สารต้านราก่อโรคพืชจากพรรณไม้ไทย ข่า *Alpinia galanga* Willd.
จันทน์ชะมด *Mansonia gagei* Drumm. และลำดวน *Melodorum fruticosum* Lour.

นางสาวรัชษาวรรณ์ มงคล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ปีการศึกษา 2557

ANTI-PHYTOPATHOGENIC FUNGAL COMPOUNDS FROM THAI PLANTS *Alpinia galanga* Willd. *Mansonia gagei* Drumm. AND *Melodorum fruticosum* Lour.

Miss Rachsawan Mongkol

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biotechnology
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Thesis Title	ANTI-PHYTOPATHOGENIC FUNGAL COMPOUNDS FROM THAI PLANTS *Alpinia galanga* Willd. *Mansonia gagei* Drumm. AND *Melodorum fruticosum* Lour.
By	Miss Rachsawan Mongkol
Field of Study	Biotechnology
Thesis Advisor	Assistant Professor Warinthorn Chavasiri, Ph.D.

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รัชษาวรรณ์ มงคล : สารต้านราก่อโรคพืชจากพรรณไม้ไทย ข่า *Alpinia galanga* Willd. จันทน์ชะมด *Mansonia gagei* Drumm. และลำดวน *Melodorum fruticosum* Lour. (ANTI-PHYTOPATHOGENIC FUNGAL COMPOUNDS FROM THAI PLANTS *Alpinia galanga* Willd. *Mansonia gagei* Drumm. AND *Melodorum fruticosum* Lour.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.วรินทร ชวศิริ, หน้า.

การทดสอบฤทธิ์ต้านเชื้อราเบื้องต้นจากพรรณไม้ไทย 46 ชนิด พบว่าเหง้าข่า *Alpinia galangal* Willd. เนื้อไม้จันทน์ชะมด *Mansonia gagei* Drumm. และดอกลำดวน *Melodorum fruticosum* Lour. ในส่วนสกัดจากไดคลอโรมีเทนมีผลการยับยั้งการเจริญของเชื้อรา ทั้ง 4 ชนิด ประกอบด้วย *Alternaria porri*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum* และ *Phytophthora parasitica* จึงศึกษาหาสารออกฤทธิ์โดยใช้ชีววิธีในการติดตามฤทธิ์ในการแยกสาร จากการพิสูจน์เอกลักษณ์ของสารด้วยสมบัติทางกายภาพและเทคนิคทางโครมาโทกราฟี พบสารในส่วนที่ออกฤทธิ์ในเหง้าข่า 4 ชนิด ประกอบด้วย 1′-acetoxychavicol acetate (ACA), *trans-p*-coumaryl diacetate, *trans-p*-hydroxycinnamyl acetate และ *trans-p*-hydroxycinnamaldehyde และ สาร 2 ชนิด จากปฏิกิริยา ACA solvolysis คือ *trans-p*-coumaryl alcohol ethyl ether และ *trans-p*-acetoxycinnamyl alcohol และองค์ประกอบทางเคมีของน้ำมันหอมระเหยจากเหง้าข่าสด ได้แก่ 1,8-cineole (73%), 2-acetoxy-1,8-cineol (13%) และ terpinen-4-ol (3%) สารออกฤทธิ์จากสิ่งสกัดเนื้อไม้จันทน์ชะมดประกอบด้วยสารในกลุ่ม mansonone และคูมาริน ได้แก่ mansorin A, B, C, mansonone C, E, G และ H นอกจากนี้ยังพบสารออกฤทธิ์จากสิ่งสกัดดอกลำดวน 8 ชนิด คือ 1-hexacosanol, 5-hydroxy-7-methoxyflavone, β-sitosterol, benzoic acid, 5,7-dihydroxyflavone, melodorinol, melodorinone A และ B

mansonone C ออกฤทธิ์ต้านเชื้อ *P. parasitica* ดีที่สุด ด้วยค่า IC_{50} 3.2 $\mu g \cdot mL^{-1}$ สำหรับผลยับยั้งการงอกของสปอร์รา *F. oxysporum* พบว่า ACA แสดงฤทธิ์ได้ดีที่สุดที่ความเข้มข้น 100 $\mu g \cdot mL^{-1}$ นอกจากนี้การศึกษาในระดับเนื้อเยื่อพืชโดยการทดสอบฤทธิ์ต้านเชื้อรา *P. parasitica* ซึ่งเป็นสาเหตุโรคยอดเน่าสับปะรดพบว่า ACA สามารถต้านการเกิดรอยโรคได้อย่างมีนัยสำคัญ และยับยั้งการเกิดโรคยอดเน่าในหน่อสับปะรดได้ นอกจากนี้การศึกษาผลของการรวมกันของ ACA mansonone E และ melodorinol พบว่าไม่เสริมการออกฤทธิ์ต้านเชื้อราก่อโรคพืช

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5173926723 : MAJOR BIOTECHNOLOGY
KEYWORDS: ALPINIA GALANGA / MANSONIA GAGEI / MELODORUM FRUTICOSUM / PINEAPPLE HEART ROT DISEASE / ANTIPHYTOPATHOGENIC FUNGI

RACHSAWAN MONGKOL: ANTI-PHYTOPATHOGENIC FUNGAL COMPOUNDS FROM THAI PLANTS *Alpinia galanga* Willd. *Mansonia gagei* Drumm. AND *Melodorum fruticosum* Lour.. ADVISOR: ASST. PROF. WARINTHORN CHAVASIRI, Ph.D., pp.

Forty-six Thai plants were preliminarily screened for antiphytopathogenic fungal activity. Three promising plants namely *Alpinia galanga* Willd. rhizomes, *Mansonia gagei* Drumm. heartwoods and *Melodorum fruticosum* Lour. flowers were selected for further study. The CH_2Cl_2 extracts displayed strong antifungal activity against four fungi: *Alternaria porri*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum* and *Phytophthora parasitica*. Further separation and isolation were carried out based on bioassay guided. The constituents of *A. galangal* rhizomes comprised of phenylpropanoids which were identified based on physical properties and spectroscopic evidence as 1'-acetoxy chavicol acetate (ACA), *trans-p*-coumaryl diacetate, *trans-p*-hydroxycinnamyl acetate, *trans-p*-hydroxycinnamaldehyde and two compounds from ACA solvolysis as *trans-p*-coumaryl alcohol ethyl ether and *trans-p*-acetoxycinnamyl alcohol. The major constituent in the essential oil from the fresh rhizomes are 1,8-cineole (73%), 2-acetoxy-1,8-cineol (13%) and terpinen-4-ol (3%). Mansonones and coumarins were isolated from the heartwoods of *M. gagei* and were elucidated as mansorins A, B, C, mansonones C, E, G and H. Eight compounds were isolated from *M. fruticosum* flower, including 1-hexacosanol, 5-hydroxy-7-methoxyflavone, β-sitosterol, benzoic acid, 5,7-dihydroxyflavone, melodorinol, melodorinones A and B. Among the isolated compounds, mansonone C displayed the highest antifungal activity against *P. parasitica* with IC_{50} 3.2 $\mu g \cdot mL^{-1}$. For the inhibition of spore germination, ACA displayed the highest activity against spore germination of *F. oxysporum* at 100 $\mu g \cdot mL^{-1}$, followed by mansonones E and G. ACA displayed *in vivo* complete inhibition against disease lesion caused by *P. parasitica* on pineapple leave, while it slightly inhibited the heart rot disease in pineapple suckers. Nevertheless, there was no synergistic effect observed on the combination of ACA, mansonone E and melodorinol.

Field of Study: Biotechnology Student's Signature .....

Academic Year: 2014 Advisor's Signature .....

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LIST OF ABBREVATIONS

°C	=	degree Celsius
CA	=	carrot agar
$CDCl_3$	=	deuterated chloroform
CH_2Cl_2	=	dichloromethane
^{13}C NMR	=	carbon nuclear magnetic resonance
conc.	=	concentration
cont.	=	continue
d	=	doublet
dd	=	doublet of doublet
DMSO	=	dimethylsulfoxide
Et_2O	=	diethyl ether
EtOAc	=	ethyl acetate
EtOH	=	ethanol
g	=	gram
h	=	hour
^1H NMR	=	proton nuclear magnetic resonance
H_2O	=	water
J	=	coupling constant
MeOH	=	methanol
$KMnO_4$	=	potassium permanganate
$\mu g \cdot mL^{-1}$	=	microgram per milliliter
GC-MS	=	gas chromatography-mass spectrometry
kg	=	kilogram
min	=	minute
mL	=	milliliter
mm	=	millimeter
mM	=	millimolar
MW	=	molecular weight

nm	=	nanometer
no.	=	number (No.)
NaOCl	=	sodium hypochlorite
OH	=	hydroxy
OMe	=	methoxy
PDA	=	potato dextrose agar
PDB	=	potato dextrose broth
q	=	quartet
R_f	=	retardation factor
R_t	=	retention time
RT	=	room temperature
s	=	singlet
sp.	=	species
t	=	triplet
TLC	=	thin layer chromatography
UV	=	ultraviolet
v/v	=	volume by volume
w/w	=	weight by weight

CHAPTER 1
INTRODUCTION

Thailand is an agricultural country with large varieties of delicious fruits and vegetables. More than 200 kinds of vegetables have been produced and exported with high value. The country was ranked for the 7th world exporter of agricultural products and food sectors with value of $ 24,681 million or 2.52% of world export. Thailand is also the largest exporter of rice, rubber, tapioca and pineapple [1]. However, plant diseases were a serious problem for the extensive loss in agricultural products. Temperature, soil nutrients, soil structure, pH, air humidity and light are environmental factors causing plant diseases. Those diseases derived from fungi are the largest problem to all plant stages of their lives both pre- and post-harvest. Especially fruits containing a lot of water with sweet taste are favored to be attacked by fungi. Each parasitic fungus can attack one or many kinds of plants.

1.1 Plant disease caused by fungi and control

The agricultural problems from plant diseases caused by fungi were extensively caused of yield loss. Both of pre- and post-harvest losses due to fungal disease in world crop protection even higher in developing countries [2]. Fungi are widely distributed and highly variable organisms which did not contain chlorophyll and conductive tissues. Most of them have filamentous vegetative structures (mycelium) and spore was used for their reproduction. More than 8,000 species of fungi caused plant diseases. The specific identification of fungal disease is recognized by the characteristic symptoms on host plants, their mycelium, spores and fruiting body [3].

In this research, four fungi including *Alternaria porri* (purple blotch), *Colletotrichum gloeosporioides* (anthracnose), *Fusarium oxysporum* (Fusarium wilt) and *Phytophthora parasitica* (heart or root rot) are selected as tested organisms since they severely damage economic agricultural products.

1.1.1 Plant disease caused by *Alternaria porri*

Kingdom: Fungi
Phylum: Ascomycota
Class: Dothideomycetes
Order: Pleosporales
Family: Pleosporaceae
Genus: *Alternaria*
Species: *A. porri*

This fungus has dark color mycelium and produces short, simple, vertical conidiophores which generate single or branched chains of conidia. The conidia are large, dark, long or pear shape and multicellular with both horizontal and longitudinal cross walls. These conidia are easily detached and carried by air. It is a pathogen caused purple blotch disease in *Allium* sp. such as shallot (*Allium cepa* L. var. *ascalonicum*), leek (*A. porrum*), garlic (*A. sativum*) and onion (*A. cepa* L. var. *cepa*). Moreover, this fungus caused disease in potato, tomato, pear and peach. The spores of *Alternaria* sp. develop in high humidity and spread to onion leaves and bulbs in the field or storage shed by wind, water splash, implements and insects or workers [4].

The lesions of disease started from the spores germinate on onion leave and produce a small, water soaked spot and then the spot turns brown, lesion enlarges and become zonate (target spot) and purplish. The margin of this disease could be reddish to purple and surrounded by yellow zone. When this disease occurred during moist weather, the surface of the lesion may be covered by brown to black masses of fungal spores. After that, the leaves become wilt downward two to four weeks after initial infection. The purple blotch disease can form on seed stalks and floral parts of seed onions and effect to seed growth. The *Alternaria* spores may be infecting the outer scales of bulbs through wounds or the neck tissue. The sign of disease show a yellow to wine-red, semi-watery decay and the tissue turns brown to

black and dried out in the field or in the storage. **Figure 1.1** shows the disease cycle of *Alternaria* sp. initiates from the mycelium and spores infest in plant debris, on seeds and tubers and germination direct penetration or penetration through wound and leaf or stem are attacked and the symptom of disease may be present as collar rot damping off, stem rot, leaf rot and fruit rot.

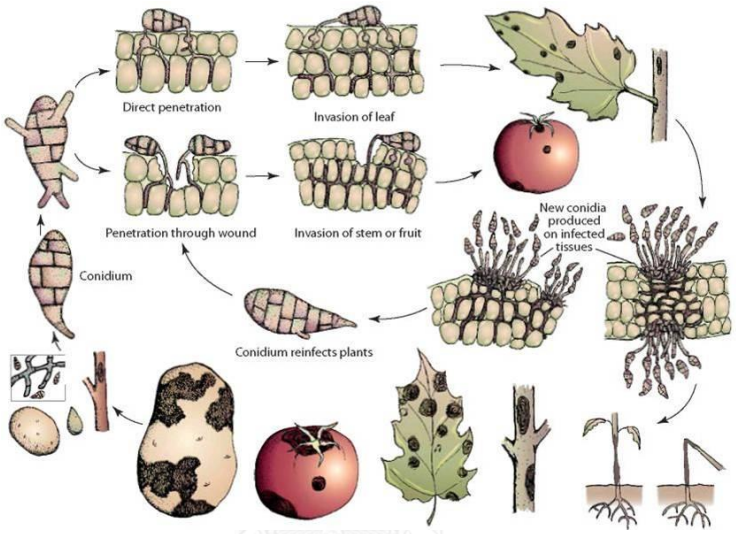


Figure 1.1 Disease cycle of *Alternaria* sp. [5]

1.1.2 Plant disease caused by Colletotrichum gloeosporioides

Kingdom: Fungi
Phylum: Ascomycota
Class: Sordariomycetes
Order: Phyllachorales
Family: Phyllachoraceae
Genus: *Colletotrichum*
Species: *C. gloeosporioides*

The synonym of this fungus is *Glomerella cingulata* in a perfect (sexual) stage. *C. gloeosporioides* produces hyaline, one-celled ovoid to oblong and slightly curved or dumbbell in shaped conidia with pink or salmon color, 10-15 and 5-7 µm

in length and width respectively. They produced the waxy acervuli in subepidermis infected tissue of host and typically with setae, simple, short and erect conidiophores. Anthracnose is a serious disease caused by *C. gloeosporioides*, so the common name is anthracnose fungus. This fungus can infect a wide variety of host especially in economic plants such as banana, chili, guava, olive, papaya, pepper, pomegranate, tomato, mango and strawberry [3]. The disease cycle shows in **Figure 1.2**.

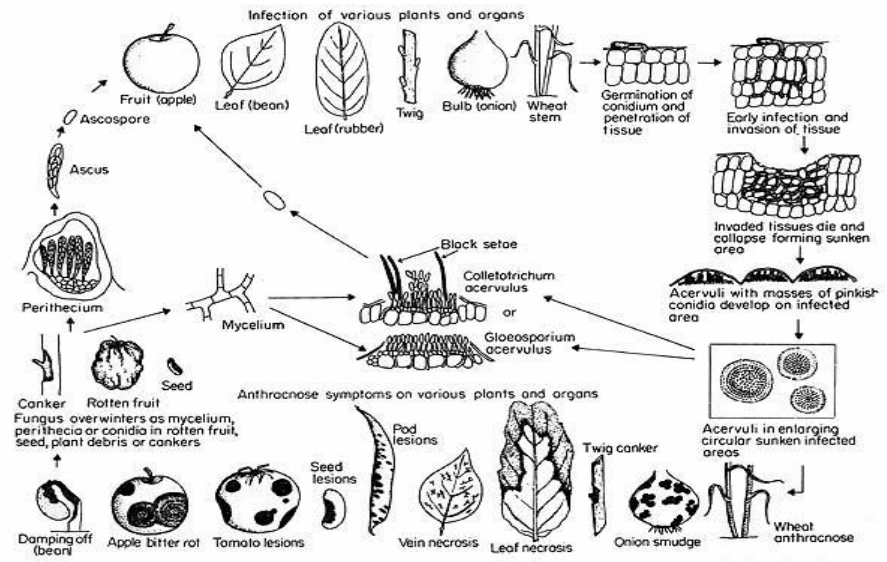


Figure 1.2 Disease cycle of *C. gloeosporioides* [3]

The disease cycle of *C. gloeosporioides* survives the winter as seed like structures called sclerotia and as thread like strands called hyphae in infested tomato or plants debris. After that, this fungus produced perithecium which contained ascus and each ascus produced many ascospores. In late spring the lower leaves and fruit may become infected by germinating sclerotia and ascospores in the soil debris. Infections of the lower leaves of plants are important sources of spores for secondary infections throughout the growing season. After the germinated spore penetrated into host tissues using its appressorium by enzymatic penetration, invaded tissues would die and collapse forming sunken area. The symptoms of

anthracnose disease are round, water soaked and sunken spots on the ripening host plants. The pinkish-orange lesions are formed by acervuli masses that covered the center and produced a concentric ring pattern. The environmental conditions favoring the pathogen are high temperature (28°C) and high humidity. The spores need free water to germinate and release from acervuli when the moisture is abundance and splashing water in the form of rain or overhead irrigation favors the spread of the disease [4].

Since anthracnose is more prevalent on poorly drained soils, crops should be planted on well-drained land. Three- to four-year crop rotations excluding crops in the Solanaceae family are recommended to prevent a buildup of the fungus in soil. Several fungicides are registered for use to control anthracnose. Fungicides such as benomyl or carbendazim should be applied when fruit are formed on the first cluster [6].

1.1.3 Plant disease caused by *Fusarium oxysporum*

Kingdom: Fungi
Phylum: Ascomycota
Class: Sordariomycetes
Order: Hypocreales
Family: Nectriaceae
Genus: *Fusarium*
Species: *F. oxysporum*

Fusarium oxysporum shows the colorless of mycelial however it becomes cream or pale yellow with age. This fungus will produce a pale pink or purplish colony when they grow in optimum conditions. There are three kinds of asexual spores. Microconidia are one or two cells with rod shape and produced under all conditions, while macroconidia are three to five cells with curve shape.

Chlamydospores are one or two cells with thick wall, round shape and produced terminally on old mycelium. These fungus complex diverse soil-born fungi, survival of a pathogen from year to year in soil including plant pathogens causing vascular wilt more than 100 cultivated plants [7]. It is very injurious and frightening plant disease appearing as more of less rapid wilting, browning and dying of leave, shoots followed by final death of whole plant.

The symptoms of wilts occur from the presence of the pathogen in xylem vascular tissues or some vessels of plant may be clogged with its mycelium, spores or polysaccharide produced by this fungus. The clogging will increase by accumulation of breakdown products of plants cells digested by fungal enzyme. The oxidation and translocation of breakdown products responsible for the brown discoloration of affected vascular tissue. This pathogen continues to spread through the xylem vessels until the entire plants are killed. The fungus infects the plants through the roots *via* direct penetration or *via* wounds, after the xylem tissue is colonized after infection; the parts above of plant may be dying within a week [3]. *F. oxysporum* can attack at all stages of plant development and the symptoms are noticeable if the crop is exposed to unfavorable conditions such as drought, heat (wilt disease) or water logging. This fungus causes a disease in tulip bulb, mustard (*Brassica campestris* L.), rice (*Oryza sativa* L.), spinach (*Spinacia oleracea* L.) and tomato (*Lycopersicum esculentum* Mill.) [8].

The *Fusarium* wilts disease cycle shows in **Figure 1.3**. The infested soils contain mycelium or spores and spread by water or contaminated in farm equipment or farmer. When the plants grow in contaminated soil, spores germinate or mycelium sink in the root through the root cortex and enter to vessels. The ring of discolored vessels in secondary xylem is present after enlargement mycelium of cells is collapsed, vessels are distorted and plants will die.

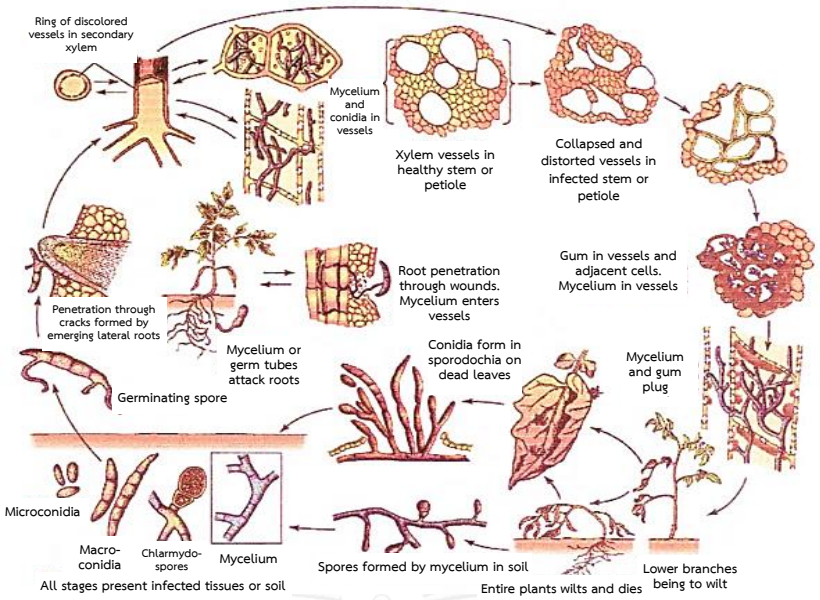


Figure 1.3 Disease cycle of *Fusarium* wilt of tomato caused by *F. oxysporum* [3]

1.1.4 Plant disease caused by *Phytophthora parasitica*

Kingdom: Chromalveolata
Phylum: Oomycota
Class: Oomycetes
Order: Pythiales
Family: Pythiaceae
Genus: *Phytophthora*
Species: *P. parasitica*

Phytophthora parasitica is a cosmopolitan Oomycete of warm temperature, subtropical and tropical environments and it has a broad host range including herbaceous (tobacco, tomato, carnation, pineapple) and woody (citrus, eucalyptus). The disease in many economic plants caused by *Phytophthora* sp. such as root rot, heart rot, rot of lower stems, tubers or rot of buds or fruits, foliar blights and damping off. The distinctive symptoms of Phytophthora root rot are brown lesions on roots of all sizes and the xylem of roots above the lesions often turns yellowish or brown and girdled or rotted off. The infected plants at aboveground are slow

growing and may wilt and die in hot weather. Fruits in contact with the ground are infected and the disease is called buckeye rot.

Phytophthora root rot can occur when the soil becomes wet, high moisture and humidity, poorly drained area and the temperature remains fairly low. The seedling may be killed within a few days. Whereas in the older plant, the roots are killed rapidly or slowly depending on the amount of fungus present in the soil and environment conditions. The development of the disease presents in **Figure 1.4**. In winter, oospores, chlamydospores, or mycelium in soil infect roots. In spring, spores germinate and mycelium further grows and produces zoosporangia which release zoospores. The zoospores swim around infected root of susceptible hosts. Moreover, mycelium spreads up the stem in the cortical region causing discoloration and collapse of cells. When mycelium invades the aerial part of host plants, it produces sporangiophores, which emerge through the stomata of leave and extend into air. The sporangia produced on the sporangiophores become dispersed by rain and start next cycle when the sporangia land on wet soil [3].

