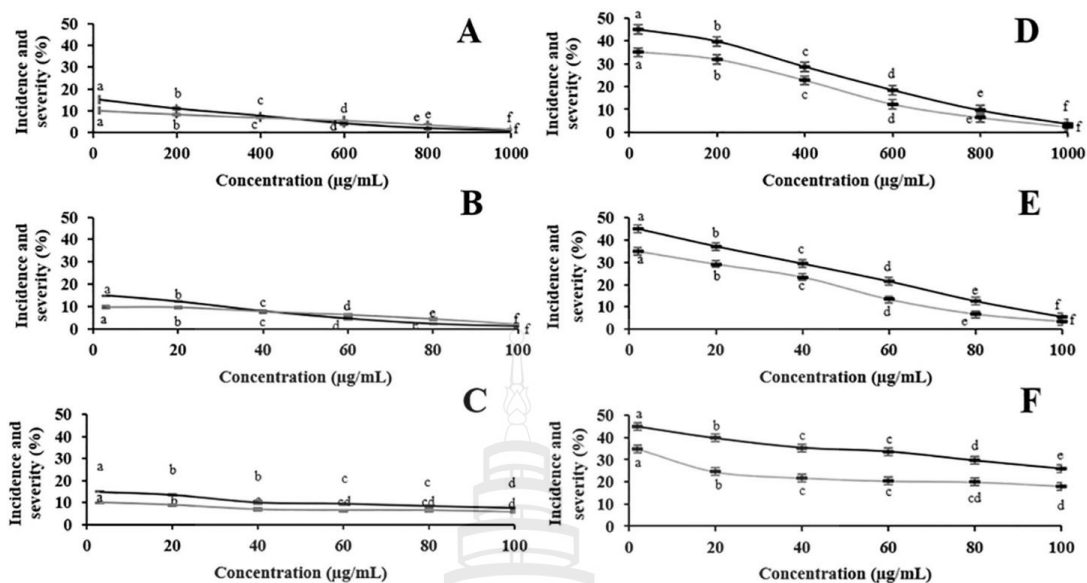
Note Grape berries obtained from various treatments: CC (A), *C. cyminum* NC (B), cumin aldehyde NC (C), α -terpinen-7-al NC (D), PC (E), *C. cyminum* TE (F-J) at concentrations of 200, 400, 600, 800 and 1,000 $\mu\text{g/mL}$, cumin aldehyde TE (K-O) and α -terpinen-7-al TE (P-T) at concentrations of 20, 40, 60, 80 and 100 $\mu\text{g/mL}$, respectively.

Figure 6.3 *In vivo* assay of the antifungal activity of the *C. cyminum* essential, cumin aldehyde, and α -terpinen-7-al against *A. aculeatus* infections after 10 days



Note Grape berries obtained from various treatments: CC (A), *C. cyminum* NC (B), cumin aldehyde NC (C), α -terpinen-7-al NC (D), PC (E), *C. cyminum* TE (F-J) at concentrations of 200, 400, 600, 800 and 1,000 $\mu\text{g/mL}$, cumin aldehyde TE (K-O) and α -terpinen-7-al TE (P-T) at concentrations of 20, 40, 60, 80 and 100 $\mu\text{g/mL}$, respectively.

Figure 6.4 *In vivo* assay of the antifungal activity of the *C. cyminum* essential, cumin aldehyde, and α -terpinen-7-al against *A. aculeatus* infections after 20 days



Note Data are mean $\pm$ standard deviation. Different letters indicate significant difference within each essential oil concentrations determined in each day according to Duncan's post-hoc test ($P < 0.05$).

Figure 6.5 Severity (■) and incidence (□) percentage of *A. aculeatus* in grape berries treated with different concentrations of *C. cyminum* essential oil (A and D), cumin aldehyde (B and E), and α -terpinen-7-al (C, F) after 10 days and 20 days, respectively