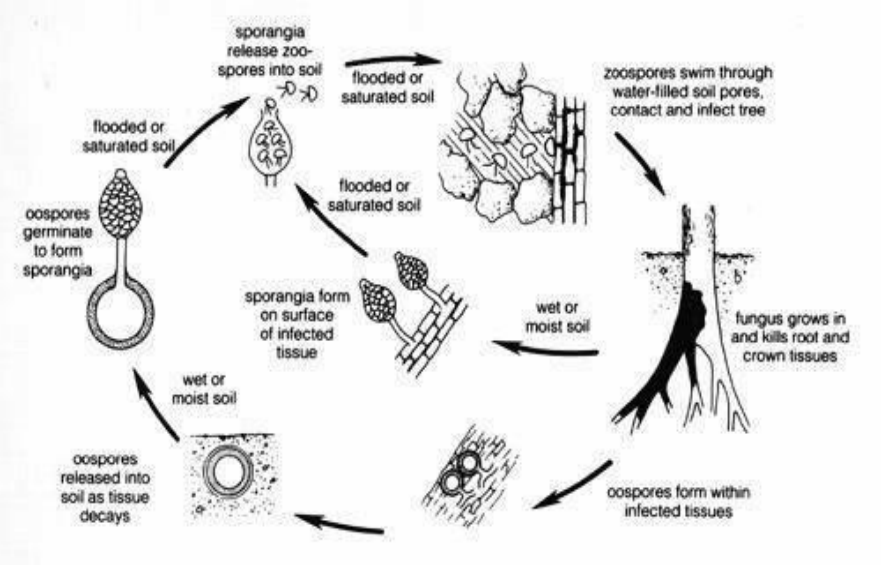


Figure 1.4 Disease cycle of *Phytophthora* sp. [3]

1.2 Natural products from plants

Thailand imported pesticide approximate 7,000 tons in 1999 and has a tendency to increase annually. In 2012, Thailand imported pesticides in the value of 10,813 million Baht [9]. Fungicides are the most popular use to control severity of plant diseases; however, a frequent use of fungicides can also lead to the formation of resistant pathogen strains [10]. There are certain reports concerning *Phytophthora* sp. resistant to fungicide such as metalaxyl, cyprofuram, benalaxyl and oxadixyl [11-14]. Besides, the pesticide residues on agricultural products also create problems for exporting the products aboard because of legislation in some countries. Therefore, the desire for safer agrochemicals with less environmental and mammalian toxicity is a major concern. In addition, health and environmental concerns generate a strong demand for biological fruits and vegetables as organic crops. Thus, an integrated method for pest management such as biological control treatment, cultural practices or natural chemicals from plants has been an alternative method to use for plant disease control instead of synthetic fungicides.

The researches concerning plant-derived fungicides and their applications in agriculture are now being intensified. The secondary metabolites from plants have evolved to protect them from attack by microbial pathogens [15] and phytophagous insects [16]. Thus, there has been a growing interest on the research of the possible use of the plant extracts, which are less damage for pest and disease control in agriculture. The safety of antimicrobial agents has been an increase in naturally developed substances, which has use of naturally derived compounds such as essential oil and plant extracts from the various parts of the medicinal plants [17]. The plants have long been recognized to provide a potential source of biological active compounds or more commonly products, known as phytochemicals. Moreover, the natural products are simply by their nature and safe relative to the environment or the organism targeted for benefit. However, their occurrence in nature is a strong indication that they existed for a long period without harmful biological or environmental effects [18].

Thailand is located in tropical region where a vast biodiversity and abundance of natural products. A large number of medicinal plants have been used for traditional treatment in the primary health care and used for agrochemicals. Therefore, natural products research is targeted toward the goal of studying biological activity and chemical constituents.

1.3 Objective of this research

The main objectives of this research are to evaluate antiphytopathogenic fungal activity from plant extracts, to isolate, purify and characterize the structures of active compounds from potential plant extracts, and to evaluate active principles *in vitro* and *in vivo* antifungal activity.

CHAPTER 2

PRELIMINARY ANTIPHYTOPATHOGENIC FUNGI SCREENING

2.1 Plant materials

Forty-six Thai plants were collected and investigated for anti-phytopathogenic fungi screening. Medicinal plants and weeds were collected from Cha-Am district, Phetchaburi province in 2010-1012 and some medicinal dried plants were purchased from “Chow Krom Per” and “Thai-Hua-Chan” medical herbal shop, Bangkok Thailand in 2010-2012. The selected plants and parts used are summarized in **Table 2.1**.

Table 2.1 The list of Thai plants for preliminary anti-phytopathogenic fungi screening

No.	Family	Scientific name	Common name	Plant parts
1	Acanthaceae	*Acanthus ilicifolius* L. เหงือกปลาหมอ	Sea holly	leaf
2		*Andrographis paniculata* (Burm.f.) Nees ฟ้าทะลายโจร	King of bitters, Kirayat	leaf
3	Annonaceae	*Melodorum fruticosum* Lour. ลำดวน	Davil tree, White heesewood	flower
4	Araceae	*Acorus calamus* L. ว่านน้ำ	Myrtlegrass, Sweetflag	rhizome
5	Bignoniaceae	*Oroxylum indicum* Vent. เพกา	Broken Bones Tree	bark/wood
6	Boraginaceae	*Heliotropium indicum* L. หญ้างวงช้าง	Eye bright , Indian heliotrope	whole plant
7	Caesalpiniaceae	*Cassia siamea* Britt. ขี้เหล็ก	Cassod tree, Thai copper pod	leaf/stem
8	Compositae	*Ageratum conyzoides* L. สาบแร้งสาบกา	Goat Weed	whole plant
9		*Eupatorium odoratum* L. สาบเสือ	Bitter bush, Siam weed	leaf/stem
10		*Grangea maderaspatana* (L.) Poir. พญามุตติ	Phayaa mutti	whole plant

Table 2.1 (cont.)

No.	Family	Scientific name	Common name	Plant parts
11		*Tagetes erecta* L. ดาวเรือง	Marigold	flower
12	Cucurbitacease	*Momordica charantia* L. มะระขี้นก	Bitter Cucumber, Balsum Pear	leaf/stem
13		*Momordica cochinchinensis* Spreng. ฟักข้าว	Spring bitter cucumber	leaf/seed
14	Dipterocarpaceae	*Vatica diospyroides* Symington. จันทร์กระพ้อ	Resak	leaf
15	Euphorbriaceae	*Croton bonplandianus* L. เปล้าทุ่ง	Jungle tulsi	leaf
16	Flacourtiaceae	*Hydnocarpus anthelminthicus* Pieere ex Laness. กระเบา	Chaulmoogra	seed
17	Gentianaceae	*Fagraea fragrans* Roxb. กันเกรา	Anan, Tembusu	flower/brark/ wood
18	Gramineae	*Cymbopogon nardus* (L.) Rendel. ตะไคร้หอม	Citronella grass	leaf
19	Lauraceae	*Cinnamomum porrectum* Kosterm เทพธาโร	Citronella laurel	wood
20	Leguminasae	*Erythrina variegata* L. ทองหลาง	Variegated- coral tree	leaf
21	Loranthaceae	*Helixanthera cylindrica* Dans. กาฝาก	Parasite, Kafak	leaf
22	Malraceae	*Hibicus sabdariffa* L. กระเจี๊ยบแดง	Jamaican Sorel, Roselle	fruit
23	Moraceae	*Artocapus heterophyllus* Lamk. ขนุน	Jack fruit tree	leaf/root
24	Myrtaceae	*Syzygium cumini* L. หว้า	Jambolan Plum	leaf/bark
25	Passifloraceae	*Passiflora foetida* L. กระทกรก	Fetid passionflower	leaf/stem
26	Piperaceae	*Piper betle* L. พลู	Betle Vine	leaf

Table 2.1 (cont.)

No.	Family	Scientific name	Common name	Plant parts
27		*Piper ribesoides* สะคร้าน	Cocculus, Fishberry	leaf
28		*Piper nigrum* L. พริกไทยดำ	Black pepper	fruit
29	Plamae	*Areca catechu* L. หมาก	Betel nut , Areca palm	seed
30	Plantaginaceae	*Plantago major* L. หมอน้อย	Greater plaintain	leaf
31	Polygonaceae	*Polygonum odoratum* Lour. ผักแพว	Vietnamese mint	whole plant
32	Pteridaceae	*Acrostichum aureum* L. ปรงทะเล	Leather fern	leaf
33	Rubiaceae	*Gardenia angusta* (L.) Merr. พุดซ้อน	Cape jasmine	flower/leaf/ branch
34		*Gardenia jasminoides* L. พุด	Gerdenia crape jasmine	flower
35	Rutaceae	*Zanthoxylum limonella* Alston. ต้นมะแข่น	Ma-khan	stem
36	Sapindaceae	*Nephelium lappaceum* L. เงาะ	Rambutan	seed
37	Scrophulariaceae	*Limnophila aromatica* (Lamk) Merr. ผักแขยง	Finger grass	whole plant
38	Stercubaceae	*Mansonia gagei* Drumm. จันทน์ชะมด	chan-cha-mod	wood
39	Verbenaceae	*Clerodendrum inerme* (L.) Gaertn. สำมะงา	Garden Quinine	leaf
40		*Tectona grandis* L. ไม้แดง	Iron wood	wood
41	Zigiberaceae	*Alpinia galanga* (L.) Willd. ข่า	Galanga	rhizome
42		*Boesenbergia rotunda* (Roxb.) Schltr. กระชายเหลือง	Kaempfer	rhizome
43	Zigiberaceae	*Curcuma longa* L. ขมิ้นชัน	Turmeric	rhizome

Table 2.1 (cont.)

No.	Family	Scientific name	Common name	Plant parts
44		*Kaempferia galanga* L. เปราะหอม	Aromatic ginger	rhizome
45		*Kaempferia parviflora* Wall.Ex Baker กระชายดำ	Belamcanda chimensis	rhizome
46		*Zingiber cassumunar* Roxb. ไพล	Cassumunar ginger	rhizome

2.2 Plant extraction

Fresh plants were cut into small pieces and dried. The dried plants were milled to powder and extracted by Soxhlet. The extraction started with hexane for leaf and for the other parts of samples with dichloromethane (CH_2Cl_2) and the residue of the extract was further extracted by methanol (MeOH). The extracts were filtered and evaporated by rotatory evaporator. In the case of essential oil, fresh samples were hydro-distillated. The distillate was extracted with Et_2O, dried over anhydrous Na_2SO_4 and finally evaporated. The % yields of crude extracts are displayed in **Table 2.2**.

2.3 Preliminary antiphytopathogenic fungi screening

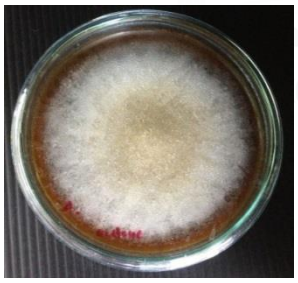
The hexane, CH_2Cl_2 and MeOH extracts from several species of Thai plants were investigated for antiphytopathogenic screening against four fungi: *Alternaria porri* DOAC 1601 (DOAC: Department of Agriculture Collection), *Colletothrichum gloeosporioides* DOAC 2047, *Fusarium oxysporum* DOAC 1258 and *Phytophthora parasitica* DOAC 2052 (**Figure 2.1**). These fungi were supplied by Division of Plant Disease and Microbiology, Department of Agriculture, Ministry of Agriculture and Cooperative, Bangkok, Thailand. Each fungus (*A. porri*, *C. gloeosporioides* and *F. oxysporum*) was cultured and maintained on potato dextrose agar (PDA) slant stored at 4°C until used whereas, *P. parasitica* were cultured on carrot agar (CA) at RT (25-28°C) [19]. The preliminary antiphytopathogenic screening using agar incorporation method (dilution on a solid medium) followed the method described by

Tangjitcharoenkul (2010). All fungi were cultivated on PDA in Petri dish for 7 days before using [20]. The crude extracts from plants were dissolved in DMSO or 1% tween 80 after that, 100 µL of each solution extract was aseptically added and mixed with 9.9 mL of PDA (liquefied and maintained at melting point in water bath) to obtain the final concentration of 1,000 µg·mL^{-1}. After well-mixed with PDA, it was poured into Petri dish, cooling and solidification. The disc mycelium growth of tested fungi (8 mm diameter) was picked up from the culture and placed in the center of Petri dish. The agar plates amend with DMSO and 1% tween 80 as a negative control while two commercial fungicides: metalaxyl and benomyl as a positive control. After the tested plates were incubated for 5-7 days at 27°C, the radial mycelial measurements of growth were taken when the fungi of control plate reached the edge of plate. The colony diameters were measured and calculated as percentage mycelial growth inhibition according to the formula [21]. All treatments were replicated three times. The results are displayed in **Table 2.2**.

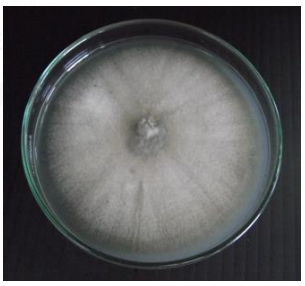
Percentage inhibition of mycelial growth (%) = [(dc-dt)/ dc × 100]

dc: average diameter of the fungal colony of control plate

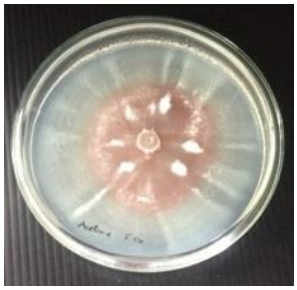
dt: average diameter of the fungal colony of treatment plate



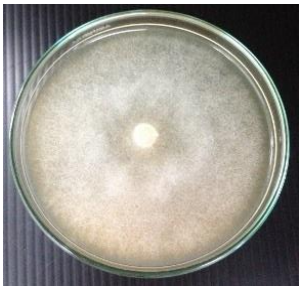
A. porri DOAC 1601



C. gloeosporioides DOAC 2047



F. oxysporum DOAC 1258



P. parasitica DOAC 2052

Figure 2.1 Phytopathogenic fungi

The % yield and % inhibitions of plant extracts against mycelial growth of phytopathogenic fungi are displayed in **Table 2.2**.

Table 2.2 %yield and %inhibition against mycelial growth of phytopathogenic fungi from Thai plant extracts

Scientific name	Part	Fraction	Yield %	%Inhibition[a,b]			
				A. por[c]	*C. glo*	*F. oxy*	*P. par*
A. ilicifolius	leaf	CH_2Cl_2	*	4.5±1.0	3.7±1.2	37.8±1.0	38.9±0.0
เหงือกปลาหมอ							
A. paniculata	leaf	Hexane	*	5.0±0.0	22.4±1.0	19.0±0.6	0.0±0.0
ฟ้าทะลายโจร		CH_2Cl_2		18.3±0.0	31.6±0.6	24.1±0.0	21.1±0.0
		MeOH		28.3±1.5	18.4±2.6	9.2±2.0	20.6±1.0
M. fruticosum	flower	CH_2Cl_2	3.2	49.5±1.5	31.1±0.0	74.4±1.2	83.3±3.7
ลำดวน		MeOH	11.3	47.1±1.5	11.1±0.0	26.7±1.2	51.4±1.6
A. calamus	rhizome	CH_2Cl_2	3.4	50±0.0	43.7±1.2	66.7±0.0	85.6±8.9
ว่านน้ำ		MeOH	9.2	26.7±1.2	10.3±1.2	13.8±3.5	32.2±1.0
O. indicum	bark	CH_2Cl_2	0.63	23.1±1.2	0.0±0.0	45.5±7.5	58.9±2.3
เพกา		MeOH	3.8	40±1.7	3.3±0.0	44.1±3.2	51.4±1.0
	wood	CH_2Cl_2		10.0±0.0	0.0±0.0	41.4±11.9	43.3±3.7
		MeOH		2.5±0.0	0.0±0.0	22.2±1.0	5.5±1.6
H. indicum	whole plant	Hexane	*	0.0±0.0	0.0±0.0	5.3±2.1	10.8±6.8
หญ้างวงช้าง		CH_2Cl_2		3.1±1.2	2.3±1.5	7.4±3.1	17±1.5
		EtOAc		5.2±2.2	4.5±3.3	6.7±1.0	24.4±0.0
		MeOH		4.6±3.1	3.2±3.1	11.4±2.1	12.2±1.0
C. siamea	leaf	CH_2Cl_2	*	-8.7±1.2	0.9±1.5	33.3±0.0	3.5±0.0
ขี้เหล็ก		MeOH		-7.7±1.0	-1.4±1.6	32.2±0.0	14.4±1.2
	stem	MeOH	*	43.3±3.2	24.1±2.1	13.8±1.2	0.0±0.0
A. conyzoides	whole plant	Essential oil	0.06				
สาบแร้งสาบกา		Hexane	2.4	32.2±1.6	48.9±2.1	15.2±3.2	50.0±0.0

Table 2.2 (cont.)

Scientific name	Part	Fraction	Yield	Inhibition (%)[a,b]			
			%	*A. por*[c]	*C. glo*	*F. oxy*	*P. par*
		CH_2Cl_2	1.3	33±0.0	45.6±2.2	26.7±1.4	51.9±0.6
		MeOH	10.3	17±1.2	40.0±0.0	19.7±2.1	17.4±0.6
E. odoratum	leaf	Essential oil	0.2				
สาบเสือ		Hexane	2.7	57.0±1.2	57.8±0.0	48.1±0.6	56.7±1.0
E. odoratum	leaf	CH_2Cl_2	5.6	58.1±0.6	56.7±0.0	50.0±0.0	70.0±0.0
สาบเสือ		MeOH	13.6	34.8±0.6	40.0±0.0	16.7±0.0	12.6±1.6
	stem	Hexane	1.05	38.9±1.7	46.7±0.0	27.0±4.0	35.6±0.0
		CH_2Cl_2	0.3	44.1±1.5	50.0±0.0	26.3±0.6	59.2±0.0
		MeOH	4.5	17.4±0.6	31.1±0.0	13.3±0.0	0.0±0.0
G. maderaspatana	whole plant	CH_2Cl_2	*	9.0±1.5	-9.3±1.5	-3.1±0.0	0.0±0.0
พญามุติ							
T. erecta	flower	CH_2Cl_2	*	31.4±1.5	16.1±1.0	54.4±1.2	20.0±0.0
ดาวเรือง		MeOH		21.7±1.0	21.2±1.0	19.5±1.2	0.0±0.0
M. charantia	leaf	CH_2Cl_2	8.6	18.1±0.0	28.8±1.0	10.0±5.4	-15.2±0.7
มะระขี้นก		MeOH	22.7	25.6±0.0	16.7±12.9	30.6±1.0	-13.3±0.0
	stem	CH_2Cl_2	13.2	27.3±2.1	14.4±1.6	0.0±0.0	-16.3±1.2
		MeOH	15.7	22.2±0.0	15.3±0.0	8.9±0.0	-13.0±1.5
M. cochinchinensis	leaf	CH_2Cl_2	1.9	-12.8±1.6	3.7±1.5	22.2±0.0	8.9±0.0
ฟักข้าว		MeOH	7.1	-3.7±1.2	17.0±6.1	11.1±0.0	27.8±0.0
	seed	CH_2Cl_2	32.3	0.0±0.0	28.9±	17.2±4.7	14.4±0.0
		MeOH	10.1	22.2±0.0	24.8±2.5	11.1±0.0	13.9±2.6
C. bonplandianus	leaf	CH_2Cl_2	*	-15.7±2.0	-1.8±2.1	30.0±1.0	0.0±0.0
เปล้าทุ่ง							
H. sabdariffa	leaf	CH_2Cl_2	*	21.4±2.1	12.8±0.0	60.6±8.9	20.0±1.6
จันทร์กระพ้อ		MeOH		23.9±2.1	28.9±0.0	50.0±0.6	50.0±2.4
H. anthelminthicus	seed	CH_2Cl_2	*	47.1±0.0	17.8±0.0	51.7±1.0	7.8±1.6
กระเบา							

Table 2.2 **(cont.)**

Scientific name	Part	Fraction	Yield	Inhibition (%)[a,b]			
			%	*A. por*[c]	*C. glo*	*F. oxy*	*P. par*
F. fragrans	flower	CH_2Cl_2	****	45.6±0.6	17.8±1.0	18.9±2.6	16.7±0.0
กันเกรา		MeOH		52.2±0.6	26.11.0	17.0±0.6	16.7±0.0
	bark	CH_2Cl_2	****	62.6±1.5	29.2±1.5	49.7±1.0	34.4±0.0
		MeOH		57.8±1.2	28.1±1.0	22.2±0.0	21.9±0.6
	wood	CH_2Cl_2	****	58.0±3.5	26.7±1.0	15.6±1.7	19.2±0.6
		MeOH		51.9±1.2	18.9±0.0	10.0±1.0	17.0±0.6
C. winterianus	leaf	CH_2Cl_2	*	-6.8±2.1	22.2±1.0	37.8±0.0	23.3±0.0
ตะไคร้หอม							
C. porrectum	wood	Essential oil	**	0.0±0.0	0.0±0.0	24.0±0.0	53.3±0.0
เทพธาโร		CH_2Cl_2		26.2±3.1	20.7±0.0	13.8±2.3	86.7±0.0
		MeOH		26.6±2.3	8.9±0.0	6.7±2.0	79.3±0.6
E. variegata	leaf	CH_2Cl_2	*	27.9±1.6	17.1±1.0	56.1±3.0	12.2±1.6
ทองหลาง		MeOH		24.3±0.6	14.4±1.6	57.2±1.2	13.3±0.6
H. cylindrica	leaf	CH_2Cl_2	*	37.9±1.6	25.0±0.0	30.0±0.0	0.0±0.0
กาฝาก		MeOH		-19.1±2.5	-13.9±2.6	28.9±0.0	25.6±1.0
H. sabdariffa	fruit	MeOH	*	36.8±1.0	-6.9±2.0	40.0±2.0	68.9±0.0
กระเจี๊ยบแดง							
A. heterophyllus	leaf	CH_2Cl_2	13.8	16.7±13.2	17.8±1.5	-21.4±1.6	-15.3±0.2
ขนุน		MeOH	16.5	-10.5±1.2	-1.8±2.0	-28.6±0.6	-17.1±0.6
	root	CH_2Cl_2	3.2	3.3±0.6	5.6±0.0	0.0±0.0	0.0±0.0
		MeOH	4.8	22.6±4.5	16.7±0.0	0.0±0.0	0.0±0.0
S. cumini	leaf	Hexane	*	40.4±2.1	20.0±0.0	55.6±1.0	26.7±2.1
หว้า		CH_2Cl_2		27.1±4.7	19.4±0.6	55.6±1.2	21.7±1.5
		MeOH		35.4±1.5	17.8±1.0	49.4±2.3	15.6±1.6
	bark	CH_2Cl_2	*	51.4±0.0	18.9±2.1	55.0±4.7	25.4±0.6
		MeOH		52.1±0.6	12.8±1.2	53.3±2.6	15.6±0.6
P. foetida	leaf	CH_2Cl_2	9.6	41.4±4.5	41.4±0.0	51.9±0.6	56.7±0.6
กระทกรก		MeOH	26.5	53.3±0.0	28.7±2.1	53.3±0.0	44.8±0.5