Table 6.4 Quality of grape berries obtained from all treatments after 10 days

Treatments		% Weight loss	Firmness (N)	Total phenolic content (mg GAE/g oil)	Antioxidant activity (μ M Trolox/g oil)	pH	TSS (°Brix)
CC		2.08±0.01 hi	0.45±0.01 cd	7.07±0.01 k	29.83±0.07 j	3.41±0.00 ab	19.66±0.03 bc
PC		3.32±0.02 o	0.31±0.01 i	3.92±0.01 l	18.63±0.07 l	3.24±0.01 f	17.70±0.19 g
<i>C. cyminum</i> NC		2.11±0.01 j	0.41±0.01 ef	7.33±0.03 j	27.42±0.23 k	3.42±0.01 ab	18.63±0.06 e
Cumin aldehyde NC		2.08±0.01 i	0.42±0.01 de	7.38±0.01 j	28.40±0.51 k	3.41±0.02 ab	18.83±0.10 e
α -terpinen-7-al NC		2.06±0.01 h	0.40±0.01 f	7.35±0.03 j	28.55±0.09 k	3.42±0.02 ab	18.87±0.05 e
<i>C. cyminum</i> TE							
concentration (μ g/mL)	200	2.25±0.01 l	0.46±0.03 bc	9.52±0.03 d	38.87±0.11 c	3.42±0.01 ab	19.28±0.07 d
	400	1.96±0.01 g	0.47±0.02 b	9.54±0.02 d	38.32±0.03 cd	3.42±0.03 ab	19.24±0.14 d
	600	1.84±0.01 e	0.47±0.02 bc	9.67±0.02 cd	39.56±0.31 bc	3.42±0.01 ab	18.77±0.08 e
	800	1.95±0.01 g	0.48±0.01 b	9.87±0.03 b	40.62±0.33 b	3.43±0.03 a	18.72±0.23 e
	1,000	1.77±0.01 c	0.51±0.01 a	10.28±0.03 a	43.51±0.26 a	3.43±0.03 a	18.71±0.04 e
Cumin aldehyde TE							
concentration (μ g/mL)	20	2.06±0.02 h	0.44±0.01 d	8.45±0.16 g	35.28±0.80 fg	3.42±0.01 ab	18.29±0.17 f
	40	1.81±0.02 d	0.44±0.01 cd	8.69 ±0.01 f	36.45±1.79 ef	3.42±0.03 ab	18.64±0.50 e

Table 6.4 (Continued)

Treatments		% Weight loss	Firmness (N)	Total phenolic content (mg GAE/g oil)	Antioxidant activity (μM Trolox/g oil)	pH	TSS (°Brix)
α-Terpinen-7-al TE concentration (μg/mL)	60	1.86±0.01 f	0.45±0.01 cd	8.38±0.38 gh	36.95±1.85 e	3.42±0.01 ab	18.70±0.11 e
	80	1.66±0.01 b	0.46±0.01 bc	9.79±0.01 bc	37.11±0.63 de	3.43±0.03 a	19.40±0.34 cd
	100	1.53±0.01 a	0.48±0.01 b	9.84±0.01 b	37.35±1.48 de	3.43±0.03 a	19.27±0.39 d
	20	3.34±0.01 o	0.34±0.03 h	7.85±0.06 i	32.93±0.21 i	3.33±0.02 e	19.71±0.20 bc
	40	3.22±0.01 n	0.35±0.01 h	7.76±0.02 i	33.54±0.19 hi	3.37±0.01 cd	19.77±0.03 b
	60	2.15±0.01 k	0.36±0.01 gh	8.26±0.03 h	33.88±0.08 hi	3.35±0.01 de	20.12±0.04 a
	80	2.30±0.01 m	0.35±0.02 h	9.26±0.03 e	34.27±0.25 gh	3.37±0.02 cd	20.20±0.10 a
	100	2.16±0.01 k	0.38±0.01 g	9.55±0.05 d	34.65±0.12 gh	3.39±0.01 bc	20.22±0.08 a

Note Data are mean ± standard deviation. Different letters indicate significant difference within each group according to Duncan's post-hoc test (P<0.05).

Table 6.5 Quality of grape berries obtained from all treatments after 20 days

Treatments		% Weight loss	Firmness (N)	Total phenolic content (mg GAE/g oil)	Antioxidant activity (μ M Trolox/g oil)	pH	TSS (°Brix)
CC		5.08±0.13 h	0.21±0.00 bc	3.30±0.01 g	13.88±0.00 i	3.39±0.01 cd	18.39±0.28 bcd
PC		8.39±0.22 r	0.13±0.01 g	1.89±0.00 h	8.50±0.02 j	3.22±0.01 h	16.50±0.22 h
<i>C. cyminum</i> NC		5.16±0.48 i	0.19±0.01 bcd	3.46±0.01 de	12.91±0.02 i	3.40±0.01 bc	17.58±0.14 efg
Cumin aldehyde NC		5.28±0.09 j	0.19±0.02 bcd	3.44±0.01 fg	13.38±0.01 i	3.40±0.01 bc	17.75±0.16 def
α -terpinen-7-al NC		5.36±0.08 k	0.17±0.04 cde	3.42±0.01 fg	13.51±0.02 i	3.39±0.01 cd	17.69±0.14 def
<i>C. cyminum</i> TE							
concentration (μ g/mL)	200	5.69±0.26 n	0.21±0.01 bc	4.41±0.02 b	18.19±0.01 bcd	3.39±0.02 a	17.57±0.11 efg
	400	5.09±0.19 h	0.21±0.01 ab	4.43±0.02 b	17.90±0.01 bcd	3.40±0.01 a	17.60±0.22 efg
	600	4.76±0.49 f	0.22±0.01 ab	4.49±0.03 b	18.50±0.01 bc	3.40±0.01 ab	17.64±0.21 efg
	800	4.91±0.20 g	0.22±0.01 ab	4.60±0.02 ab	18.99±0.01 b	3.42±0.02 a	17.98±0.24 cde
	1,000	4.37±0.16 c	0.24±0.01 a	4.79±0.01 a	20.46±0.02 a	3.42±0.02 a	18.06±0.26 cde
Cumin aldehyde TE							
concentration (μ g/mL)	20	5.09±0.11 h	0.20±0.01 bcd	3.94±0.01 cd	16.50±0.01 fgh	3.38±0.01 d	17.14±0.33gh
	40	4.51±0.25 d	0.20±0.02 bcd	4.06±0.02 c	17.08±0.02 efg	3.39±0.01 cd	17.43±0.34 fg

Table 6.5 (Continued)

Treatments		% Weight loss	Firmness (N)	Total phenolic content (mg GAE/g oil)	Antioxidant activity (μ M Trolox/g oil)	pH	TSS ($^{\circ}$ Brix)
α -Terpinen-7-al TE concentration (μ g/mL)	60	4.68 $\pm$ 0.28 e	0.21 $\pm$ 0.01 bc	4.02 $\pm$ 0.01 cd	17.24 $\pm$ 0.02 def	3.39 $\pm$ 0.01cd	17.47 $\pm$ 0.33 efg
	80	4.24 $\pm$ 0.39 b	0.21 $\pm$ 0.01 bc	4.57 $\pm$ 0.01 ab	17.35 $\pm$ 0.01 def	3.40 $\pm$ 0.01 bc	18.17 $\pm$ 0.25 cde
	100	3.88 $\pm$ 0.15 a	0.22 $\pm$ 0.011 ab	4.59 $\pm$ 0.02 ab	17.70 $\pm$ 0.01 cde	3.40 $\pm$ 0.00 bc	18.22 $\pm$ 0.30 bcd
	20	8.21 $\pm$ 0.15 q	0.16 $\pm$ 0.02 f	3.65 $\pm$ 0.02 ef	15.44 $\pm$ 0.01 h	3.30 $\pm$ 0.01 g	18.40 $\pm$ 0.18 bcd
	40	8.01 $\pm$ 0.44 p	0.17 $\pm$ 0.01 ef	3.62 $\pm$ 0.02 ef	15.65 $\pm$ 0.01 h	3.35 $\pm$ 0.01 f	18.47 $\pm$ 0.21 abc
	60	5.84 $\pm$ 0.29 o	0.17 $\pm$ 0.02 f	3.84 $\pm$ 0.04 cde	15.82 $\pm$ 0.02 gh	3.34 $\pm$ 0.01 f	18.83 $\pm$ 0.18 ab
	80	5.65 $\pm$ 0.36 m	0.18 $\pm$ 0.02 cde	4.35 $\pm$ 0.02 b	16.06 $\pm$ 0.02 fgh	3.35 $\pm$ 0.01 ef	18.90 $\pm$ 0.22 ab
	100	5.42 $\pm$ 0.47 l	0.18 $\pm$ 0.03 cde	4.47 $\pm$ 0.02 b	16.17 $\pm$ 0.02 fgh	3.36 $\pm$ 0.01 e	19.01 $\pm$ 0.31 a

Note Data are mean $\pm$ standard deviation. Different letters indicate significant difference within each group according to Duncan's post-hoc test ($P < 0.05$).