Table 2.2 **(cont.)**

Scientific name	Part	Fraction	Yield	Inhibition (%)[a,b]			
			%	*A. por*[c]	*C. glo*	*F. oxy*	*P. par*
		MeOH	15.3	45.0±0.5	17.2±2.1	60.5±1.2	25.9±1.2
A. catechu	seed	CH_2Cl_2	2.2	44.3±1.6	21.1±1.0	53.3±1.5	22.2±2.1
หมาก		MeOH	38.9	10.0±3.8	5.8±1.0	9.2±0.0	21.1±6.2
P. betle	leaf	Hexane	3.4	26.4±1.0	18.3±1.6	54.4±0.6	40.0±2.1
พลู		CH_2Cl_2	2.4	46.7±0.6	29.9±9.4	44.8±0.0	65.6±4.0
P. ribesoides	leaf	CH_2Cl_2	*	30.8±0.0	48.3±1.0	47.7±1.0	5.0±1.0
สะคร้าน		MeOH		40.0±0.6	31.0±1.0	23.0±0.0	0.0±0.0
P. nigrum	seed	CH_2Cl_2	2.7	57.1±0.0	51.7±0.0	49.7±0.0	55.6±0.6
พริกไทยดำ		MeOH	14.1	57.6±1.0	52.2±0.6	54.4±1.6	48.9±1.2
P. major	leaf	CH_2Cl_2	*	-7.8±1.0	-11.5±0.6	33.3±0.0	0.0±0.0
หมอน้อย							
P. odoratum	whole plant	Essential oil	*	40.9±0.6	3.0±2.5	46.4±2.1	23.3±1.0
ผักแพว							
A. aureum	leaf	CH_2Cl_2	*	-22.8±2.0	0.9±0.6	30.0±1.5	2.2±2.0
ปรงทะเล							
G. angusta	flower	CH_2Cl_2	***	8.5±0.0	35.1±0.0	-12.7±0.0	36.2±4.4
พุฒช้อน		MeOH		-8.5±1.0	5.2±0.6	1.6±0.6	19.4±2.3
	leaf	CH_2Cl_2	***	0.0±0.0	8.2±0.6	-23.8±0.0	5.9±1.0
		MeOH		-26.8±0.0	14.3±0.0	-11.1±0.0	11.2±2.3
	branch	CH_2Cl_2	***	-7.0±0.0	22.1±0.0	-10.6±0.0	48.2±7.1
		MeOH		-3.8±0.0	14.3±0.0	-13.8±0.0	4.5±0.6
G. jasminoides	flower	CH_2Cl_2	***	1.4±0.0	19.5±0.0	-6.3±0.0	0.0±0.0
พุด		MeOH		9.4±1.2	-14.3±0.0	-8.4±0.0	17.1±1.6
Z. limonella	stem	CH_2Cl_2	0.4	55.4±0.6	31.9±0.0	44.4±0.0	37.8±1.0
ต้นมะแข่น		MeOH	2.1	54.7±1.5	26.9±3.6	33.3±0.0	48.9±1.0
N. lappaceum	seed	hexane	*	12.2±1.0	15.2±0.6	5.0±0.0	0±0.0
เงาะ		CH_2Cl_2		26.7±1.0	16.7±0.0	2.6±0.6	3.3±1.0
		EtOAc		44.4±1.0	21.1±1.0	15.6±1.0	11.1±1.0

Table 2.2 **(cont.)**

Scientific name	Part	Fraction	Yield	Inhibition (%)[a,b]			
			%	*A. por*[c]	*C. glo*	*F. oxy*	*P. par*
		MeOH		50.0±0.6	25.9±3.5	13.0±0.6	13.7±3.5
L. aromatica	whole plant	Essential oil	*	70.3±0.6	11.1±0.0	46.3±0.6	54.4±5.5
ผักแขยง							
M. gagei	wood	CH_2Cl_2	3.2	53.3±1.3	25.3±1.5	54.0±2.3	73.3±1.0
จันทน์ชะมด		MeOH	4.3	48.3±1.7	21.8±1.1	50.6±2.1	26.7±1.0
C. inerme		CH2Cl2	*	35.0±3.5	28.7±1.2	23.0±1.0	22.2±20.2
สำมะงา		MeOH		31.7±6.0	23.0±2.1	11.5±2.1	0.0±0.0
T. grandis	wood	CH_2Cl_2	****	53.3±0.0	42.2±1.0	20.0±0.0	60.8±2.1
ไม้แดง		MeOH		49.7±2.5	37.4±1.5	19.7±0.6	55.2±0.6
A. galanga	rhizome	Essential oil	0.22				
ข่า		CH_2Cl_2	2.3	42.5±1.0	33.3±2.0	70.1±1.5	100.0±0.0
		MeOH	19.8	28.3±0.6	8.1±0.0	3.4±0.0	0.0±0.0
B. rotunda	rhizome	CH_2Cl_2	*	51.7±1.0	43.7±0.0	46.0±1.5	20.0±2.0
กระชายเหลือง		MeOH		48.3±0.0	42.5±1.2	51.7±1.0	80.6±2.5
C. longa	rhizome	CH_2Cl_2	*	50.0±0.0	39.1±1.0	64.4±1.6	64.4±14.2
ขมิ้นชัน							
K. galanga	rhizome	CH_2Cl_2	*	61.7±0.0	44.8±0.2	46.0±0.0	81.7±1.5
เปราะหอม		MeOH		45.0±1.0	27.6±1.6	37.9±1.0	42.2±1.0
K. parviflora	rhizome	CH_2Cl_2	*	39.2±1.0	57.5±0.0	59.2±4.9	46.7±11.4
กระชายดำ		MeOH		38.3±0.6	24.1±2.5	47.1±2.1	32.2±1.0
Z. cassumunar	rhizome	CH_2Cl_2	*	31.7±0.0	24.1±1.0	47.1±1.6	0.0±0.0
ไพล		MeOH		27.5±1.0	28.1±1.0	20.7±0.0	0.0±0.0

Kindly provide from: *Saipin Boonkerd, **Suekanya Jarupinthusophon, ***Pragatsawat Chanprapai, ****Veerachai Pongkittipan

[a] Final concentration at 1000 μg·mL^{-1}

[b] Values, an average ± standard deviation (SD) of 3 replicates of the mean mycelial growth inhibition

[c] *A. por*: *A. porri*, *C. glo*: *C. gloeosporioides*, *F. oxy*: *F. oxysporum*, *P. par*: *P. parasitica*

As results presented in **Table 2.2**, the antifungal activity screening of the extracts of 46 Thai plants showed different extents depend on the phytopathogenic fungi; the plant in family Scrophulariaceae, the essential oil of *L. aromatica* displayed the highest activity against *A. porri* with 70% inhibition. Eighteen species of collected plants (*A. calamus*, *M. fruticosum*, *E. odoratum*, *H. anthelminthicus*, *P. foetida*, *A. catechu*, *P. betle*, *P. nigrum*, *N. lapaceum*, *M. gagei, Z. limonella, T. grandis, A. galanga, B. rotunda, C. longa* and *K. galanga*) moderately inhibited against *A. porri* (40.9%-61.7% inhibition). In addition, 12 species (*A. paniculata*, *C. siamea*, *T. erecta*, *C. porrectum*, *E. variegate*, *S. cumini*, *P. ribesoides*, *G. jasminoides, H. sabdariffa*, *C. inerme*, *K. parviflora* and *Z. cassumunar*) displayed weakly against *A. porri* with 9.4%-39.2% inhibition. Whereas, all tested parts (flower, leaf and stem) from *G. angusta* crude extract stimulated the growth of *A. porri* faster than control group (-3.8 to -26.8% inhibition).

In case of the antiphytopathogenic activity against the mycelial growth of *C. gloeosporioides*, the CH_2Cl_2 extract from family Compositae (*E. odoratum* and *A. conyzoides*) showed moderate activity against this tested organism, followed by *K. palviflora*, *P. nigrum*, *P. betle*, *K. galanga*, *B. rotunda*, *A. calamus*, *T. grandis* and *P. foetida*, respectively. The other plants showed low activity against *C. gloeosporioides* (16.7-39.1%). The extracts from *O. indicum*, *H. indicum*, *C. perrectum*, *P. odoratum* and *A. aureum* did not show the effect on *C. gloeosporioides* at 1,000 $\mu g \cdot mL^{-1}$. However, some of collected plants (*C. siamea*, *G. maderaspatana*, *C. bonplandianus*, *H. sabdariffa*, leaf of *A. heterophyllus*, *P. major* and *G. angusta*) stimulated the mycelial growth of tested fungi.

For *F. oxysporum*, the CH_2Cl_2 extract of family Annonaceae (*M. fruticosum*) and family Zingiberaceae (*A. galanga*) showed strong inhibition against this fungus with 74.4 and 70.1% inhibition, respectively, followed by *A. calamas*, *C. longa*, *H. sabdariffa*, *P. foetida*, *P. paviflora*, *E. variegate*, *S. cumini*, *P. betle*, *P. nigrum*, *T. erecta*, *M. gagei*, *A. catechu*, *B. rotunda*, *H. anthelminthicus*, *E. odoratum*, *F. fragrans*, *P. ribesoides*, *Z. cassumunar*, *P. odoratum*, *L. aromatia*, *K. galanga*, *O. indicum* and *Z. limonella* (44.4-66.7% inhibition). Eleven species (*A. ilicifolius*, *C. siamea*, *M. charantia*, *E. odoratum*, *A. conyzoides*, *A. paniculata*, *C. inerme*, *M. cochinchinensis*,

T. grandis, *N. lappaceum and H. indicum*) displayed weak inhibition less than 40.0%. On the other hand, four species including *A. heterophyllus*, *Gr. manderaspatana*, *Ga. angusta* and *Ga. jasminoides* stimulated the mycelium growth of this fungus.

Family Zingiberaceae showed the highest activity against phytopathogenic fungi. Especially, the CH_2Cl_2 extract of *A. galanga* completely exhibited against the mycelial growth of *P. parasitica*, followed by *C. porrectum* (Lauraceae), *A. calamus* (Araceae), *M. fruticosum* (Annonaceae), *K. galanga*, *B. rotunda* (Zingiberaceae), *M. gagei* (Stercubaceae) and *E. odoratum* (Compositae). These plants revealed strong activity against *P. parasitica* with 70-86.7% inhibition. Eleven species (*O. indicum, A. conyzoides, P. betle, P. nigrum, G. amgista, L. aromarica, H. sabdariffa, Z. limonella, T. grandis, C. longa* and *K. parviflora*) exhibited moderate inhibition (46.7-68.9%) against *P. parasitica*. Fifteen plant extracts (*A. paniculata, H. indicum, C. siamea, T. erecta, M. cochinchinensis, C. winterianus, H. anthelminthicus. E. variegate. H. cylindrical, S. cumini, A. catechu, P. osoratum, G. jasminoides, F. fragrans* and *C. inerme*) showed low activity (7.8-34.4%) against *P. parasitica*. Whereas, *C. siamea* (stem), *M. chrantia, C. bonplandianus, A. heterophyllus, P. major, G. maderaspatana* and *Z. cassumunar* did not reveal any activity against *P. parasitica*.

The percent yield of crude extract from MeOH part was higher than that of CH_2Cl_2 extract; however, the MeOH extract showed the antifungal activity less than CH_2Cl_2 extract. Since the MeOH extract contained high polar compounds such as monosaccharides (fructose, glucose) and polysaccharide [22, 23]. These compounds could be served as nutrient for supporting the growth of fungi [24].

In this research, the antifungal activity against four phytopathogenic fungi was compared. The efficiency of plants extracts was different because of the variety of plant species, crude extracts from different organic solvents and variety of fungi. Sati and Joshi (2011) reported that different compounds such as essential oil, alkaloids, saponins, terpenes, flavonoids, peptide and protein exhibited antimicrobial activity [25]. However, it cannot be attributed which plant compound is responsible to what extent for its effectiveness towards the tested fungi until isolation of phytochemicals active compound after it gives preliminary data and followed the activity using bioassay-guided fractionation.

In **Figure 2.2**, *A. calamus* displayed broad spectrum antifungal potential against all tested fungi. There are many reports about this useful plant, for example herbal medicine [26], perfume [27], antioxidant [28], antifungal [4], antimicrobial [29], insecticidal [30] and adipogenesis inhibition [31]. However, the products derived from *A. calamus* especially asarone were banned in 1968 as food additives and medicines by the United Stated Food and Drug Administration (FDA). FDA banned this plant because the report about supplementing diets of lab animals over a prolonged period of time with massive doses of isolated chemical (***β***-asarone), the animals developed tumors, and the plant was labeled procarcinogen [32]. From this reason, *A. calamus* did not select for further study in this research.

Various biological activities of *L. aromatic* (Syn. *L. gratissima*) were reported. The essential oil of this plant showed good antimicrobial activity against *S. aureus*, *B. subtilis*, *E. coli*, *P. auruginosa* [33] and antifungal activity [34]. Moreover, Brahmachari (2008) reported the chemical constituents and pharmaceutical activities aspects of *Limnophila* species (Scrophulariaceae) such as anti-inflammatory, antioxidant, wound healing, antitubercular, cytotoxic and anthelmintic [35].

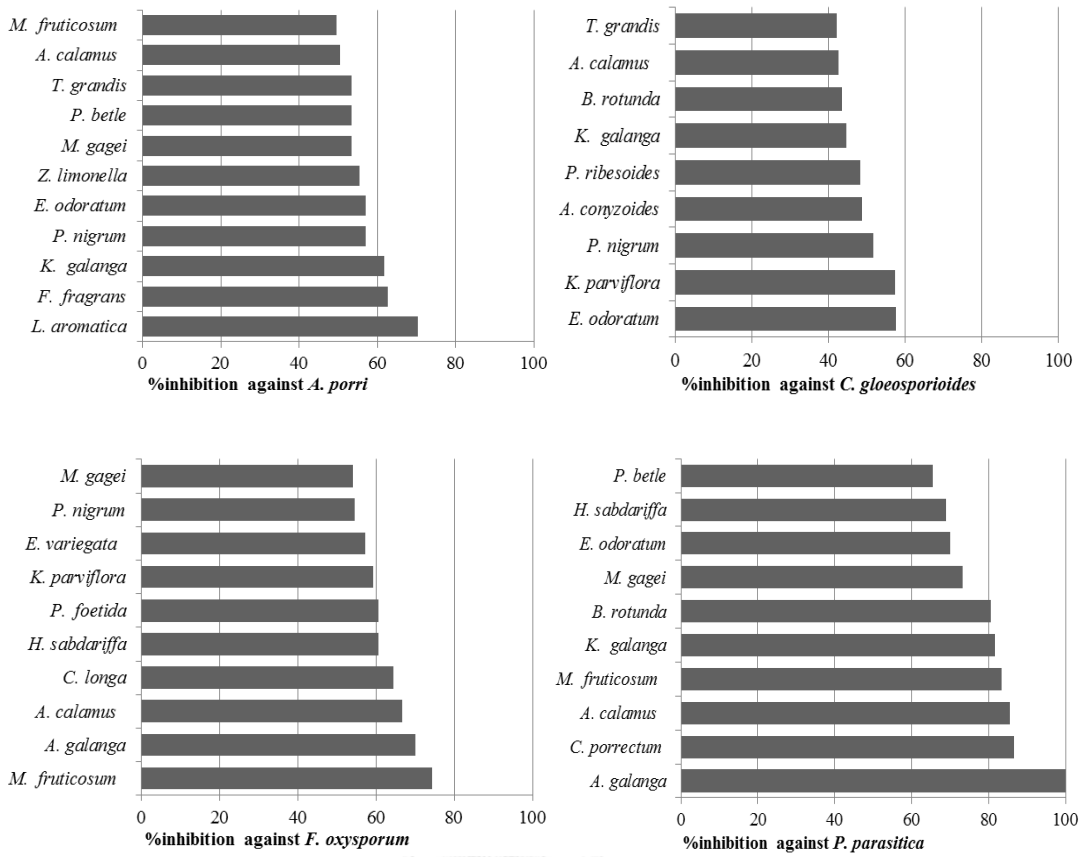


Figure 2.2 Ten plants displayed strong antifungal activity against four phytopathogenic fungal at 1000 μg·mL^{-1}

According to the preliminary antiphytopathogenic fungal screening of Thai plants, the CH_2Cl_2 extract of *A. galanga*, *M. fruticosum* and *M. gagei* displayed strong inhibition against the mycelium growth of *P. parasitica* and other tested fungi (**Figure 2.2**). Moreover, there was no report of these plants on antiphytopathogenic fungi especially against *A. porri*, *C. gloeosporioides*, *F. oxysporum* and *P. parasitica*. Therefore, the CH_2Cl_2 extract of these plants were selected to evaluate for the active compounds against phytopathogenic fungi.

CHAPTER 3

ANTIPHYTOPATHOGENIC FUNGAL AGENTS FROM THE RHIZOMES OF *Alpinia galanga* (L) Willd. AND THEIR PHYTOTOXIC ACTIVITY

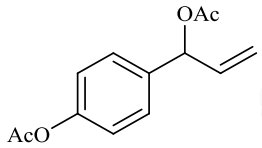
3.1 Introduction of *Alpinia galanga* (L) Willd.

Alpinia galanga (L) Willd. (Family Zingiberaceae) syn. *Languas galanga* an herbaceous perennial, 1.5-2 m tall with white flower (**Figure 3.1**) [34]. This plant is widely cultivated in India, China, and Southeast Asian countries, such as Indonesia, Malaysia, Philippines and found abundantly in Thailand [36]. It is also called Thai galanga or greater galanga and Kha [37]. The rhizomes of galangal are extensively used for foods, especially Tom Yom Kung because of its pungent, hot and spicy taste with aromatic ginger like odor. Moreover, galangal rhizomes are also used as traditional medicine for several purposes such as stomachic in China for carminative, antiflatulent, antifungal and anti-itching in Thailand.

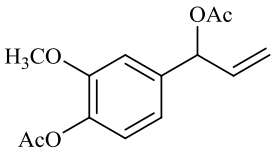
This plant was rich in phenolic compounds and the rhizomes were not only lowest in fat, but also richest in carbohydrate [38]. The chemical constituents of *A. galanga* rhizomes have been widely investigated (**Figure 3.2**). Their major constituents are phenylpropanoids such as (1′*S*′)-1′-acetoxychavicol acetate (ACA) (**3-1**).

Figure 3.1 The shrub, flowers and rhizomes of *A. galanga* (L) Willd.

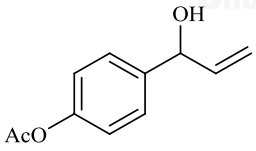
Other identified compositions include (1′*S*′)-1′-acetoxyeugenol acetate (**3-2**), (1′*S*′)-1′-hydroxychavicol acetate (**3-3**), *trans-p*-coumaryl alcohol (**3-4**), *trans-p*-hydroxycinnamaldehyde (**3-5**), *trans-p*-hydroxycinnamyl acetate (**3-6**), *trans*-3,4-dimethoxycinnamyl alcohol (**3-7**), *trans-p*-coumaryl diacetate (**3-8**), *p*-hydroxybenzaldehyde (**3-9**), galangin (**3-10**), *trans-p*-coumaric acid (**3-11**) and methyleugenol (**3-12**) [39]. Galangogalloside (**3-13**), galangoflavonoid (**3-14**), β-sitosterol diglucosyl caprate (**3-15**) and galanganol B (**3-16**) were isolated from *A. galanga* rhizomes) [40-43]. Furthermore, Zhu and co-workers (2008) reported new phenyl propanoids as 4-[(*E*)-3-hydroxyprop-1-enyl]phenyl acetate (**3-17**), *trans-p*-hydroxy cinnamaldehyde acetate (**3-18**), 5-hydroxy-7-(4″-hydroxy-3″-methoxyphenyl)-1-phenyl-3-heptanone (**3-19**), 7-(4″-hydroxy-3″-methoxyphenyl)-1-phenylhep-4-en-3-one (**3-20**) [44]. Moreover, Zhao *et al*. (2012) reported two new phenylpropanoids as (*S*)-1′-ethoxy chavicol acetate (**3-21**) and (*E*)-4-acetoxy cinnamyl ethyl ether (**3-22**) [45].



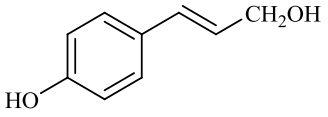
(1′*S*′)-1′-acetoxychavicol acetate (ACA) (**3-1**)



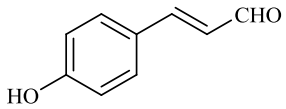
(1′*S*′)-1′-acetoxyeugenol acetate (**3-2**)



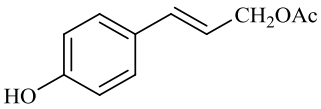
(1′*S*′)-1′-hydroxychavicol acetate (**3-3**)



trans-p-coumaryl alcohol (**3-4**)

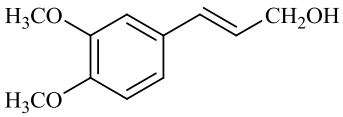


trans-p-hydroxycinnamaldehyde (**3-5**)

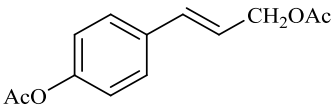


trans-p-hydroxycinnamyl acetate (**3-6**)

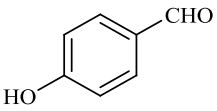
Figure 3.2 Chemical constituents isolated from *A. galanga* rhizomes



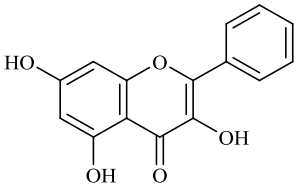
trans-3,4-dimethoxycinnamyl alcohol (**3-7**)



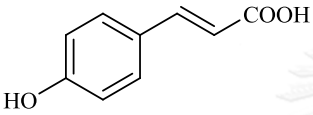
trans-p-coumaryl diacetate (**3-8**)



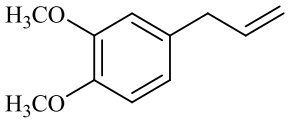
p-hydroxybenzaldehyde (**3-9**)



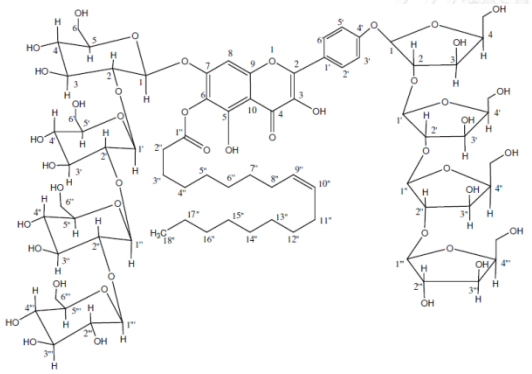
galangin (**3-10**)



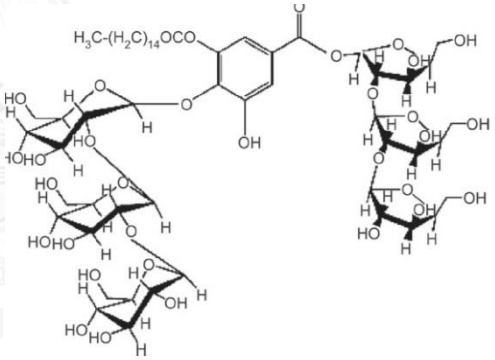
trans-p-coumaric acid (**3-11**)



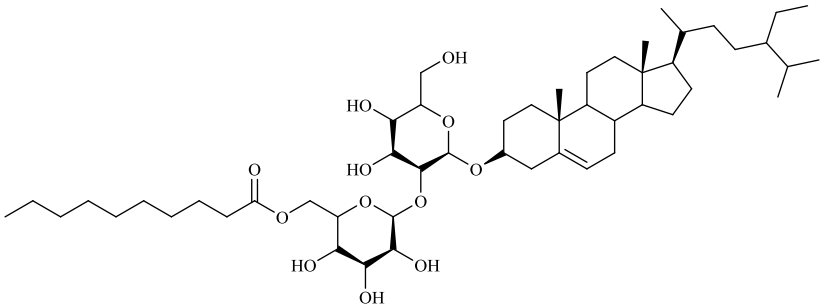
methyleugenol (**3-12**)



galangogalloside (**3-13**)

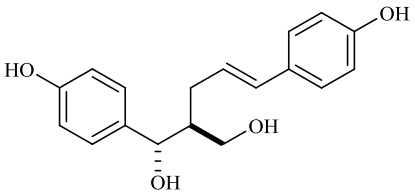


galangoflavonoid (**3-14**)

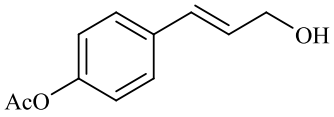


β-sitosterol diglucosyl caprate (**3-15**)

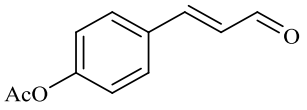
Figure 3.2 (cont.)