6.3.7 Quality of Grape Berries After Treatments

The effects of *C. cyminum* oil, cumin aldehyde, and α -terpinen-7-al essential oils on the quality of grape berries after 10- and 20-day incubation in terms of weight loss percentage, firmness, total soluble solids, pH, total phenolic content, and antioxidant activity are depicted in Tables 6.4 and 6.5, respectively. After ten days of treatments, no significant difference was observed for pH of the grape berries from all treatments (3.24–3.43) while other qualities including % weight loss, firmness, total phenolic content, antioxidant activity and total soluble solids were significance among these treatments. Weight loss percentage, firmness, total phenolic content, antioxidant activity and total soluble solids among these treatments were ranged from 1.53– 3.34%, 0.31–0.51 N, 3.92–10.28 mg GAE/g oil, 18.63–43.51 μ M Trolox/g oil, 17.70–20.22°Brix, respectively. Furthermore, treatments by *C. cyminum* essential oil and cumin aldehyde did not have a negative effect on grape quality. After 20 days of treatments, similar pH values were detected with on significance from all treatments (3.22–3.42) while other qualities were significance among these treatments. Weight loss percentage, firmness, total phenolic content, antioxidant activity and total soluble solids among these treatments were ranged from 3.88–8.39%, 0.16–0.24 N, 1.89–4.79 mg GAE/g oil, 8.50–20.46 μ M Trolox/g oil, 16.50–19.01°Brix, respectively.

6.4 Discussion

Essential oils from five plant species were selected for this study due to their broad-spectrum antimicrobial activity. According to previous studies, these essential oils were reported to produce volatile compounds showing antimicrobial activity against *Aspergillus niger* and *Candida species* (Naeini et al., 2014; Pawar & Thaker, 2006). Moreover, they have been evaluated to inhibit some phytopathogens such as *Penicillium lilacinum*, *A. niger*, *A. flavus* and *Botrytis cinerea* (Khosravi et al., 2011; Kro et al., 2017; Tripathi et al., 2008; Zare-Shehneh et al., 2014). Therefore, essential oils extracted from these plant species may be capable of producing volatile compounds that suppress the bunch rot disease caused by *A. aculeatus* in postharvest grapes.

Among these essential oils, *C. cyminum* essential oil showed high antifungal activity inhibiting the *A. aculeatus* mycelial growth and its infections on postharvest grapes. Based on *in vitro* assay, the volatile compounds produced by *C. cyminum* essential oil only showed high antifungal activity and their effectiveness gradually decreased over time (Table 6.1). This result was good agreement to the reports (Khosravi et al., 2011; Phuangsri et al., 2017) in which essential oils of *C. cyminum* oil showed potent inhibitory activity against *A. fumigatus*. In this study, *C. cyminum* essential oil was effective in controlling *A. aculeatus* at all concentrations, while low concentration of *C. sativum*, *Z. montanum* (J. Koenig) Link ex A. Dietr., *Z. bungeanum* Maxim., and *Z. rhetsa* essential oils (200 and 400 µg/mL) were sufficient to control *A. aculeatus*.

Stronger inhibitory effect on mycelial growth and conidia germination of *A. aculeatus* were observed in *C. cyminum* oil than the pure volatile compounds treatment. This result was similar to the study from Edris & Farrag (2003) reporting high antifungal activity of sweet basil essential oil compared to those obtained by pure major compounds. Moreover, Cavaleiro et al. (2006). also reported that *Juniperus* essential oil showed greater antimicrobial activity than those found in their major compounds such as α -pinene, β -phellandrene, and myrcene.