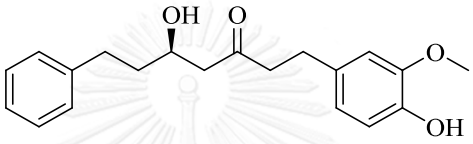
galanganol B (**3-16**)



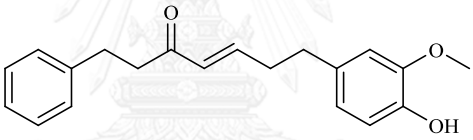
4-[(*E*)-3-hydroxyprop-1-enyl]phenyl acetate (**3-17**)



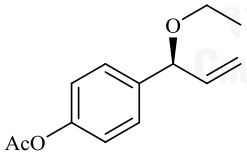
trans-p-hydroxycinnamaldehyde acetate (**3-18**)



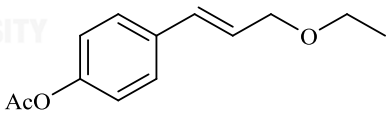
5-hydroxy-7-(4″-hydroxy-3″-methoxyphenyl)-1-phenyl-3-heptanone (**3-19**)



7-(4″-hydroxy-3″-methoxyphenyl)-1-phenylhep-4-en-3-one (**3-20**)



(*S*)-1′-ethoxy chavicol acetate (**3-21**)



(*E*)-4-acetoxy cinnamyl ethyl ether (**3-22**)

Figure 3.2 (cont.)

The constituents of the essential oil from dried and fresh rhizomes were characterized as 1,8-cineole (**3-23**), chavicol (4-allylphenol) (**3-24**), chavicol acetate (**3-25**), eugenol (**3-26**), eugenol acetate (**3-27**) and methyleugenol (**3-28**), α-pinene (**3-29**), β-pinene (**3-30**), terpinen-4-ol (**3-31**), α-terpineol (**3-32**), β-bisabolene (**3-33**), β-caryophyllene (**3-34**), β-selinene (**3-35**), linalool (**3-36**), geranyl acetate (**3-37**),

farnesyl acetate (**3-38**), limonene (**3-39**) [37, 46-50]. Moreover, Kubota *et al.* (1998) isolated the volatile from fresh rhizomes of *A. galanga* by stream distillation, 4 isomers of acetoxycineoles including (*trans* and *cis*)-2- and 3-acetoxy-1,8-cineoles (**3-40** to **3-43**) were identified as the odor component [51]. The structure of the chemical constituents from the essential oil of *A. galanga* rhizomes were indicated in **Figure 3.3**.

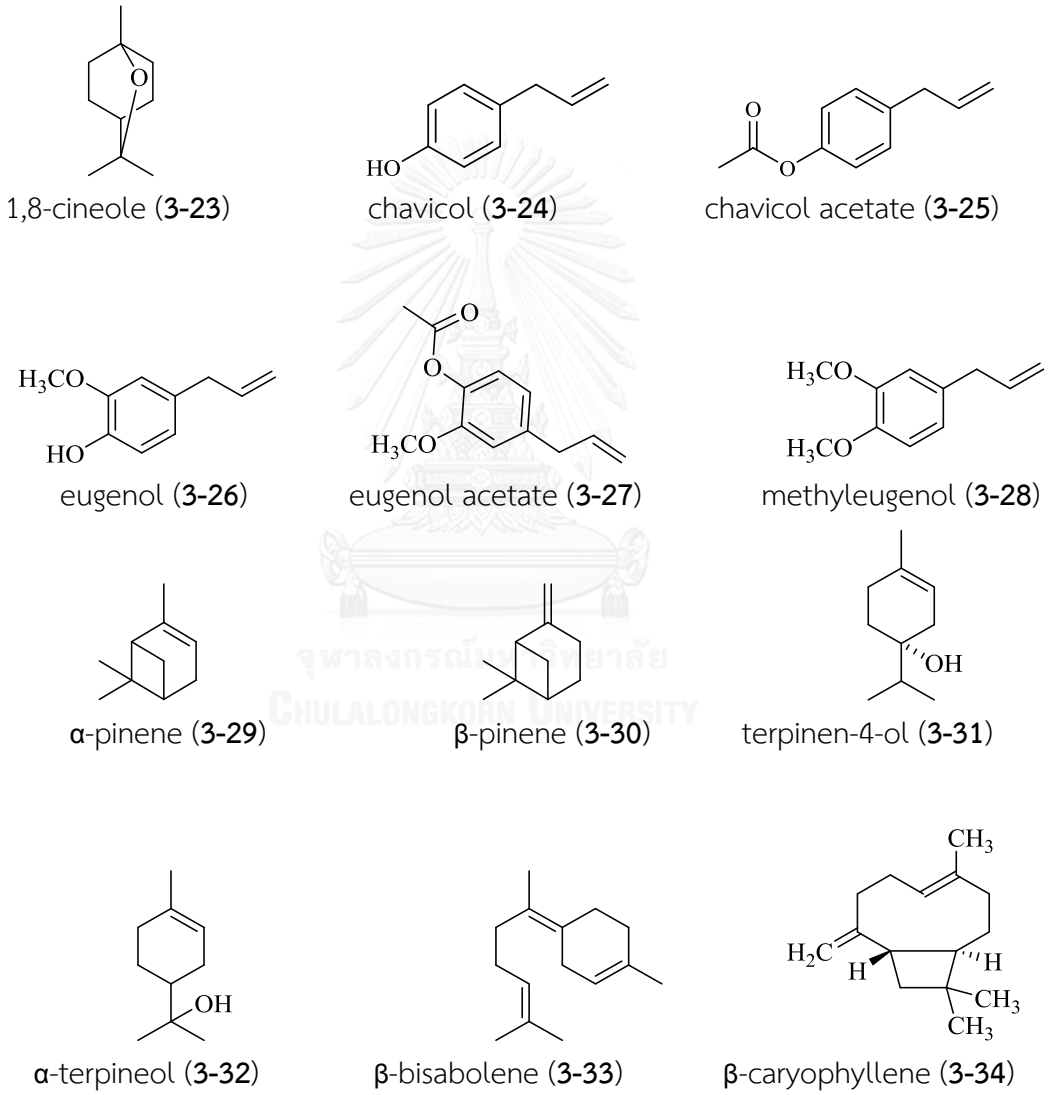
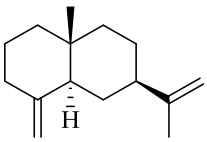
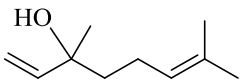


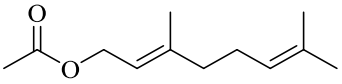
Figure 3.3 Chemical constituents from the essential oil of *A. galanga* rhizomes



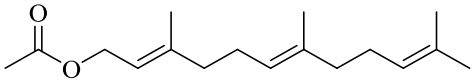
β-selinene (**3-35**)



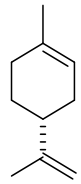
linalool (**3-36**)



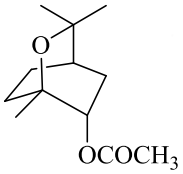
geranyl acetate (**3-37**)



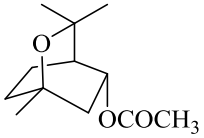
farnesyl acetate (**3-38**)



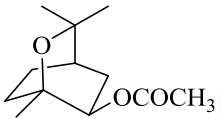
limonene (**3-39**)



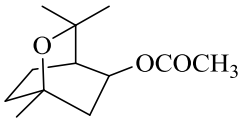
trans-2-acetoxy-1,8-cineole **(3-40)**



trans-3-acetoxy-1,8-cineole **(3-41)**



cis-2-acetoxy-1,8-cineole **(3-42)**



cis-3-acetoxy-1,8-cineole **(3-43)**

Figure 3.3 (cont.)

The biological activities of galangal have been examined. Janssen and Scheffer (1985) reported the extract of *A. galanga* rhizomes against seven fungi and the active compound as ACA showed significant antifungal activity against *Trichophyton mentagrophytes*, *T. rubrum*, *T. concentricum*, *R. stolonifer* and *A. niger* [52]. The ethanolic extracts of *A. galanga* showed good antifungal activity against *T. longifusus, Microsporum canis* and *Fusarium solani* (60, 30 and 40%, respectively) and completely inhibited phytotoxic activity against *Lemuna minor* at 1000 $\mu g \cdot mL^{-1}$. Moreover, the antibacterial activity of galanga extract was tested against *Staphylococcus aureus* (23% inhibition), *Salmonella* typhi (27% inhibition); however, this extract could not inhibit against *Escherichia coli, Bacillus subtillus, Pseudomonas*

aeruginosa and *Shigella flexnali* [53, 54]. Oonmetta-aree *et al.* (2006) studied the ethanol extracts of this plant and determined antimicrobial activity [55]. This report showed that *S. aureus* (Gram-positive) was more sensitive to galanga extract than *E. coli* (Gram-negative) with MIC and MBC as 0.325 and 1.3 mg·mL^{-1}, respectively. Besides, *S. epidermidis* and *Saccharomyces cerivisiae* also were sensitive to this extract. While, the tested microorganisms *B. circus*, *B. megaterium* and *Streptococcus lactis* were less susceptible to extract. On the other hand, this extract did not show activity against Gram negative bacteria (*Salmonella* sp., *Enterobacter aerogenes* and *P. aeruginosa*). In addition, Mayachiew and Devahastin (2008) reported the extracts of *A. galanga* against *S. aureus* with MIC and MBC of 0.78 and 2.34 mg·mL^{-1} [37]. In 2009, Latha and co-workers studied the antiplasmid activity of ACA from *A. galanga* against multi-drug resistant bacteria. The results revealed that the acetone extract exhibited antiplasmid against *S.* typhi, *E. coli* and vancomycin resistant *Enterococcus faecalis* with efficiency of 92, 82 and 8%, respectively at 400 µg·mL^{-1} [56].

In 2009, Pompimon and colleagues reported that the crude extracts of *A. galangal, Curcuma longa, Boesenbergia pandurata and Chromolaena odorata* inhibited mycelial growth of *P. capsici* with ED_{90} as 300 µg·mL^{-1} [57]. The study in the field, hexane extract of *A. galanga* (20 g·L^{-1}) significantly increased the percentage of the survival chili (43.6%) after planting in *P. capsici*-infested soil 7 weeks compared with captan and bio-control (*Trichoderma virens*).

In case of antioxidant activity, 50% EtOH in water extract of *A. galanga* was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and oxygen radical absorbance capacity (ORAC) methods. The ethanolic extract was the most effective radical scavenger with IC_{50} as 10.5 mg·mL^{-1} [58]. In addition, Mayachiew and Devahastin (2008) reported 70.3% antioxidant activity of galangal extracts using β-carotene bleaching method [59]. Butkhup *et al.* (2011) reported the antioxidant activity of *A. galanga* 78.9 % radical scavenging activity at 2.0 mg·mL^{-1} compared with ascorbic acid (96.3%) at the same concentration [60]. Meanwhile, ferrous ion-chelating value from *A. galanga* leave displayed higher than that from the rhizomes 20 times [61]. Singh *et al*. (2011) reported that the EtOH extract of *A. galanga* expressed oxidative stress induced Alzheimer's type amnesia in mice and the mice

were treated with the extract at 200 and 400 mg·kg^{-1} dose for 14 days and injected with neurotoxic Amyloid $\beta_{(25-35)}$ (Aβ) [62]. They suggested the galanga extract possess an antiamnesiac effect in Aβ induced neurodegeneration through an antioxidant property. Moreover, the concentration, pH and heat were influence on antioxidant activity [63].

For tyrosinase inhibition property, Weerapreeyakul and co-workers (2012) observed that the water and ethanolic extracts of galanga rhizome exhibited the highest mushroom tyrosinase inhibitory activity with IC_{50} as 3.33 and 14.2 μg·mL^{-1} which was higher than vitamin C (19.6 μg·mL^{-1}).

Phongpaichit *et al.* (2005) explored the extracts from Thai medicinal plants against fungal infections in AIDS patients and indicated the $CHCl_3$ extract of *A. galanga* (1 mg·disc^{-1}) against *C. albicans, Cryptococcus neoformans* and *M. gypseum* with the ranged from 9.6-27.5 mm [64]. Tamura *et al* (2009) searched for Rev-export inhibitor from *A. galanga* rhizome and founded ACA as a new inhibitor for HIV-1 viral which was similar to the report from Ying *et al* (2006) that ACA expressed blocking HIV type 1[65, 66].

In addition, antiulcer, antitumor, anticancer activities of *A. galanga* extract were examined. Mitsu *et al.* (1976) demonstrated the anti-Shay ulcer principles as ACA and 1′-acetoxyeugenol acetate and the dose of compounds at 5 mg·kg^{-1} displayed 77 and 100% inhibition, respectively [67]. Moreover, Lee and Hounghton (2005) presented that ACA which isolated from *A. galanga* of Thailand expressed cytotoxic efficiency and amount higher than that from Malaysia (against COR L23 cells, IC_{50} 7.8 μM against MCF7, IC_{50} 23.9 μM) [68].

For antiallergic activity, ACA and 1′-acetoxyeugenol acetate exhibited release of β-hexosaminidase with IC_{50} 15 and 19 μM, respectively (Matsuda *et al*., 2003b; Yasuhara *et al*., 2009). The anti-inflammatory from the rhizome of *A. galanga* was evaluated in acute and sub-acute in rat model and the extract at 1-5% w/w significant varied from control group [69, 70].

The antileishmanial activity of phenylpropanoids from *A. galanga* was demonstrated against *Leishmania donovani* and among 12 compounds, 1′-

acetoxyugenol acetate exhibited the highest activity with IC_{50} 18.9 μM [43]. The antifeedant activity of four Zingiberaceae plant extracts against *Athalia proxima* Klug was conducted and the results showed *A. galanga* weak effect among all tested plants [16].

For the essential oil of this plant, Chukanhom *et al.* (2005) reported the antifungal activity of the essential oil from galangal rhizome against Saprolegniaceae pathogen in goldenfish and platyfish [49]. According to the results, geranyl acetate, linalool and 1,8-cineole (500, 750 and 1,500 $\mu g \cdot mL^{-1}$, respectively) completely inhibited the zoospores germination.

According to the literature review, there were insufficient information of phytotoxicity and antiphytopathogens against *A. porri*, *C. gloeosporioides*, *F. oxysporum* and *P. parasitica*. Thus, this study aims to isolate the major bioactive constituents from Thai *A. galanga* rhizomes and to evaluate for their phytotoxic and antiphytopathogenic activities.

3.2 Plant materials and extraction

Fresh rhizomes were collected from Petchaburi province, Thailand in January 2011. The voucher specimen was submitted to Herbarium of the Royal Forest Department of Thailand. The rhizomes were cleaned and sliced into small pieces, and then dried. The dried galangal materials (4 kg) were extracted by maceration with CH_2Cl_2 for three times at RT. After that, the CH_2Cl_2 extract was filtered and concentrated to dryness with rotatory evaporator to obtain brown liquid with pungent-spicy odor (2.3% yield).

3.3 Separation of the CH_2Cl_2 extract of *A. galangal* rhizomes with bioassay guide

One hundred and fifty grams of the CH_2Cl_2 extract were separated by quick column chromatography using silica gel 60G Art.7729 as adsorbent. The column was initially eluted with hexane and gradually changed to a mixture of EtOAc and hexane-EtOAc. Approximately 1 L of solvent was collected for each fraction and then

concentrated by rotatory evaporator. The fractions were combined according to TLC results to obtain six fractions, **AGR1–AGR6**. The results of the separation are shown in **Table 3.1**.

Table 3.1 The separation of the CH_2Cl_2 extract from *A. galangal* rhizomes by quick column chromatography

Fraction No.	Solvent system (%v/ v)	Remarks	Weight (g)
AGR1	100% hexane	Orange oil	21.8
AGR2	5-10% EtOAc in hexane	Yellow oil	33.5
AGR3	20% EtOAc in hexane	Dark yellow oil	33.9
AGR4	30-40% EtOAc in hexane	Brown oil	35.4
AGR5	60-80% EtOAc in hexane	Brown solid	2.1
AGR6	100 % EtOAc	Dark brown solid	5.2

AGR1-4 were obtained in high yield as 21.8, 33.5, 33.9 and 35.4 g (14.5, 22.3, 22.6 and 23.6% yield), respectively, whereas **AGR5-6** gave only 2.1 and 5.2 g (1.4 and 3.5% yield), respectively. All six fractions and the essential oil were subjected to anti-*P. parasitica* test by agar incorporation method at the final concentration of 1,000 $\mu g \cdot mL^{-1}$ as activity guide to search for the active fractions (all tests were performed in triplicate as described in Chapter II). The activities of these fractions and the essential oil are shown in **Table 3.2** and **Figure 3.4**.

The results in **Table 3.2** and **Figure 3.4**, **AGR2** and **AGR3** displayed complete inhibition of the mycelial growth of *P. parasitica*, followed by **AGR4** and **AGR1** (75.5 and 5.0% inhibition). Whereas, **AGR5-6** did not inhibit the mycelial growth of the fungus. For the antifungal activity of the essential oil, moderate activity (55.6%) was observed. For further study, 250 mg of each fraction: **AGR2-4** were subjected to flash column chromatograph to isolate bioactive compounds.

Table 3.2 The percentage of mycelial growth inhibition of separated fractions and the essential oil

Fraction No.	Growth (mm)	Inhibition (%)*
AGR1	85.0±0.4	5.0±0.7
AGR2	0.0±0.0	100.0±0.0
AGR3	0.0±0.0	100.0±0.0
AGR4	22.0±1.0	75.5±1.1
AGR5	90.0±0.0	0.0±0.0
AGR6	90.0±0.0	0.0±0.0
Essential oil	40.0±1.0	55.6±1.1
DMSO	90.0±0.0	0.0±0.0

*Values, an average ± standard deviation (SD) of 3 replicates of the mean mycelial growth inhibition

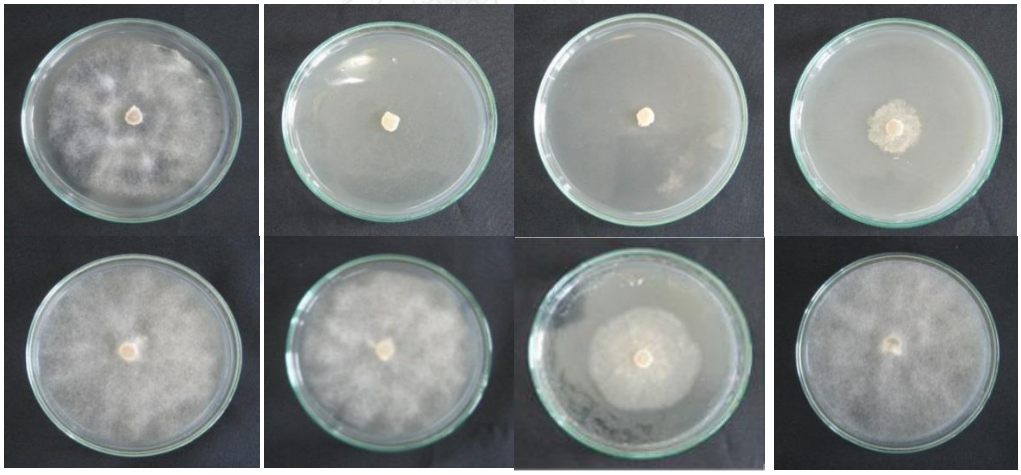


Figure 3.4 The mycelial growth inhibition zone against *P. parasitica* by separated fractions and the essential oil at the final concentration of 1,000 μg·mL^{-1}
Above (left to right): **AGR1**, **AGR2**, **AGR3**, **AGR4**
Below (left to right): **AGR5**, **AGR6**, Essential oil, DMSO

3.4 Isolation of bioactive compounds

For further study, each fraction (**AGR2**, **AGR3** and **AGR4**) was subjected to flash silica gel column (Isolera, Biotage®) using hexane and EtOAc gradient system (**Figure 3.5**, **Tables 3.3-3.5**). The collected portion was checked and combined according to TLC behavior. The isolated compounds were characterized using spectroscopic data.

The isolation of bioactive compounds from the CH_2Cl_2 extract of *A. galangal* rhizomes is summarized in **Scheme 3.1**.

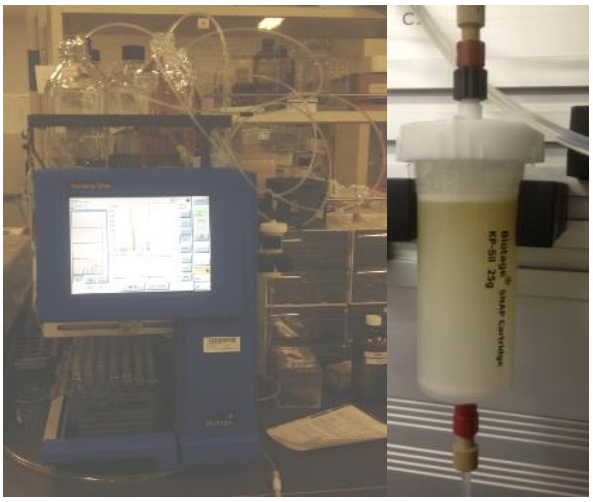
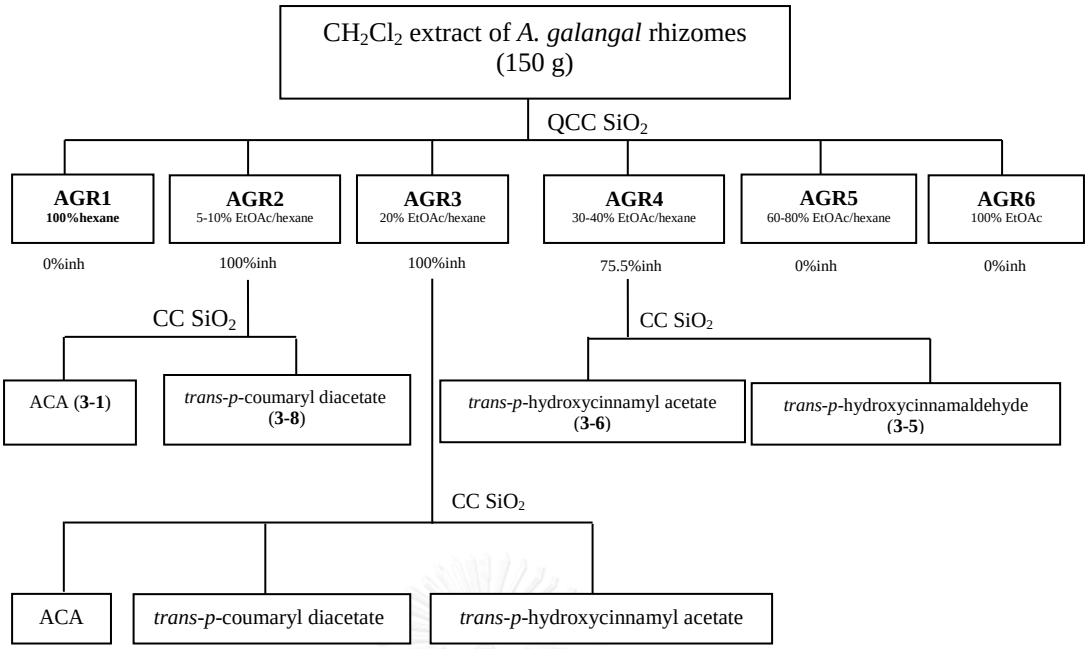


Figure 3.5 The machine for flash column chromatography and Biotage® SNAP Cartridge KP-Sil pre-pack column



Scheme 3.1 The separation of the CH_2Cl_2 extract of *A. galangal* rhizomes

Table 3.3 The separation of **AGR2** by flash column

Fractions	Solvent system (%v/v)	Remarks	Weight (mg)
AGR2/1	15-20% EtOAc in hexane	colorless oil (3-**1**)	127
AGR2/2	21-25% EtOAc in hexane	light yellow oil	84
AGR2/3	26-30% EtOAc in hexane	light yellow oil (**3-6**)	15

The separation of **AGR2** yielded three sub-fractions: **AGR2/1-2/3** (**Table 3.3**). Compound 3-**1** as colorless oil with pungent smell (127 mg, 50.8% yield based on **AGR2**) revealed a single spot on TLC with R_f 0.59 (developing system: EtOAc:hexane (1:1)). The molecular formula of this compound was proposed to be $C_{13}H_{14}O_4$, MW 234. The ^{1}H and ^{13}C NMR spectra of this compound are presented in **Figures 3.4-3.5.** The comparison of NMR spectroscopic data of the reported 1′-acetoxychavicol acetate (ACA) and compound 3-**1** is collected in **Table 3.4**.

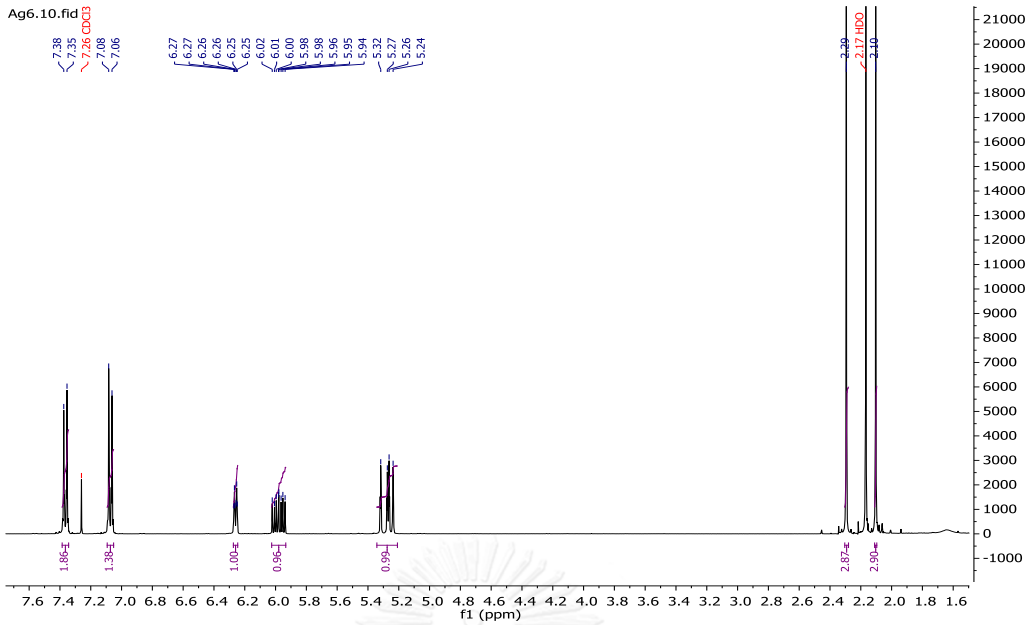


Figure 3.6 The ^{1}H NMR spectrum ($CDCl_3$) of compound **3-1**

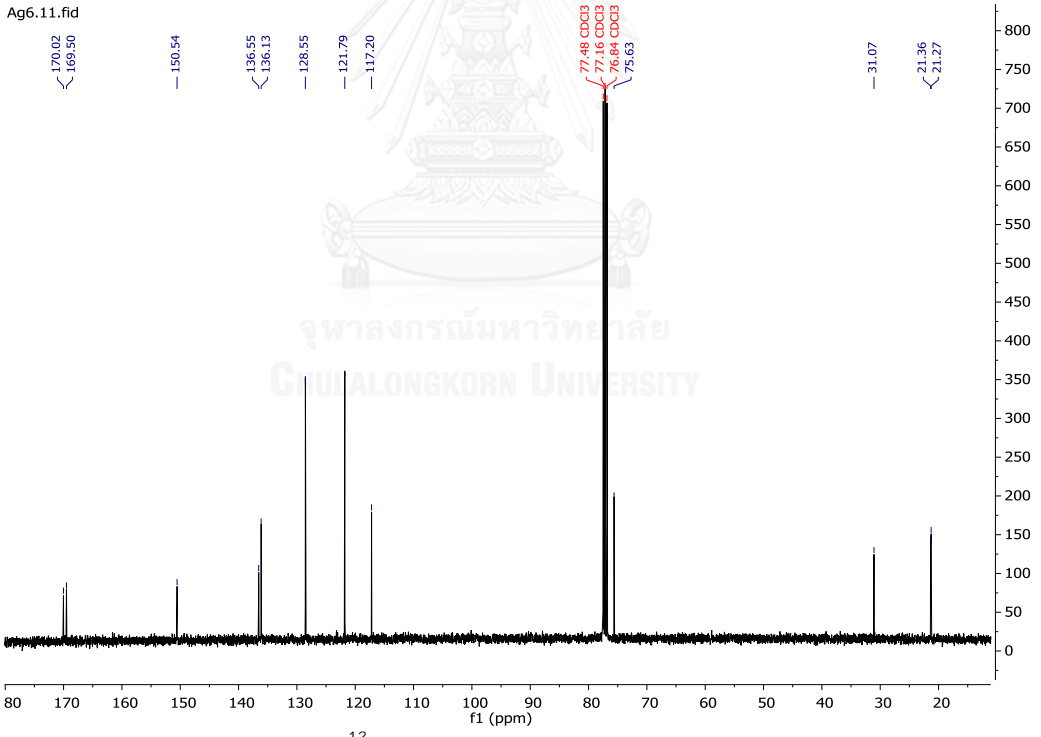


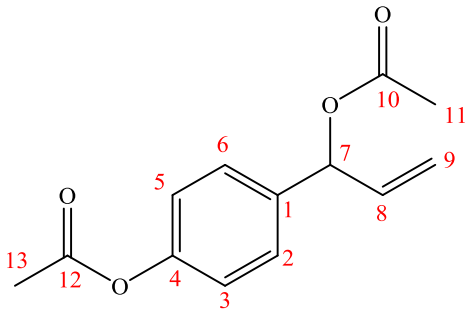
Figure 3.7 The ^{13}C NMR spectrum ($CDCl_3$) of compound **3-1**

Table 3.4 The comparison of ^{1}H and ^{13}C NMR spectral assignment of 1'-acetoxychavicol acetate (ACA) and compound **3-1**

Position	1'-acetoxychavicol acetate (ACA)*		Compound 3-1	
	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
1		150.6		150.5
2, 6	7.06 (*d*, *J* = 8.6 Hz, 1H)	121.6	7.07 (*d*, *J* = 8.6 Hz, 1H)	121.8
3, 5	7.36 (*d*, *J* = 8.6 Hz, 1H)	128.4	7.36 (*d*, *J* = 8.4 Hz, 2H)	128.6
4		136.5		136.6
1'	6.26 (*d*, *J* = 5.9 Hz, 1H)	75.7	6.26 (*d*, *J* = 5.9 Hz, 1H)	75.6
2'	5.99 (m, 1H)	136.2	5.98 (m, 1H)	136.1
3'a	5.27 (*d*, *J* = 16.3 Hz, 1H)	117.0	5.27 (*d*, *J* = 4.5 Hz, 1H)	117.2
3'b	5.23 (*d*, *J* = 9.6 Hz, 1H)	117.0	5.24 (*d*, *J* = 2.5 Hz, 1H)	117.2
$OCOCH_3$	2.09 (s, 3H)	21.1 (2)	2.10 (s, 3H)	21.3
	2.28 (s, 3H)	21.1 (2)	2.29 (s, 3H)	21.4
$OCOCH_3$		169.1		169.5
		169.7		170.0

*[71]

The optical rotation of compound **3-1** was $[\alpha]_D^{20}$ -57.2° (*c* 0.039, EtOH) [72]. GC/MS (DEI, 70eV) m/z (rel int, %): 234 (M^+, 7.2), 192 (50.7), 174 (9.0), 150 (48.6), 149 (28.1), 132 (100), 103 (15.6) and 77 (20.2). From the NMR, GC/MS data suggested that compound **3-1** be 1′-acetoxychavicol acetate (ACA).



1′-acetoxychavicol acetate (ACA) (**3-1**)

Compound **3-8** as light yellow oil (14.7 mg) was obtained from **AGR2/3** (26-30% EtOAc in hexane). It showed only a single spot on TLC with R_f 0.54 (developing

system: EtOAc: hexane (1:1)). The molecular formula was suggested as $C_{13}H_{14}O_4$, MW 234. The ^{1}H and ^{13}C NMR spectra are shown in **Figures 3.8-3.9**.

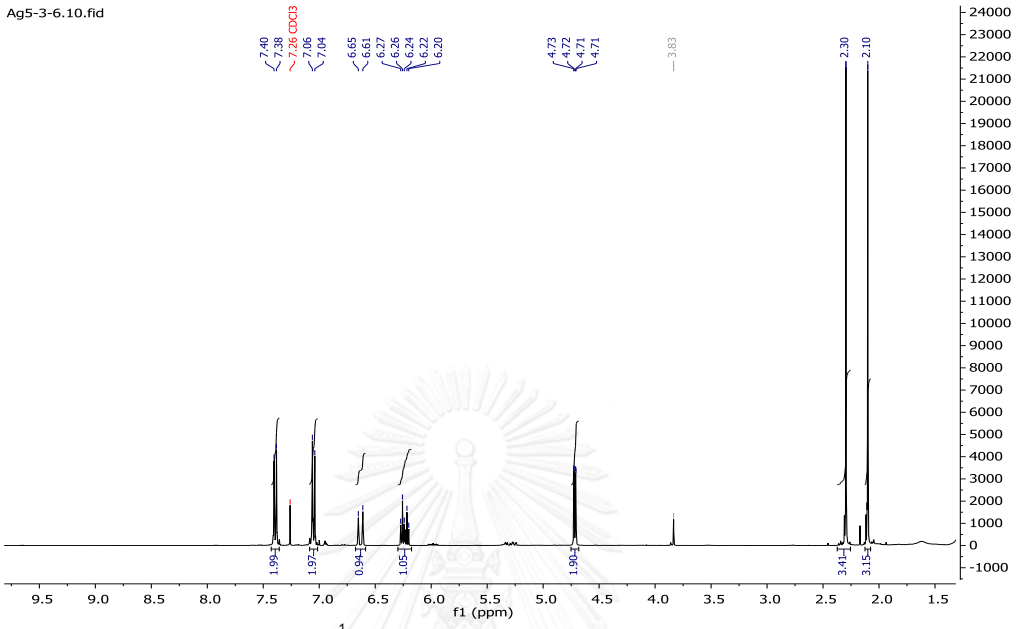


Figure 3.8 The ^{1}H NMR spectrum ($CDCl_3$) of compound **3-8**

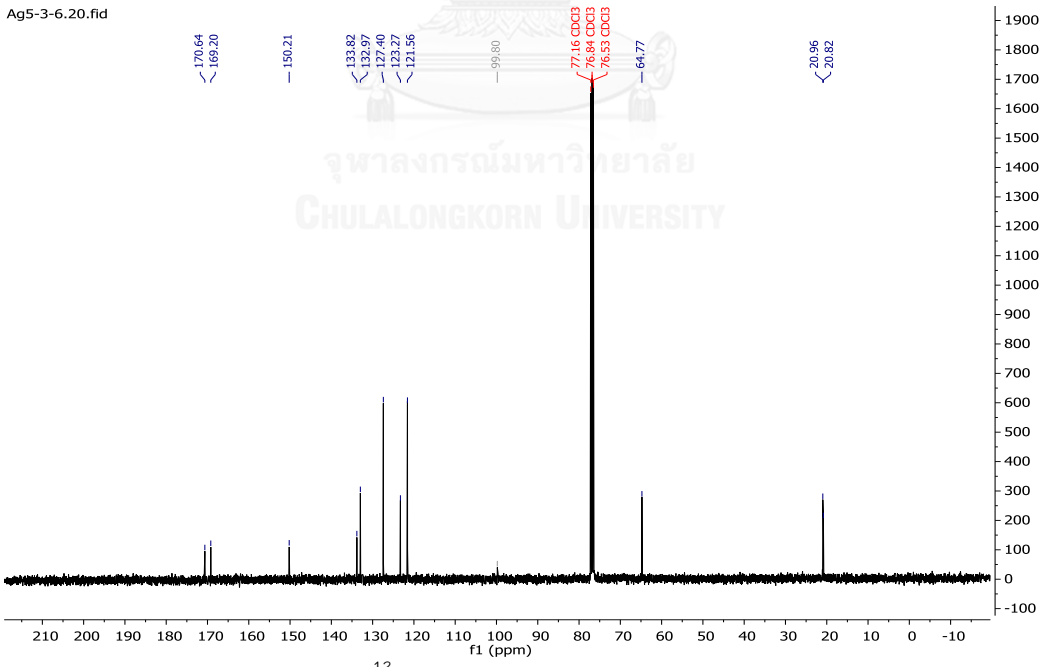


Figure 3.9 The ^{13}C NMR spectrum ($CDCl_3$) of compound **3-8**

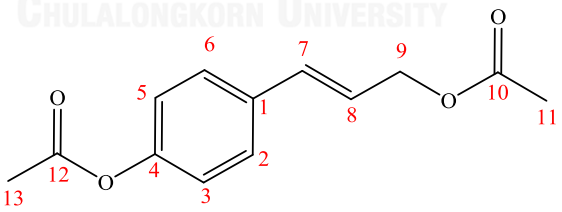
The comparison of NMR spectroscopic data of the reported *trans-p*-coumaryl diacetate and compound **3-8** is collected in **Table 3.5**.

Table 3.5 The comparison of ^{1}H and ^{13}C NMR spectral assignment of *trans-p*-coumaryl diacetate (CDA) and compound **3-8**

Position	*trans-p*-coumaryl diacetate (CDA)*		Compound 3-8	
	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
1		150.8		150.2
2, 6	7.07 (*d*, *J* = 8.5 Hz, 1H)	121.8	7.05 (*d*, *J* = 8.6 Hz, 1H)	121.6
3, 5	7.38 (*d*, *J* = 8.5 Hz, 1H)	127.7	7.39 (*d*, *J* = 8.6 Hz, 1H)	127.4
4		134.3		133.9
7	6.64 (*d*, *J* = 15.9 Hz, 1H)	133.3	6.63 (*d*, *J* = 15.9 Hz, 1H)	133.0
8	6.21 (*dt*, *J* = 15.9, 5.2 Hz, 1H)	123.8	6.24 (m, 1H)	123.3
9	4.71 (*d*, *J* = 5.2 Hz, 2H)	64.9	4.72 (*dd*, *J* = 6.5, 1.3 Hz, 2H)	64.8
10		169.0		169.2
11	2.09 (s, 3H)	20.9	2.10 (s, 3H)	20.8
12		170.6		170.6
13	2.28 (s, 3H)	21.0	2.30 (s, 3H)	21.0

*[71]

Based on the NMR data, compound **3-8** was identified as *trans-p*-coumaryl diacetate or *trans-p*-acetoxycinnamyl acetate.



trans-p-coumaryl diacetate (**3-8**)

The TLC of **AGR3** displayed at least 3 main spots. The separation of this fraction was conducted and the results are presented in **Table 3.6**.

Table 3.6 The separation of **AGR3** by flash column.

Fractions	Solvent system (%v/ v)	Remarks	Weight (mg)
AGR3/1	15-20% EtOAc in hexane	colorless oil (**3-1**)	90.2
AGR3/2	21-25% EtOAc in hexane	light yellow oil	40.3
AGR3/3	26-30% EtOAc in hexane	light yellow oil (**3-8**)	19.4
AGR3/4	31-35% EtOAc in hexane	light yellow oil	15.7
AGR3/5	36-40% EtOAc in hexane	colorless oil (**3-6**)	38.6

The separation of fractions **AGR3/1** and **AGR3/3** yielded ACA (**3-1**) and CDA (**3-8**). In addition, **AGR3/5** contained a different single spot of compound **3-6** as colorless oil 38.6 mg (15.4% yield based on **AGR3**) with R_f 0.49 (developing system: EtOAc: hexane (1:1)). The molecular formula was suggested as $C_{11}H_{12}O_3$, MW 192. The ^{1}H and ^{13}C NMR spectra are presented in **Figures 3.10-3.11**.

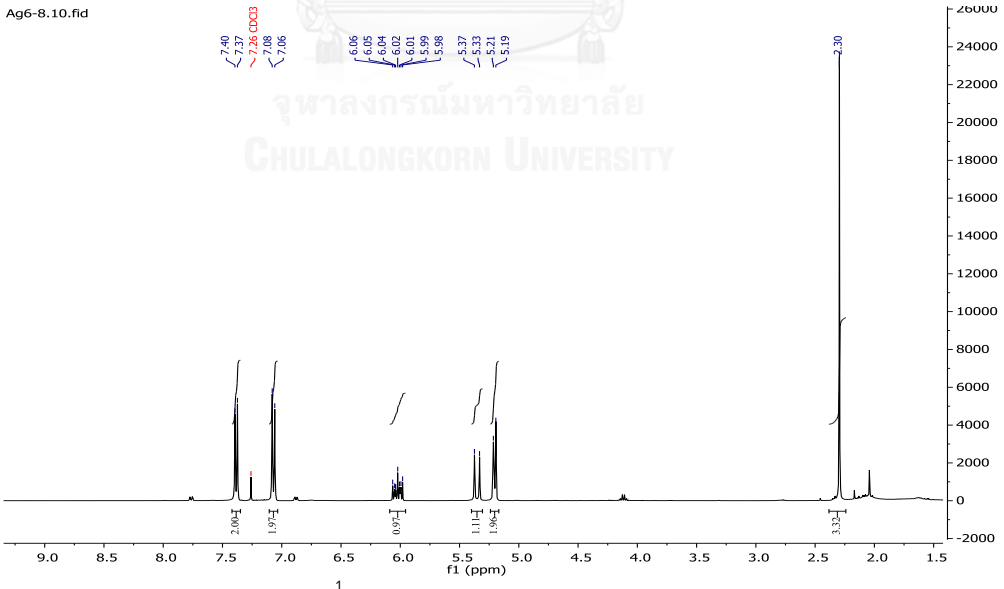


Figure 3.10 The ^{1}H NMR spectrum ($CDCl_3$) of compound **3-6**

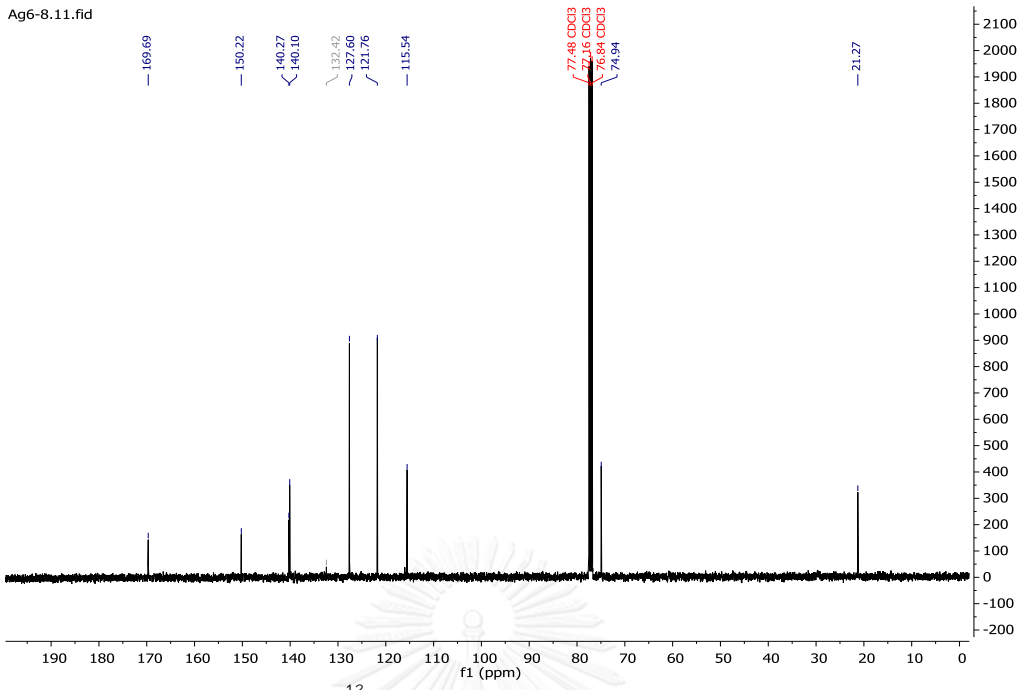
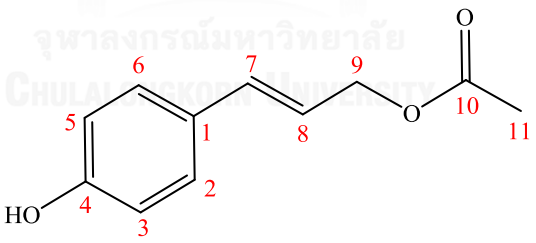


Figure 3.11 The ^{13}C NMR spectrum ($CDCl_3$) of compound **3-6**

The comparison of NMR spectroscopic data of the reported *trans-p*-hydroxy cinnamyl acetate and compound **3-6** is collected in **Table 3.7**. According to the NMR data, compound **3-6** was identified as *trans-p*-hydroxycinnamyl acetate or *trans-p*-coumaryl acetate.



trans-p-hydroxycinnamyl acetate (**3-6**)

Table 3.7 The comparison of ^{1}H and ^{13}C NMR spectral assignment of *trans-p*-hydroxycinnamyl acetate and compound **3-6**

Position	*trans-p*-hydroxycinnamyl acetate*		Compound 3-6	
	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
1		155.9		150.2
2, 6	6.80 (*d*, *J* = 8.9 Hz, 2H)	115.5	7.07 (*d*, *J* = 8.6 Hz, 2H)	115.5
3, 5	7.25 (*d*, *J* = 8.9 Hz, 2H)	128.1	7.38 (*d*, *J* = 8.6 Hz, 2H)	140.1
4		128.7		140.3
7	6.58 (br *d*, *J* = 15.9 Hz, 1H)	134.3	6.02 (br *d*, *J* = 16.5 Hz, 1H)	127.6
8	6.12 (*dt*, *J* = 15.9, 6.7 Hz, 1H)	120.4	5.35 (*d*, *J* = 17.1 Hz, 1H)	121.7
9	4.71 (*dd*, *J* = 6.7, 1.3 Hz, 2H)	65.6	5.15 (*dd*, *J* = 6.7, 1.3 Hz, 2H)	74.9
10		171.6		169.7
11	2.11 (*s*, 3H)	21.1	2.30 (*s*, 3H)	21.3
OH	5.98 (br *s*, 1H)		5.20 (d, *J* = 8.3 Hz, 1H)	

*[73]

Further investigation on the other active fraction was conducted. The separation of **AGR4** furnished 3 fractions as presented in **Table 3.8**.