The *in vivo* study, after 10 days of storage at room temperature, showed that treatments by *C. cyminum* essential oil at 1,000 µg/mL effectively reduced incidence and severity percentage of *A. aculeatus* infection in grape berries compared to other treatments from 10 and 20 days incubation. Previous studies have reported that *C. cyminum* oil could control or inhibit phytopathogens such as *B. cinerea*, *Penicillium italicum*, *P. expansum*, *P. commune*, *Rhizopus stolonifer*, and *R. lyococcus* in postharvest products (Asghari Marjanlo et al., 2009; Mohammadifar et al., 2012). In addition, the mycelial growth of *A. aculeatus* was inhibited by *Cinnamomum zeylanicum*, *Ocimum gratissimum*, *Syzygium aromaticum* and *Eugenia caryophyllata* essential oils (Javed et al., 2012; Philippe et al., 2012; Sessou et al., 2013). *In vivo* effectivity of *C. cyminum* essential oil was probably due to the antifungal activity of their volatile compounds. Generally, complexity of the essential oil composition has been reported to influence its antimicrobial property (Bajpai et al., 2013). Antifungal activity of the *C. cyminum* essential oil may be attributed to cumin

aldehyde and α -terpinen-7-al (Petretto et al., 2018; Zheljazkov et al., 2015). According to previous studies, cuminaldehyde has been reported to inhibit *Colletotrichum lindemuthianum*, *Fusarium oxysporum*, and *Vorticillium dahliae* pathogens while α -terpinen-7-al showed antifungal activities against *Candida* species (Khaledi & Hassani, 2018; Petretto et al., 2018; Sekine et al., 2007). The antifungal effect of *C. cyminum* essential oil may also be achieved via a synergistic response of minor volatile compounds such as γ -terpinene, cuminyl alcohol, and β -pinene (Miri & Djenane, 2018; Pajohi et al., 2011). A combination of terpenoid compounds has been reported to induce functional and physical decay of cell tissue of fungi, thereby causing cell membrane leakage (Serrano et al., 2005). Moreover, essential oils also have antimicrobial properties due to their disruption properties on the mycelial growth by reduction of microbial cellular metabolism (Lambert, 2011; Tian et al., 2012). Uribe et al. (1985) and Sikkema et al. (1994) reported that their antifungal action of several monoterpenes was performed at the membrane level or embedded enzymes. Changing of the fatty acid composition of the cell membrane, alteration of permeability, and inhibition of respiration were suggested as possible mechanisms for antifungal properties of monoterpenes (Cox et al., 2000; Prashar et al., 2003). Marei and Abdelgaleil (2018) also reported that the monoterpenes showed antifungal activity through the inhibition of pectin methyl esterase and cellulase enzymes. They were considered as the potent inhibitors of pectin methyl esterase modifying the degree of methylesterification of pectin, major component of fungal cell walls. Changing of pectin structure were resulted in cellular adhesion, plasticity, pH, and ionic contents of the cell wall and influenced fungi development, membrane integrity and permeability.

Evaluation of post-treatment grape berries quality indicates that *C. cyminum* essential oil having concentration of at least 400 $\mu\text{g/mL}$ could delay the ripening process in grape berries by inhibiting *A. aculeatus* infection without affecting certain grape berries qualities after 10 days. The quality of fresh fruit depends on several factors including weight loss, firmness, total soluble solids, total phenolic content, and antioxidant activity. Serrano et al. (2005) reported the efficacy of thymol and eugenol essential oils exposure on cherries and grapes without effects. Fennel and thyme oil were reported to reduce loss of grape weight (Abdollahi et al., 2012) while cinnamon

and eucalyptus essential oils do not affect weight loss in strawberries and tomato fruits (Tzortzakis, 2007). In similar reports, *Ocimum basilicum* essential oil coated on Embul banana peel to control crown rot and anthracnose did not have any significant effect on the total soluble solid (Anthony et al., 2003). Ulukanli and Oz (2014) reported that there are no significant differences in pH value in strawberries treated with myrtle essential oil. In addition, Wang et al. (2008) found that thymol, eugenol, and menthol from essential oils enhanced phenolic and antioxidant activity in strawberries.

6.5 Conclusion

This investigation suggests *C. cyminum* essential oil as an alternative biological fungicide to control bunch rot diseases caused by *A. aculeatus* on postharvest grape berries. Considering all evaluated parameters on grape quality within the scope of this study, *C. cyminum* essential oil treatment was shown to delay grape ripening. Total phenolic content and antioxidant activity assay also showed that postharvest grape berries treated with *C. cyminum* essential oil could be stored while maintaining their quality for up to ten days. However, further studies are required before applying *C. cyminum* essential oil treatments as a biocontrol agent to increase the postharvest period of grapes.