Table 3.8 The separation of **AGR4** by flash column

Fractions	Solvent system (%v/ v)	Remarks	Weight (mg)
AGR4/1	36-40% EtOAc in hexane	yellow oil (cpd **3-6**)	76.8
AGR4/2	41-45% EtOAc in hexane	dark yellow oil	88.7
AGR4/3	46-50% EtOAc in hexane	yellow semi-solid (cpd **3-5**)	59.7

By comparing with TLC and ^{1}H NMR sprectrum, the compound containing in **AGR4/1** was identical with compound **3**. Compound **3-5** (59.7 mg, 23.8% yield based on **AGR4**) from **AGR4/3** with cinnamon flavor was attained as yellow semi-solid. It showed a single spot on TLC with R_f 0.35 (developing system: EtOAc:hexane (1:1)). The molecular formula was suggested as $C_9H_8O_2$, MW 148. **Figures 3.12-3.13** show the ^{1}H and ^{13}C NMR spectra of compound **3-5**.

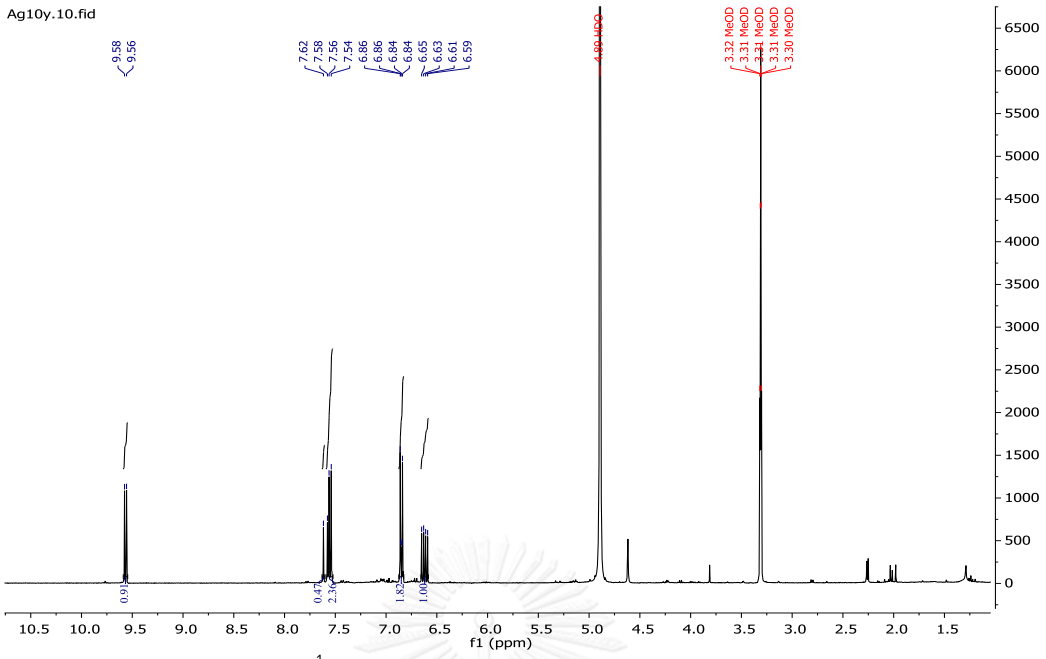


Figure 3.12 The ^{1}H NMR spectrum (methanol-d_6) of compound **3-5**

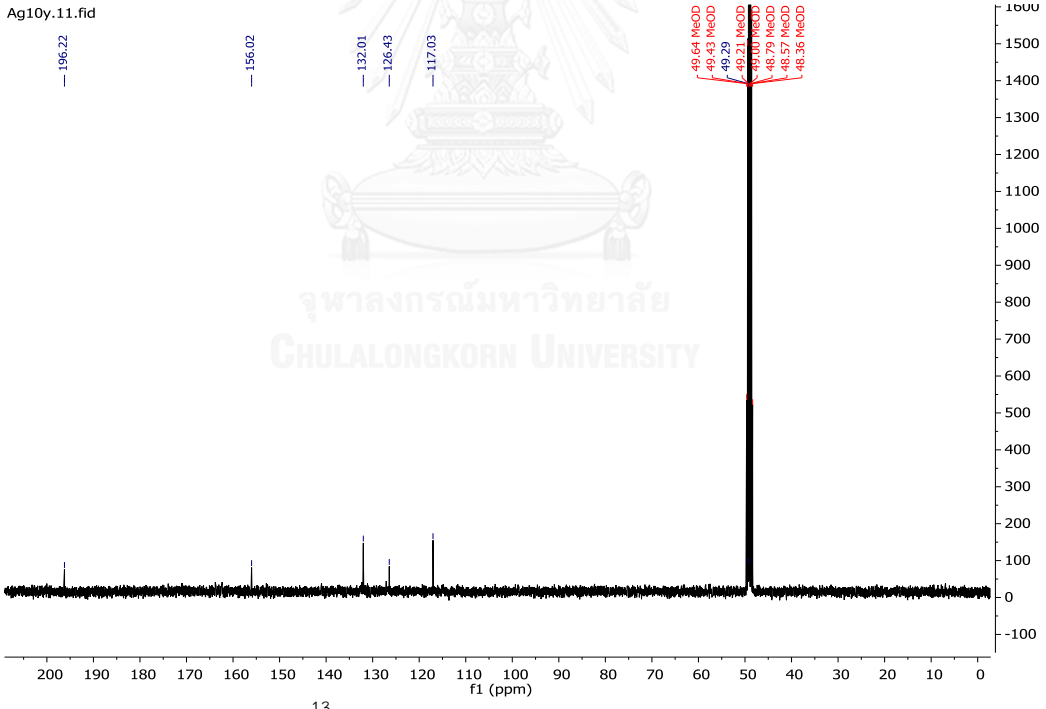


Figure 3.13 The ^{13}C NMR spectrum (methanol-d_4) of compound **3-5**

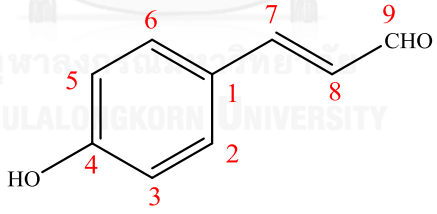
The comparison of NMR spectroscopic data of the reported *trans-p*-hydroxycinnamaldehyde and compound **3-5** is collected in **Table 3.9**.

Table 3.9 The comparison of ^{1}H and ^{13}C NMR spectral assignment of *trans-p*-hydroxycinnamaldehyde and compound **3-5**

Position	trans-p-hydroxycinnamaldehyde*		Compound 3-5	
	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
1		158.2		156.0
2, 6	7.7 (*d*, *J* = 8.7 Hz, 2H)	132.8	7.6 (*m*, 2H)	132.0
3, 5	6.9 (*d*, *J* = 8.6 Hz, 2H)	117.6	6.9 (*m*, 2H)	117.0
4		161.1		162.3
7	7.7 (*d*, *J* = 15.6 Hz, 1H)	158.2	7.6 (*s*, 1H)	156.0
8	6.7 (*d*, *J* = 15.7 Hz, 1H)	126.1	6.6 (*dd*, *J* = 15.7, 7.9 Hz, 1H)	126.4
9	9.5 (*d*, *J* = 8.2 Hz, 1H)	199.5	9.6 (*d*, *J* = 7.9 Hz, 1H)	196.2

*[74]

By comparing the NMR data with previously reported literature, compound **3-5** was identified as *trans-p*-hydroxycinnamaldehyde.



trans-p-hydroxycinnamaldehyde (**3-5**)

The structures of all bioactive compounds isolated from *A. galanga* rhizomes are displayed in **Figure 3.14**.

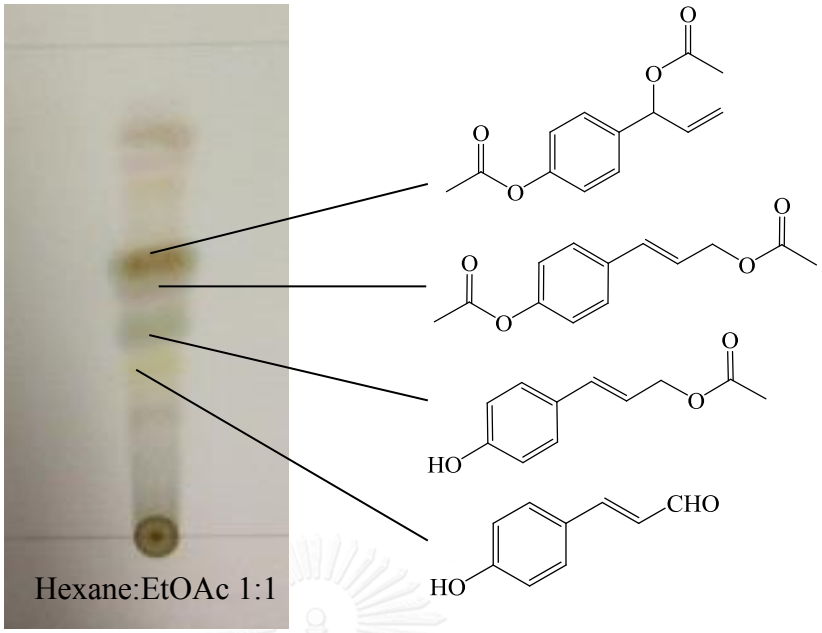


Figure 3.14 TLC of bioactive compounds from the CH_2Cl_2 extract of *A. galanga* rhizomes

Hexane:EtOAc 1:1

3.5.1 Solvolysis of ACA

The solvolysis of ACA was conducted following the procedure modified by Yang *et al.* (1999). ACA (550 mg) was dissolved in 2%EtOH-water, refluxed and stirred at 60°C; 2 h during reflux the reaction was checked by TLC at 30, 60, 90 and 120 mins. The reaction was quenched with water (30 mL). The reaction mixture was extracted with Et_2O 4-5 times to obtain 2 parts: Et_2O (308.4 mg, 56.1% yield) and water parts. The first part (Et_2O) was dried over anh Na_2SO_4. The second part (water) was adjusted the pH by 0.1 N HCl to pH 9, after that extracted with EtOAc 4-5 times to obtain EtOAc part (221.5 mg, 40.3% yield) and the water part was discarded. Both Et_2O and EtOAc parts were separated by flash column using 15%EtOAc in hexane to 100%EtOAc as eluents (**Tables 3.10 and 3.12**).

Table 3.10 The separation of Et_2O part from ACA solvolysis

Fraction	Solvent system (%v/ v)	Remarks	Weight (mg)
1	15-20% EtOAc in hexane	colorless oil (cpd 3-**1**)	127.9
2	21-25% EtOAc in hexane	light yellow oil (cpd 3-8)	32.6
3	26-30% EtOAc in hexane	yellow oil (cpd **3-24**)	48.8
4	31-35% EtOAc in hexane	dark yellow oil	22.5
5	36-40% EtOAc in hexane	dark brown oil	26.6

Using TLC, the compounds from fractions 1 and 2 could be identified as compounds **3-1** and **3-8**. Compound **3-24** as yellow oil showing a single spot on TLC with R_f 0.55 (developing system: EtOAc:hexane (1:1)) was obtained from fraction 3 (48.8 mg). **Figures 3.15-3.16** show the ^{1}H and ^{13}C NMR spectra of compound **5**.

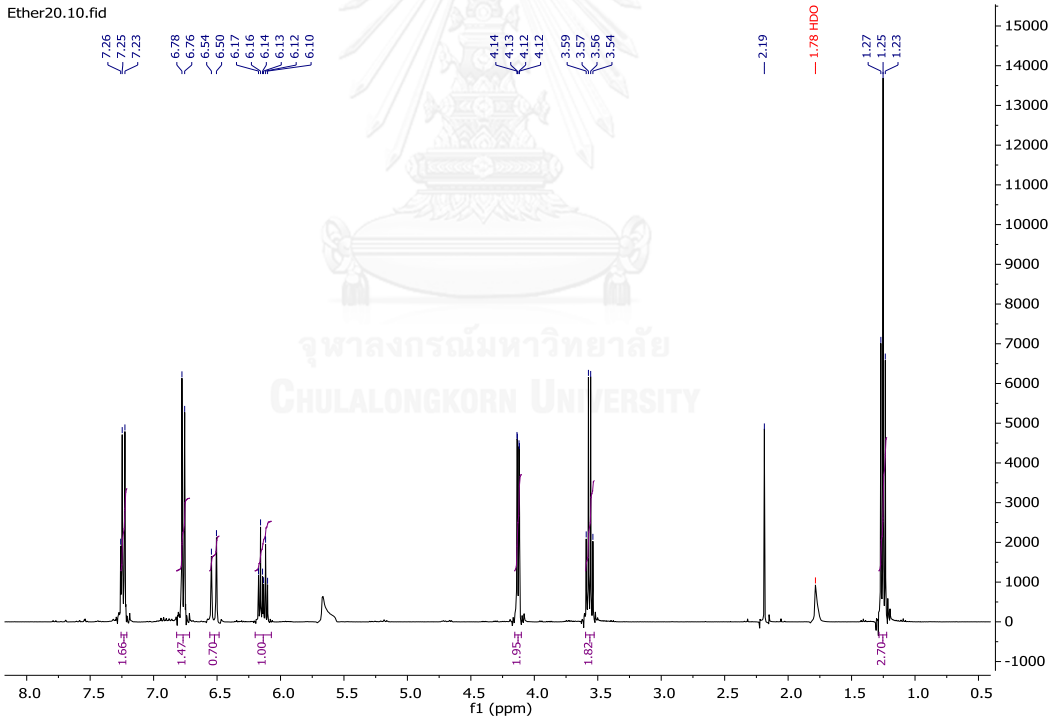


Figure 3.15 The ^{1}H NMR spectrum ($CDCl_3$) of compound **3-24**

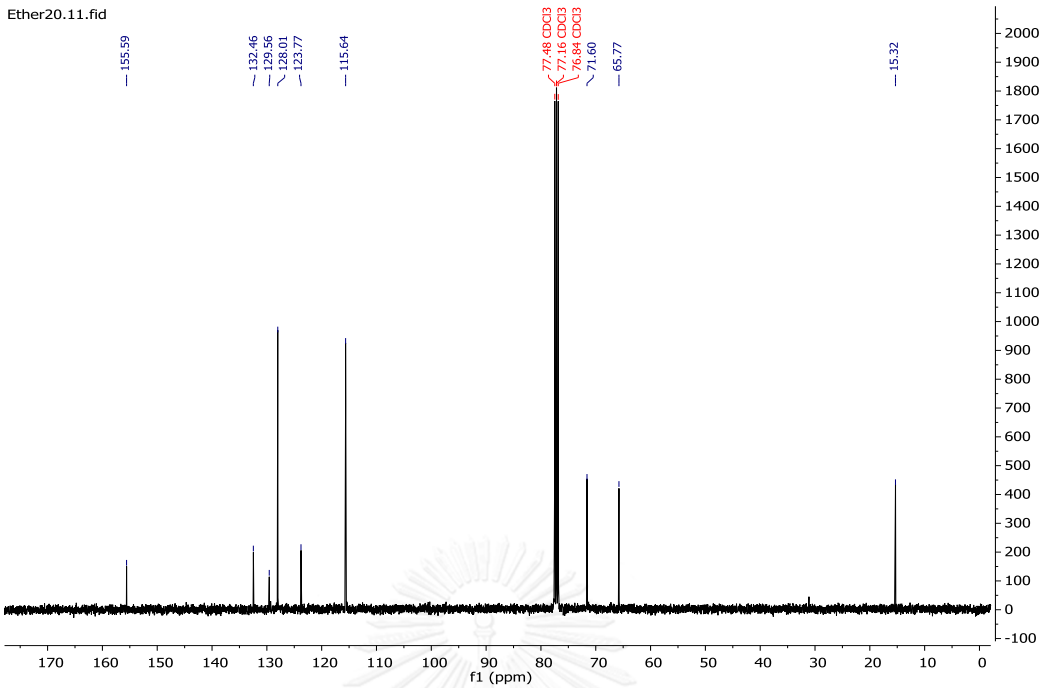


Figure 3.16 The ^{13}C NMR spectrum ($CDCl_3$) of compound **3-24**

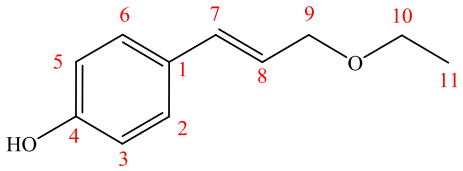
The comparison of NMR data of the reported *trans-p*-coumaryl alcohol ethyl ether and compound **3-24** is collected in **Table 3.11**.

Table 3.11 The comparison of ^{1}H and ^{13}C NMR spectral assignment of coumaryl alcohol ethyl ether and compound **3-24**

Position	*trans-p*-coumaryl alcohol ethyl ether		Compound 3-24	
	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
1		129.2		129.7
2, 6	7.23 (*d*, *J* = 8.4 Hz, 2H)	127.8	7.24 (*d*, *J* = 8.6 Hz, 2H)	128.0
3, 5	6.76 (*d*, *J* = 8.8 Hz, 2H)	115.5	6.77 (*d*, *J* = 8.6 Hz, 2H)	115.7
4		155.7		155.6
7	6.52 (*d*, *J* = 16.0 Hz, 1H)	132.5	6.52 (*d*, *J* = 15.9 Hz, 1H)	132.5
8	6.13 (*dt*, *J* = 16.0, 6.4 Hz, 1H)	123.4	6.14 (*dt*, *J* = 15.8, 6.4 Hz, 1H)	123.8
9	4.13 (*dd*, *J* = 6.4, 1.2 Hz, 2H)	71.4	4.13 (*dd*, *J* = 6.3, 1.4 Hz, 2H)	71.6
10	3.56 (q, *J* = 7.2 Hz, 2H)	65.6	3.56 (q, *J* = 7.0 Hz, 2H)	65.8
11	1.25 (*t*, *J* = 7.2 Hz, 3H)	15.1	1.25 (*t*, *J* = 7.0 Hz, 3H)	15.3

*[75]

According to the NMR data, compound **3-24** was identified as *trans-p*-coumaryl alcohol ethyl ether.



trans-p-coumaryl alcohol ethyl ether

Table 3.12 The separation of EtOAc part from ACA solvolysis

Fraction	Solvent system (%v/ v)	Remarks	Weight (mg)
1	15-20% EtOAc in hexane	colorless oil (cpd **3-1**)	112.5
2	21-25% EtOAc in hexane	light yellow oil (cpd **3-8**)	15.3
3	26-30% EtOAc in hexane	light yellow oil (cpd **3-6**)	24.7
4	36-40% EtOAc in hexane	white crystal (cpd **3-17**)	52.2

Compound **3-17** (9.5% yield based on ACA) as white crystal showed a single spot on TLC with R_f 0.36 (developing system: EtOAc:hexane (1:1)). **Figures 3.17-3.18** present the ^{1}H and ^{13}C NMR spectra of compound **3-17**.

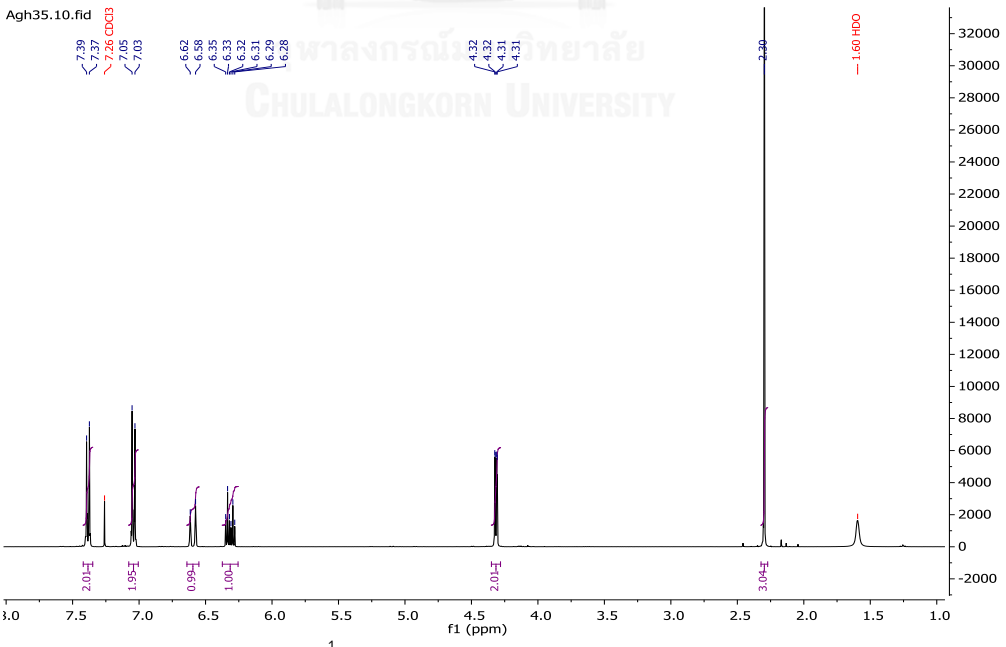


Figure 3.17 The ^{1}H NMR spectrum ($CDCl_3$) of compound **3-17**

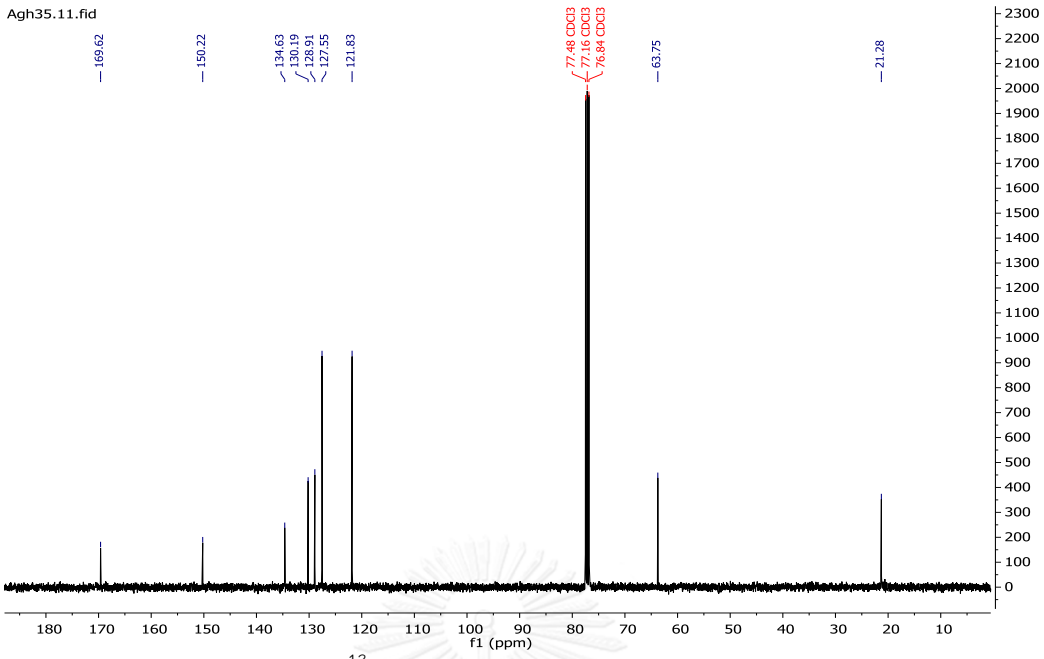


Figure 3.18 The ^{13}C NMR spectrum ($CDCl_3$) of compound **3-17**

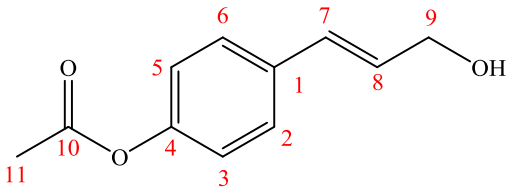
The comparison of NMR data of the reported *trans-p*-acetoxycinnamyl alcohol and compound **3-17** is collected in **Table 3.13**.

Table 3.13 The comparison of ^{1}H and ^{13}C NMR spectral assignment of *trans-p*-acetoxy cinnamyl alcohol and compound **3-17**

Position	*trans-p*-acetoxycinnamyl alcohol		Compound 3-17	
	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
1		134.5		134.6
2, 6	7.35 (*d*, *J* = 8.4 Hz, 2H)	127.4	7.38 (*d*, *J* = 8.5 Hz, 2H)	127.6
3, 5	7.02 (*d*, *J* = 8.4 Hz, 2H)	121.6	7.04 (*d*, *J* = 8.6 Hz, 2H)	121.8
4		150.0		150.2
7	6.57 (*d*, *J* = 16.0 Hz, 1H)	130	6.60 (*d*, *J* = 15.9 Hz, 1H)	130.2
8	6.28 (*dt*, *J* = 16.0, 5.6 Hz, 1H)	128.8	6.31 (*dt*, *J* = 15.9, 5.7 Hz, 1H)	128.9
9	4.29 (*dd*, *J* = 5.6, 1.6 Hz, 2H)	63.5	4.32 (*dd*, *J* = 5.7, 1.5 Hz, 2H)	63.8
10		169.4		169.6
11	2.27 (*s*, 3H)	21.1	2.30 (*s*, 3H)	21.3

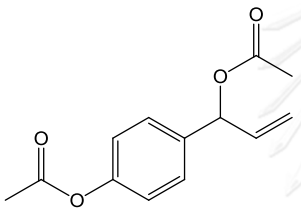
*[75]

According to the NMR data, compound **3-17** was identified as *trans-p*-acetoxycinnamyl alcohol.

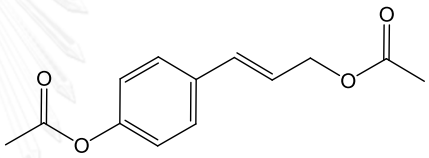


trans-p-acetoxycinnamyl alcohol (**3-17**)

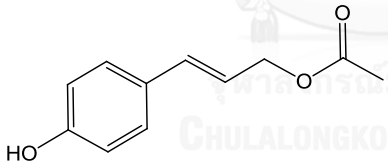
Moreover, ACA was further derivatized yielding *trans-p*-coumaryl alcohol ethyl ether (**3-24**) and *trans-p*-acetoxycinnamyl alcohol (**3-17**). The structures of isolated compounds from *A. galanga* rhizome and solvolysis of ACA are displayed in **Figure 3.19**.



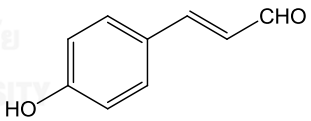
1´-acetoxychavicol acetate (**3-1**)



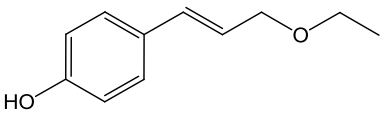
trans-p-coumaryl diacetate (**3-8**)



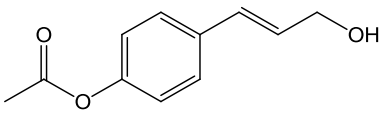
trans-p-hydroxycinnamyl acetate (**3-6**)



trans-p-hydroxycinnamaldehyde (**3-5**)



trans-p-coumaryl alcohol ethyl ether (**3-24**)



trans-p-acetoxycinnamyl alcohol (**3-17**)

Figure 3.19 Chemical constituents isolated from Thai *A. galanga* rhizomes (**3-1, 3-5, 3-6** and **3-8**) and derivatives (**3-24** and **3-17**).

ACA, the main bioactive compound isolated from the rhizomes of *A. galangal* revealed phytotoxicity and antiphytopathogenic fungal activities. The amount of ACA

was achieved in high level (more than 40 mg·g^{-1} dry weight) in old galangal rhizome from Petchaburi province [76]. This attained amount was higher than that obtained from other reports [68]. Nonetheless, no ACA was reported as a constituent in the essential oil or water extract because it was not stable in aqueous solution and hydrolyzed to other compounds [77].

3.5.2 Chemical compositions of the essential oil from the fresh rhizomes of A. galanga

According to aforementioned result, the essential oil exhibited good antifungal activity (55.6% inhibition). The further investigation was conducted by hydro-distillation of fresh rhizomes (2.7 kg) using Clevenger apparatus to gain 6.1 g (0.22% yield of fresh weight) as yellow and specific odor oil. The chemical compositions of the essential oil were analyzed by GC-MS as presented in **Figure 3.20** and **Table 3.12.**

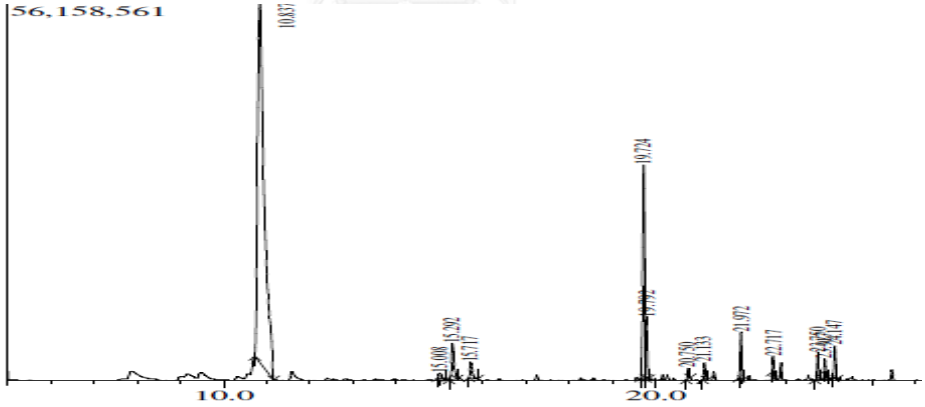


Figure 3.20 GC-MS chromatogram of the essential oil from *A. galanga* rhizomes

Table 3.14 Chemical compositions of the essential oil from fresh rhizomes of *A. galanga*

No.	R_t (min)	compounds	%composition
1	10.84	1,8-cineole	72.45
2	15.01	myrcenol	0.43
3	15.29	terpinen-4-ol	2.88
4	15.72	α–terpineol	1.24
5	19.72	2-acetoxy-1,8-cineol	12.98
6	19.79	4-allylphenyl acetate	2.57
7	20.75	geranyl acetate	0.49
8	21.13	β-elemene	0.83
9	21.97	β-caryophyllene	2.30
10	22.72	β-farnesene	0.58
11	23.75	β-selinene	1.38
12	23.93	α -selinene	0.44
13	24.15	α-bisabolene	1.42

The main composition was identified as 1,8-ceneole or eucalyptol (72.45%). Other compositions included 2-acetoxy-1,8-cineol (12.98%), terpinen-4-ol (2.88%), 4-allylphenyl acetate (2.57%), caryophyllene (2.30%), α-bisabolene (1.42%), β-selinene (1.38%), α–terpineol (1.24%), β-elemene (0.83%), β-farnesene (0.58%), geranyl acetate (0.49%), α-bisabolene (0.44%), and myrcenol (0.43%).

The biological active compound production in plants is depended on several factors: both of genetic, environment and the interactions between gene and environment [78]. The plantation areas and the environmental factors such as weather, soil, soil fertility, light, temperature, humidity and pH are also important influence [79]. The major composition of the essential oil from rhizome of *A. galanga* in the northern part of Thailand displayed the concentration of 1,8-ceneole 21.6% which less than from central part of Thailand especially in Cha-Am district, Petchaburi province that we studied. Because the samples that we used are growing

in different region which have many factor influence to chemical composition in tested plants [80]. In addition, the content of chemical concentration also depends on the age of study plant.

Moreover, compared with other reports on the major constituent and the content of 1,8-ceneole from *A. galanga* cultivated in different area of the world. Similarity of this research, the essential oil of this plant were analyzed by GC and GC-MS, the major compound as 1,8-ceneole as 58.5% [81], 40.5% [82] in Malaysia and 63.4% in north east India [83], 33.0 and 30.2% in Bangalor and Hyderabad India, respectively [84]. Together with 1,8-ceneole has been reported in many plants and also in various species of *Alpinia* (*A. galanga*, *A. officinearum*, *A. malaccensis* and *A. conchigera*) showed the different concentration [85]. However, zerumbone (44.8%) was first reported as a major compound in *A. galanga* essential oil in Sri Lanka and the content of 1,8-ceneole (6.3%) [86]. On the other hand, some research reported limonene as the composition in essential oil of *A. galanga*, however some report doesn't show limonene peak. Similarity, in this studies limonene peak didn't show as composition in *A. galanga*. Moreover, the chemical constituent of the oil depend on the method of preparation, parts and age of plant [81].

3.5.3 Antiphytopathogenic fungi activity of the essential oil and major compounds from *A. galanga* rhizomes

The essential oil and major compounds, 1,8-cineole commercial (Fluka) and terpinen-4-ol (isolated from *Z. cassumunar* essential oil) were evaluated for their antifungal activity against four phytopathogenic fungi using agar incorporation method at various concentrations (1, 10, 100, 250, 500 and 1000 $\mu g \cdot mL^{-1}$). The results are demonstrated as shown in **Tables 3.13-15** and **Figures 3.21-23**.

Table 3.15 The percent inhibition and IC_{50} values of the essential oil of *A. galanga* against phytopathogenic fungi

Conc.*	*A. porri*		*C. gloeosporioides*		*F. oxysporum*		*P. parasitica*	
	growth (mm)	%inh	growth (mm)	%inh	growth (mm)	%inh	growth (mm)	%inh
control	78.0±2.6	0.0	84.3±0.6	0.0	90.0±0.0	0.0	90.0±0.0	0.0
1	77.0±1.7	1.3	84.3±0.6	0.0	90.0±0.0	0.0	90.0±0.0	0.0
10	73.3±2.9	6.0	83.3±0.6	1.1	88.3±1.5	1.9	90.0±0.0	0.0
100	70.3±1.5	9.8	79.0±1.0	6.3	86.3±0.6	4.1	85.0±0.0	5.6
250	69.3±1.2	11.1	78.0±0.0	7.5	81.0±3.6	10	82.3±2.1	8.5
500	68.3±2.5	13.7	71.3±1.5	15.4	77.7±2.5	13.7	69.7±1.5	22.6
1000	60.0±3.6	23.1	61.3±0.6	27.2	58.0±6.1	35.6	40.3±0.6	55.2
IC_{50}[a]	>1000		>1000		>1000		840	

*concentration (μg·mL^{-1}), [a]Inhibition percentage of each concentration was calculated as IC_{50} using graphically

Table 3.16 The percent inhibition and IC_{50} values of the 1,8- cineole against phytopathogenic fungi

conc.*	*A. porri*		*C. gloeosporioides*		*F. oxysporum*		*P. parasitica*	
	growth (mm)	%inh	growth (mm)	%inh	growth (mm)	%inh	growth (mm)	%inh
control	78.3±0.6	0	90.0±0.0	0	80.3±0.6	0	90.0±0.0	0
1	77.7±0.6	0.8	90.0±0.0	0	80.0±0.0	0.4	85.0±1.0	5.6
10	76.7±0.6	2.1	90.0±0.0	0	72.7±2.5	9.5	84.0±1.0	6.7
100	79.3±0.6	2.4	90.0±0.0	0	78.7±2.3	12.6	82.7±2.5	8.1
250	78.7±0.6	3.2	87.7±0.6	2.6	77.7±2.5	13.7	73.7±0.6	18.1
500	73.7±1.2	5.9	79.3±1.2	3.6	75.0±0.0	16.7	69.7±0.6	22.6
1000	71.0±0.0	9.3	85.7±1.2	4.8	62.7±1.2	22.0	66.3±2.3	26.3
IC_{50}[a]	>1000		>1000		>1000		>1000	

*concentration (μg·mL^{-1}), [a]Inhibition percentage of each concentration was calculated as IC_{50} using graphically

Table 3.17 The percent inhibition and IC_{50} values of terpinen-4-ol against phytopathogenic fungi

conc.*	*A. porri*		*C. gloeosporioides*		*F. oxysporum*		*P. parasitica*	
	growth (mm)	%inh	growth (mm)	%inh	growth (mm)	%inh	growth (mm)	%inh
control	78.3±0.6	0	90.0±0.0	0.0	89.0±0.0	0	90.0±0.0	0.0
1	75.3±2.9	3.6	90.0±0.0	0.0	88.0±0.0	1.1	90.0±0.0	0.0
10	74.0±1.7	5.1	88.7±1.5	1.5	84.0±3.6	5.6	90.0±0.0	0.0
100	73.7±1.2	5.9	87.3±0.6	3.3	70.0±0.0	21.3	90.0±0.1	0.0
250	73.3±0.6	6.3	75.7±1.2	8.1	65.3±0.6	27.4	79.3±1.5	11.9
500	68.0±0.0	13.2	63.7±3.2	22.6	57.7±2.5	35.9	69.7±0.6	22.6
1000	34.7±2.5	55.7	64.7±3.5	42.2	24.3±4.6	72.7	29.3±0.6	67.4
IC_{50} [a]	925		>1000		675		755	

*concentration ($\mu g \cdot mL^{-1}$), [a]Inhibition percentage of each concentration was calculated as IC_{50} using graphically

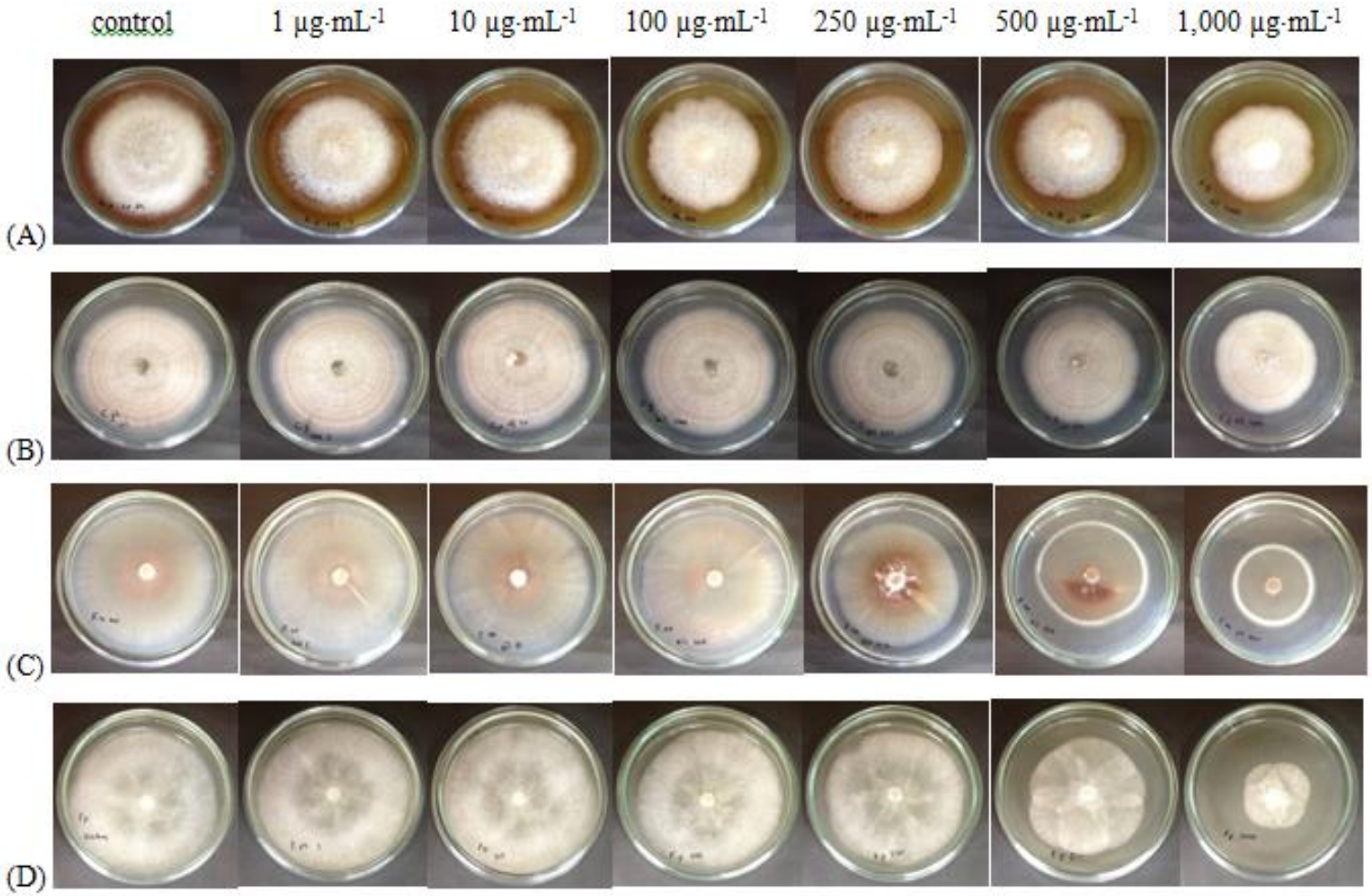


Figure 3.21 Antifungal activity of essential oil of *A. galanga* against mycelial growth of phytopathogenic fungi (A): *A. porri*, (B): *C. gloeosporioides*, (C): *F. oxysporum*, (D): *P. parasitica*

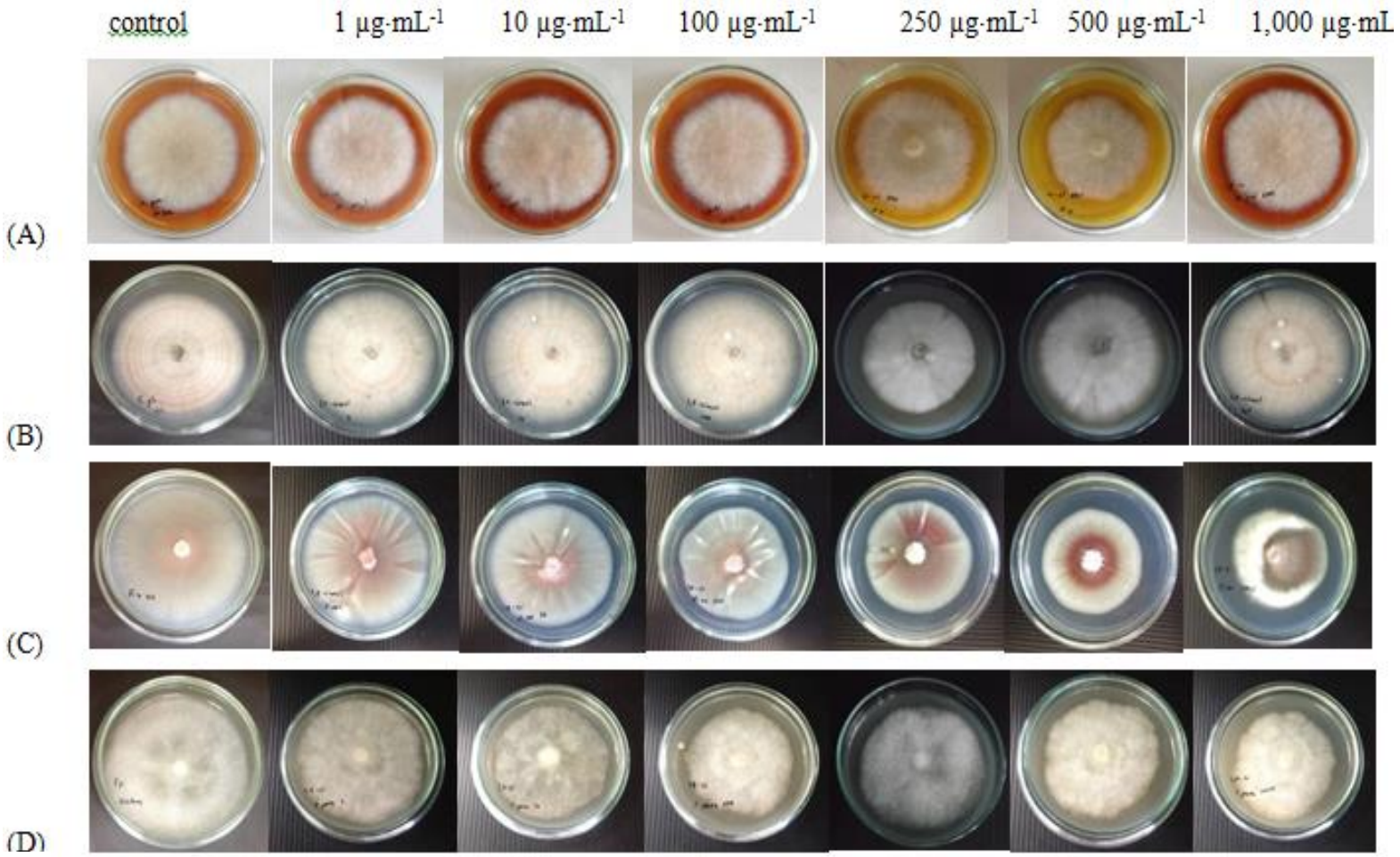


Figure 3.22 Antifungal activity of 1,8-cineol of *A. galanga* against mycelial growth of phytopathogenic fungi (A): *A. porri*, (B): *C. gloeosporioides*, (C): *F. oxysporum*, (D): *P. parasitica*