CHAPTER 7

CONCLUSION

The possibility of a new bioactive compound derived from medicinal plants was found which may provide new biological activity to replace antibiotics from natural product. Secondary metabolites in the selected plants including *Cinnamomum loureiroi*, *Polyscias fruticosa*, *Barleria prionitis*, *Cuminum cyminum*, were demonstrated the phytochemical and biological activities. Biological activities including antibacterial, antifungal, *in vitro* antagonism, and cytotoxicity were investigated through the isolation and identification of novel compounds.

Firstly, crude fungal extract isolated from *Cinnamomum loureiroi* which are *Neopestalotiopsis* sp. MFLUCC15-1130 and *Diaporthe* sp. MFLUCC15-1131 possessed notable bactericidal and fungicidal activities against all test bacteria and fungal pathogens. However, all crude extracts were explored for the first time to study on the antioxidant in this plant species. Eugenol, lauric acid, myristaldehyde, and caprylic acid were predominant as the majority in both endophytic fungi. Secondly, 8-methoxynaphthalen-1-ol was isolated from crude fungal extract obtained from *P. fruticosa* leaves, exhibited antifungal activity against *A. rolfsii* with MIC value of 250 µg/mL. This compound showed no phytotoxicity potential against *S. lycopersicum* on tomatoes. Thirdly, antibacterial and cytotoxic potential of crude extracts of fungal endophyte of *B. prionitis* leaves were studied. *Diatrypella* sp. MFLUCC19-0492 was identified and possessed antibacterial against various human pathogenic and cancer cell lines on a highly active level. Lastly, *C. cyminum* essential oil inhibited the fungal growth of *A. aculeatus* which causes in bunch rot in grape berries. In addition, *C. cyminum* essential oil could be stored while preserving the overall quality without affecting within ten days. This finding recommended that the selected plants and new endemic fungi could be useful for further applications including agricultural and pharmacological.



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APPENDIX

ABSTRACTS OF PUBLICATIONS



Production of eugenol from fungal endophytes *Neopestalotiopsis* sp. and *Diaporthe* sp. isolated from *Cinnamomum loureiroi* leaves

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ABSTRACT

Endophytic fungi, which colonize within a host plant without causing any apparent diseases, have been considered as an important source of bioactive secondary metabolites containing antimicrobial and antioxidant activities. The aim of this research was to isolate the endophytic fungi of *Cinnamomum loureiroi* and then to screen their antimicrobial and antioxidant activities. A total of 11 fungal endophytes were isolated from healthy leaves of *Cinnamomum loureiroi* belonging to six genera: *Botryosphaeria*, *Colletotrichum*, *Diaporthe*, *Fusarium*, *Neopestalotiopsis*, and *Pestalotiopsis*. All isolated strains were cultured and further extracted with ethyl acetate solvent. Antimicrobial activity of all crude endophytic fungal extracts was analyzed using disc diffusion assay against six bacterial and two fungal pathogens. Crude extracts of strains MFLUCC15-1130 and MFLUCC15-1131 showed broad-spectrum antimicrobial activity against all tested pathogens. Activity against *Bacillus cereus* and *Staphylococcus epidermidis* was notable, showing the lowest minimum inhibitory concentration at 3.91 µg/mL. Antioxidant activity of all crude endophytic fungal extracts was also evaluated based on 2,2-diphenyl-1-picrylhydrazyl assay. Significant antioxidant activity was detected in the crude extracts of fungus MFLUCC15-1130 and MFLUCC15-1131 with IC₅₀ of 22.92 ± 0.67 and 37.61 ± 0.49 µg/mL, respectively. Using molecular identification, MFLUCC15-1130 and MFLUCC15-1131 were identified as *Neopestalotiopsis* sp. and *Diaporthe* sp., respectively. The major chemical constituents produced by both crude extracts were identified by gas chromatography-mass spectrometry. Eugenol, myristaldehyde, lauric acid, and caprylic acid were the primary antimicrobial and antioxidant compounds in both crude extracts. This is the first report of eugenol being a biologically active compound of *Neopestalotiopsis* sp. and *Diaporthe* sp. fungal endophytes. Eugenol has been reported as antimicrobial and antioxidant agents with agronomic applications. Thus the two newly-isolated endophytes may be used for eugenol production, which in turn can be used in a variety of applications.

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Antifungal activity of 8-methoxynaphthalen-1-ol isolated from the endophytic fungus *Diatrype palmicola* MFLUCC 17-0313 against the plant pathogenic fungus *Athelia rolfsii* on tomatoes

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ABSTRACT

Thirty-four endophytic fungal isolates were obtained from the leaves of the medicinal plant *Polyscias fruticosa*, and their antagonistic activities against the growth of the common tomatoes plant pathogenic fungus *Athelia rolfsii* were initially screened using a dual culture assay. The endophytic fungus MFLUCC 17-0313, which was later molecularly identified as *Diatrype palmicola*, displayed the highest inhibition percentage (49.98%) in comparison to the others. This fungus was then chosen for further evaluation. Its culture broth and mycelia from a 10 L scale were separated and extracted using ethyl acetate, methanol, and hexane. Each extract was tested for antifungal activity against the same pathogen using a disc diffusion assay. Only the crude hexane extract of fungal mycelium showed antifungal activity. The hexane extract was fractionated using sephadex gel filtration chromatography and each fraction was tested for antifungal activity until the one with the highest inhibition percentage was obtained. The bioactive compound was identified as 8-methoxynaphthalen-1-ol using nuclear magnetic resonance spectroscopy and mass spectrometry. The minimum inhibition concentration of 8-methoxynaphthalen-1-ol was demonstrated at 250 µg/mL against the selected pathogen. Using the leaf assay, the solution of 8-methoxynaphthalen-1-ol was tested for phytotoxic activity against *A. rolfsii* and was found to have no phytotoxic effects. These results showed that 8-methoxynaphthalen-1-ol has the potential for controlling the growth of *A. rolfsii*, the cause of Southern blight disease on tomatoes. This study may provide the foundation for future use of this compound as a biofungicide.

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Antibacterial and cytotoxic activities of endophytic fungus *Diatrypella* sp. MFLUCC 19-0492

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Abstract

Endophytic fungi are microorganisms living inside plant tissues without causing any disease symptoms in the plant. Various bioactive compounds have been produced and may be used as alternative drugs for controlling human pathogens. The aim of this work was to investigate antibacterial and cytotoxic activity potential of fungal crude extracts isolated from *Barleria prionitis* leaves. A total of 34 endophytic fungi were isolated. The crude ethyl acetate extracts of all fungi were preliminarily screened for their antibacterial activity. The acetate extract of isolate ANG4 showed significant antibacterial activity.

This isolate was further cultured to determine its minimum inhibition concentration (MIC). The crude ethyl acetate extracts of mycelia and culture broth from isolate ANG4 were active against all tested pathogens with low MIC values ranging from 0.39 mg/mL to 25.00 mg/mL when compared to the crude hexane extract of its mycelia. The cytotoxic effects of all crude extracts of isolate ANG4 were also determined using tetrazolium-based colorimetric (MTT and XTT) assays against T-lymphoblast, cholangiocarcinoma, liver hepatocellular and lung carcinoma cell lines. The crude ethyl acetate extract of mycelia showed the most significant activity inhibiting T-lymphoblast and lung carcinoma cell lines with 100 and 71% respectively whereas the crude hexane extract of mycelia showed good cytotoxic activity against the T-lymphoblast cell line with 74%. The fungus ANG4 was also identified by molecular and phylogenetic methods as *Diatrypella* sp. MFLUCC19-0492. Therefore, this study indicates that crude extracts of *Diatrypella* sp. MFLUCC19-0492, isolated from *B. prionitis* leaves, are potential sources of novel antibacterial compounds with good cytotoxic activity.

Keywords: Antibacterial activity, *Barleria prionitis*, cytotoxic activity, *Diatrypella*, endophytic fungi.

Introduction

Medicinal plants have played an important role as the principal natural product sources of bioactive compounds. They have been widely used in traditional treatments and studied extensively for their antimicrobial properties. Medicinal plants are also reported to host various bioactive

compound-producing endophytic fungi^{1,2}. Endophytic fungi are microorganisms that live inside host plants without causing any visible manifestation of diseases³. They reside in the host plant tissues by producing secondary metabolites to protect and resist external biotic and abiotic stress to the host plant by producing secondary metabolites with antagonistic activity⁴.

New and novel bioactive compounds with various antimicrobial, anticancer, antimalarial and antioxidant activities, isolated from endophytic fungi of medicinal plants, have been reported such as alkaloids, terpenoids, steroids, quinones, flavonoids, phenols, phenolic acids and peptides^{5,6}. Therefore, endophytic fungi are evaluated as alternative important sources of bioactive compounds that can be applied as new or novel drugs⁷.

Antimicrobial resistance has been a major health problem and still poses a concern to health care systems globally. Many studies have shown that microbial pathogens have developed resistance to antibiotics via various molecular mechanisms⁸. Therefore, this global problem has led to the search for a new or novel drug from endophytic fungi, particularly those isolated from medicinal plants for their antimicrobial properties⁹.

Barleria prionitis belongs to the Acanthaceae family and is a well-known medicinal plant and Ayurvedic herb. It is distributed in tropical Africa and Asia including India, Malaysia, the Philippines, Sri Lanka and Thailand¹⁰. This medicinal plant has been used for the treatment of fevers, toothaches, bronchial asthma, rheumatic affections, inflammation, glandular swelling, dysrhythmia and gastrointestinal disorders^{11,12}. Several phytochemicals such as balarenone, pipataline, lupeol, prioniside A, prioniside B and prioniside C have been reported to possess the following biological activities: antibacterial, antiviral, antioxidant, antifertility, anthelmintic, cytoprotective, antidiabetic, anti-diarrheal, hepatoprotective and antifungal activity¹³⁻¹⁵.

Due to the reported antimicrobial potential of various endophytic fungi, this research focused on the antibacterial and cytotoxic potential of crude extracts of endophytic fungi isolated from *B. prionitis* leaves where the most potent endophytic fungus could be used as an antibacterial agent against human pathogenic bacteria. These fungi, which are suggested to be associated with the production of compounds with medical value, could be used as alternative antibiotics.

RESEARCH ARTICLE

In vitro and *in vivo* antifungal activity of *Cuminum cyminum* essential oil against *Aspergillus aculeatus* causing bunch rot of postharvest grapes

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Abstract

Bunch rot in grapes is an aggressive disease and needs to be controlled during the postharvest period. We investigate the antifungal potential of *Zanthoxylum bungeanum* Maxim., *Zanthoxylum rhetsa*, *Cuminum cyminum*, *Coriandrum sativum*, and *Zingiber montanum* (J. Koenig) Link ex A. Dietr. essential oils against *Aspergillus aculeatus* that cause bunch rot disease on postharvest grapes. *C. cyminum* essential oil exhibited stronger significantly inhibition percentage of 95.08% than other treatments in *in vitro* assay. Cumin aldehyde (33.94%) and α -terpinen-7-al (32.20%) were identified as major volatile compounds in *C. cyminum* oil. Antifungal potential of *C. cyminum* oil was then tested in conidia germination and *in vitro* tests compared to cumin aldehyde and α -terpinen-7-al. Their EC_{50} values against the conidial germination were also estimated. Significant reduction of conidia germination was also detected in *C. cyminum* essential oil and cumin aldehyde at a concentration of 1,000 and 100 μ g/mL, respectively. EC_{50} values of the *C. cyminum* essential oil, cumin aldehyde, and α -terpinen-7-al were 67.28 μ g/mL, 9.31 μ g/mL, and 13.23 μ g/mL, respectively. *In vivo* assay, the decrease of the disease severity (0.69%) and incidence (1.48%) percentage of *A. aculeatus* on grape berries treated at 1,000 μ g/mL of *C. cyminum* essential oil was significantly greater than that obtained from other treatments after 10 days incubation. In addition, grape berries treated with *C. cyminum* essential oil decreased weight loss and retained fruit firmness. The changing of total soluble solids, total phenolic content, and antioxidant activity are also delayed in treated fruits. Therefore, essential oil of *C. cyminum* may be applied as a biological antifungal agent to control *A. aculeatus* in postharvest grapes without any negative effects on its quality.

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1. Introduction

Grape (*Vitis vinifera* L.) is one of the world's most abundant fruit crops with various varieties [1]. FAO (2019) reported that grapes are produced at least 50 million metric tons in 2018. It