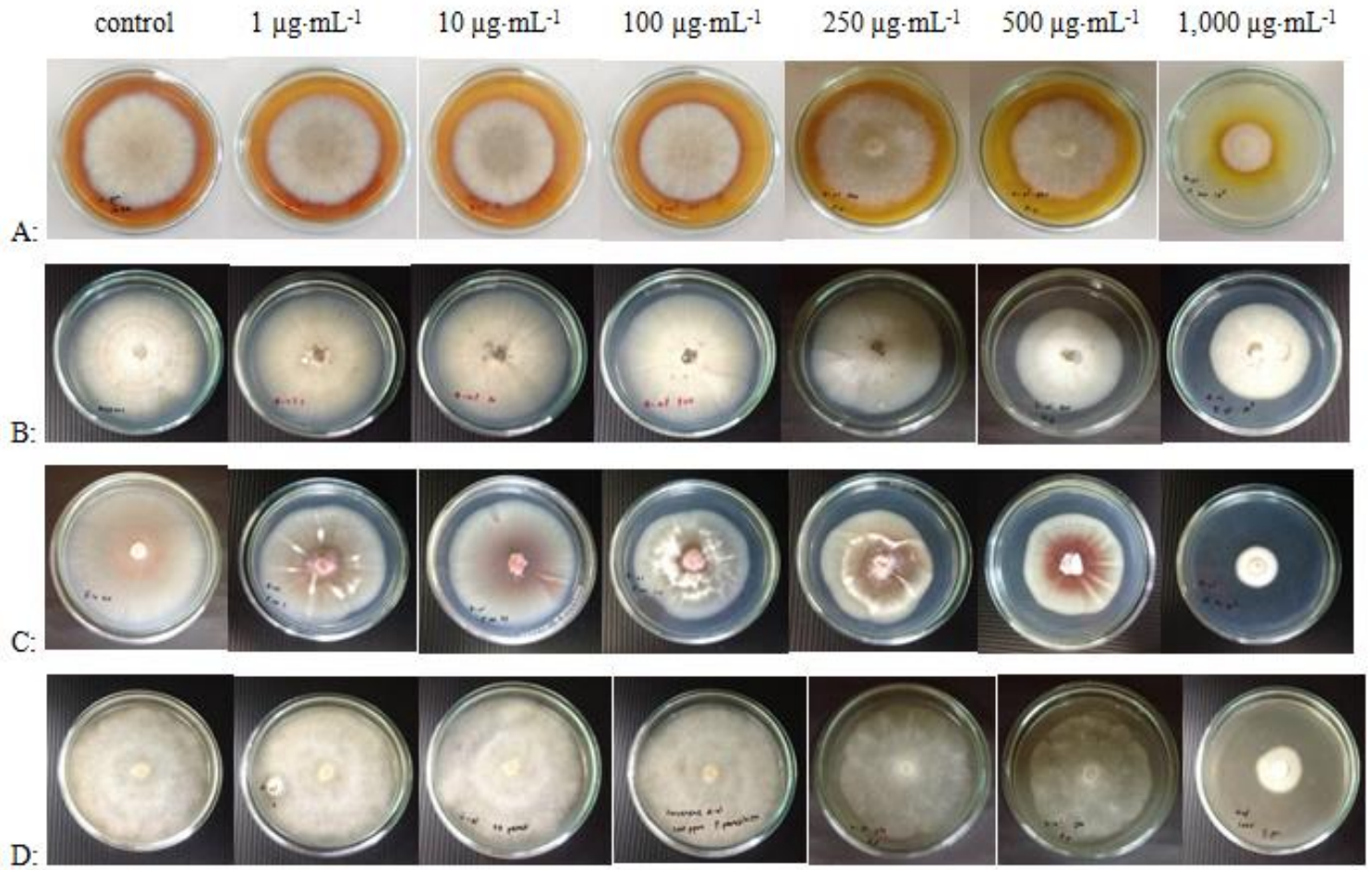


Figure 3.23 Antifungal activity of terpinene 4-ol of *A. galanga* against mycelial growth of phytopathogenic fungi (A): *A. porri*, (B): *C. gloeosporioides*, (C): *F. oxysporum*, (D): *P. parasitica*

The antifungal activity of essential oil and pure compounds including and terpinen-4-ol were showed in **Tables 3.13-15** and **Figures 3.19-21**. According to the results, terpinen-4-ol displayed antifungal activity against all tested fungi higher than 1,8-cineol. Which terpinen-4-ol showed the IC_{50} against *A. porri*, *F. oxysporum* and *P. parasitica* as 925, 675 and 755 μg·mL^{-1}, respectively.

Previously, essential oil and 1,8-cineole were reported as antimicrobial, Tangjitjaroenkun (2010), studied the antifungal activity of terpinen-4-ol and the result showed percent inhibition against *F. oxysporum* and *F. oxysporum f.sp. vasinfectum* with IC_{50} 1,671 and 3,038.3 μg·mL^{-1}, respectively [20]. The essential oil of *A. galanga* has been reported as antibacterial activity against *Salmonella typhimurium*, *S. enteritidis*, *E. coli*, *Clostridium perfringens*, *Campylobacter jejuni* [87].

3.6 *In vitro* antiphytopathogenic fungi activity of isolated compounds and their derivatives

3.6.1 Minimum inhibition concentration (MIC)

Alternaria porri (NBRC 9762), *Colletotrichum gloeosporioides* (NBRC 104617), *Fusarium oxysporum* (NBRC 7152) and *Phytophthora nicotianae* (NBRC 4873) were four selected fungi. The antiphytopathogenic activities were conducted by microwells dilution method using 96 well-microtitre plates. Compounds were dissolved in DMSO and added at two-fold serial dilutions (7.8 to 1000 μg•mL^{-1}) to sterilized potato dextrose broth (PDB). Then 100 μL of fungal spore suspensions (10^8 spore·mL^{-1}) was inoculated. DMSO (0.4% v/v) using as a control had no effect on the fungal growth. After incubation at 27°C for 48 h, turbidity of PDB medium was measured to determine a minimum inhibition concentration (MIC, μg•mL^{-1}) and repeatable of triplicates. The MICs of CH_2Cl_2 extract and isolated compounds from *A. galangal* against four selected phytopathogenic fungi are presented in **Table 3.18**.

Table 3.18 MICs of CH_2Cl_2 extract and isolated compounds against selected phytopathogenic fungi

Extract/Compounds	MICs (μg•mL^{-1})			
	A. por	*C. glo*	*F. oxy*	*P. nic*
CH_2Cl_2 extract	31.5	250	500	31.5
1´-acetoxychavicol acetate (ACA) (**3-1**)	31.5	250	250	15.6
trans-p-coumaryl diacetate (**3-8**)	500	500	1000	500
trans-p-hydroxycinnamyl acetate (**3-6**)	250	250	250	31.5
trans-p-hydroxycinnamaldehyde (**3-5**)	250	500	500	125
trans-p-coumaryl alcohol ethyl ether (**3-24**)	250	500	500	125
p-acetoxycinnamyl alcohol (**3-17**)	250	500	1000	125

ACA displayed the highest antifungal activity against *P. nicotianae* and *A. porri* with MICs 15.6 and 31.5 μg•mL^{-1}, respectively. This compound exhibited moderate activity against *C. gloeosporioides* and *F. oxysporum* with MICs 250 μg•mL^{-1}. In addition, *trans-p*-hydroxycinnamyl acetate (**3-6**) revealed strong inhibition against *P. nicotianae* (MIC: 31.2 μg•mL^{-1}), and other three fungi with MICs of 250 μg•mL^{-1}. *Trans-p*-coumaryl diacetate (**3-8**) showed weak inhibition against all selected phytopathogenic fungi (MICs 500-1000 μg•mL^{-1}). For the antiphytopathogenic activity of CH_2Cl_2 extract, *P. nicotianae* and *A. porri* were suppressed more than other fungi, followed by *C. gloeosporioides* and *F. oxysporum* with MICs: 31.5, 250 and 500 μg•mL^{-1}, respectively.

3.6.2 IC_{50} of ACA against phytopathogenic fungi

ACA (**3-1**) was tested in triplicate against phytopathogenic fungi by agar incorporation method at various concentrations (0, 1, 10, 100 and 1,000 μg•mL^{-1}). After incubated at 27°C for 5 days, the mycelium growths of fungi were measured and calculated for %inhibition and IC_{50}. The results are presented in **Table 3.19** and **Figure 3.24**.

Table 3.19 Inhibitory effect of ACA (**3-1**) on the mycelium growth of phytopathogenic fungi

Fungi	Inhibition (%)				IC_{50} [a]
	1 μg·mL^{-1}	10 μg·mL^{-1}	100 μg·mL^{-1}	1,000 μg·mL^{-1}	
A. porri	4.2±2.3	12.3±1.1	19.2±0.5	52.7±1.0	900
C. gloeosporioides	3.0±2.5	7.4±0.6	9.2±1.5	46.3±0.6	>1000
F. oxysporum	0.0±0.0	8.6±4.1	14.1±2.5	64.8±2.9	700
P. parasitica	5.9±2.5	11.1±0.0	38.9±1.0	100.0±0.0	200

*Values, an average ± standard deviation (SD) of 3 replicates of the mean mycelial growth inhibition

[a]Inhibition percentage of each concentration was calculated as IC_{50} using graphically

ACA completely inhibited radial mycelial growth of *P. parasitica* at 1,000 μg·mL^{-1}, followed by *F. oxysporum*, *A. porri* and *C. gloeosporioides* with %inhibition of 64.8, 52.7 and 46.3%, respectively. This compound also showed antifungal activity against phytopathogenic fungi with IC_{50} of 200, 700, 900 and over 1,000 μg·mL^{-1}, respectively. Jamkratoke and colleagues (2002) reported the antifungal activity against *C. gloeosporioides* with 87.6% inhibition at 10,000 μg·mL^{-1} [88].

Previous studies of *Alpinia* species, especially *A. galanga*, displayed board spectrum inhibitory activity against microorganisms [36, 89]. However, Aziz and co-workers (2013) found that the CH_2Cl_2 extract from *A. conchigera* displayed antifungal activity against dermatophytes stronger than an individual compound which may cause by the synergism of each compound [90]. However, ACA as a pure compound in this study showed antiphytopathogenic activity higher than the CH_2Cl_2 extract.

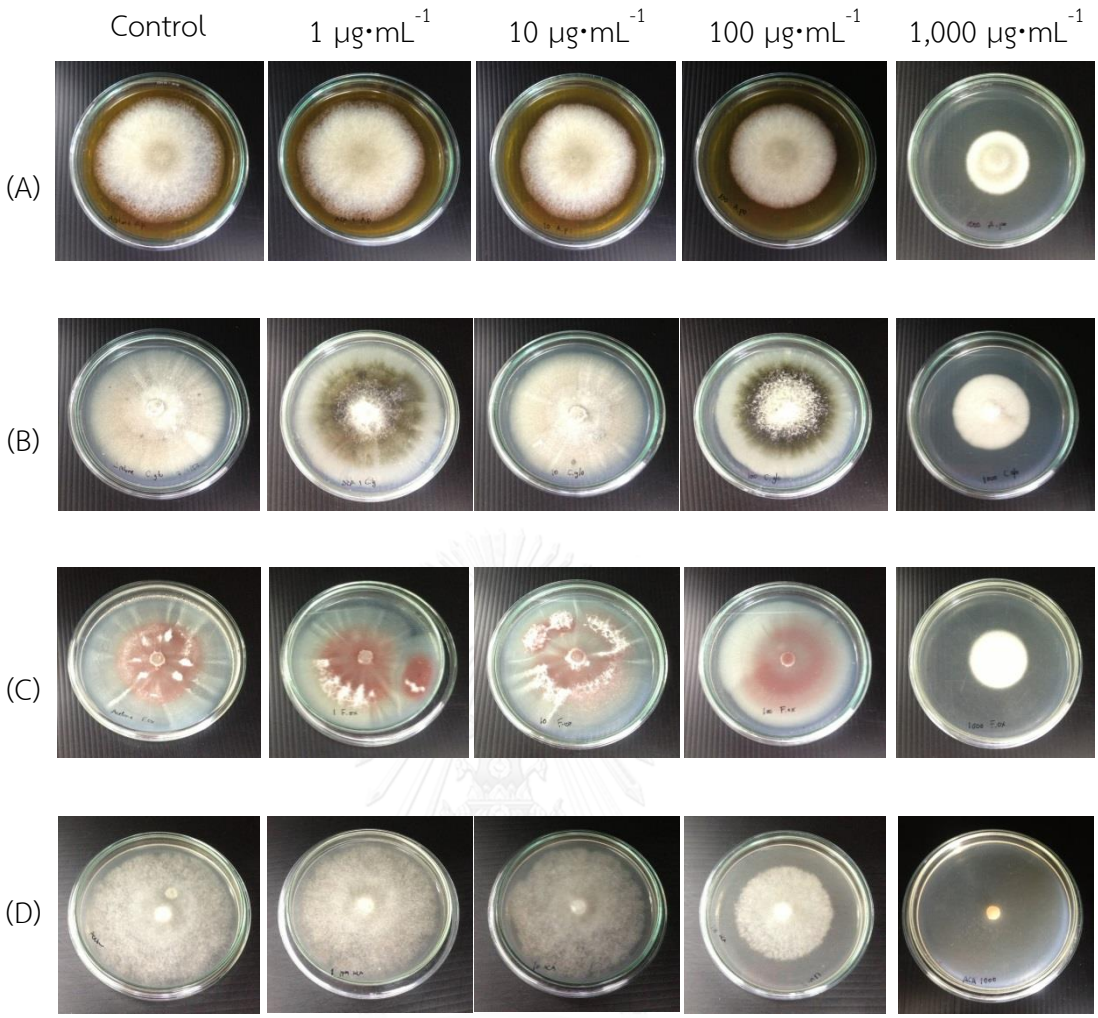


Figure 3.24 The mycelial growth inhibitions zone by ACA (**3-1**) various concentrations against *A. porri* (A), *C. gloeosporioides* (B), *F. oxysporum* (C), *P. parasitica* (D).

3.6.3 Antifungal activity of ACA (3-1) against spore germination

Spore suspension was prepared using mycelial of *F. oxysporum* (7 day old) added sterilized water and mix. After that, filtrated and adjusted the concentration of spore suspension at 10^3 spore·mL^{-1} by potato dextrose broth. ACA **(3-1)** solution was prepared at various concentrations (10^3, 10^4, $5x10^4$ and 10^5). ACA **(3-1)** solution 50 μL were added into 450 μL of spore suspension for final concentration of ACA at 10, 100, 500 and 1000 μg·mL^{-1}. The results are presented in **Figure 3.25**.

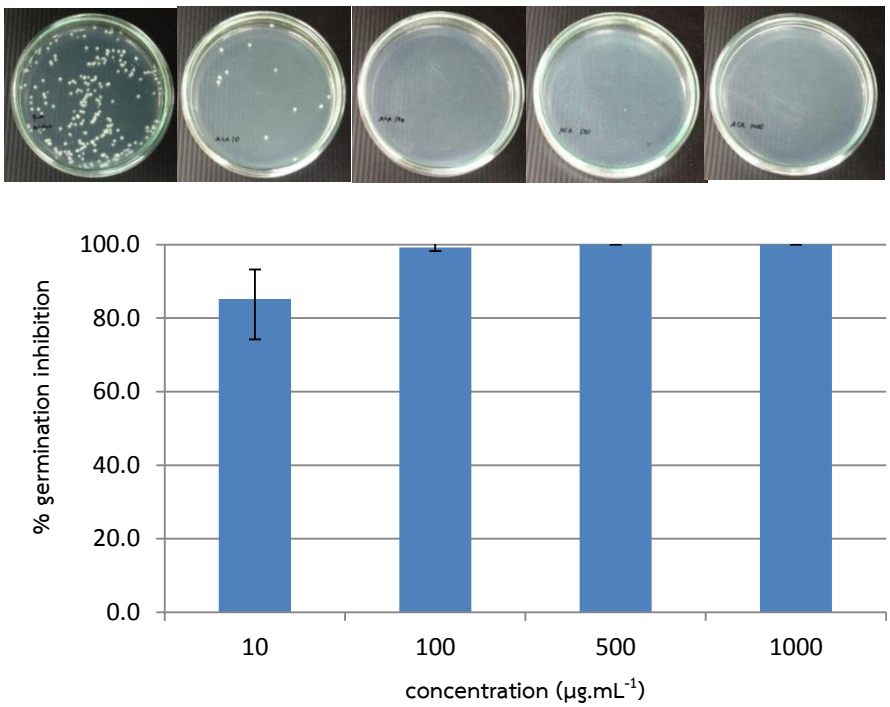


Figure 3.25 Antifungal activity of ACA against spore germination of *F. oxysporum*. Bar graphs are indicated as means ± SD of the mean (n=3).

As the results in Table 3.18, the higher spore germination could be observed when the concentration of ACA **(3-1)** increased. The complete spore germination was inhibited at 500 µg·mL^{-1}. This compound strongly inhibited at the lowest concentration (85.2%). When compared the antifungal activity between against the mycelial growth and spore germination, the concentration of ACA using against spore germination less than mycelial growth was detected. Since, the cell wall of spore and mycelial are different. The cell wall of mycelial consists of cellulose except in case of *P. parasitica* the cell wall contained chitosan and the mycelial cell wall thicker than spore [3].

3.7 Phytotoxicity of isolated compounds and their derivatives against lettuce (*Lactuca sativa* L.cv. Great Lake) and Italian ryegrass (*Lolium multiflorum* Lam. cv. Wasefudou)

The phytotoxic effects of tested compounds and CH_2Cl_2 extract against lettuce seedling (*L. sativa*) and Italian ryegrass (*L. multiflorum*) were assayed in Petri dishes (P2: 45 mm internal diameter, 10 mm height). A tested compound was dissolved in acetone, applied using a concentration range between 10-1000 $\mu g \cdot mL^{-1}$ and poured on 40 mm diameter of filter paper (No.2; Advantec, Tokyo, Japan). The solvent was allowed to completely dry. After adding 2 mL of distilled water, 10 germinated lettuce seedlings and Italian ryegrass were placed on the filter paper for each treatment with three replicates. Acetone was used as control. After incubating for 6 days for lettuce and 4 days for Italian ryegrass at 28°C under light condition, the seedling growth in terms of shoot and root for Italian ryegrass and length of cotyledon, shoot and radical length for lettuce and the total fresh weight were recorded as plant growth indices. IC_{50} was calculated in terms of $\mu g \cdot mL^{-1}$ and mM. The results are shown in **Figures 3.26-3.27** and **Table 3.20**.

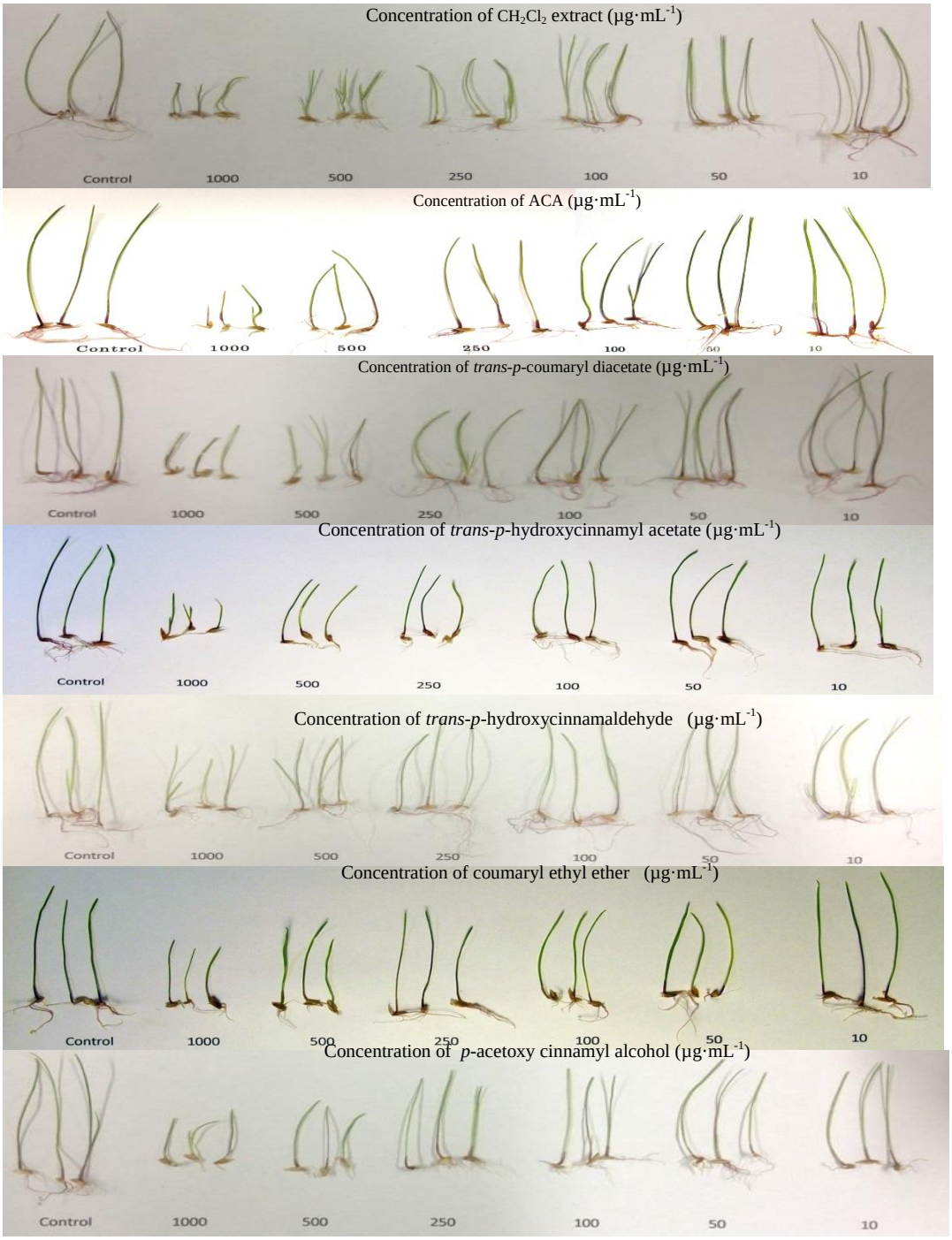


Figure 3.26 The phytotoxicity of isolated compounds and their derivatives against Italian ryegrass after incubation for 4 days.

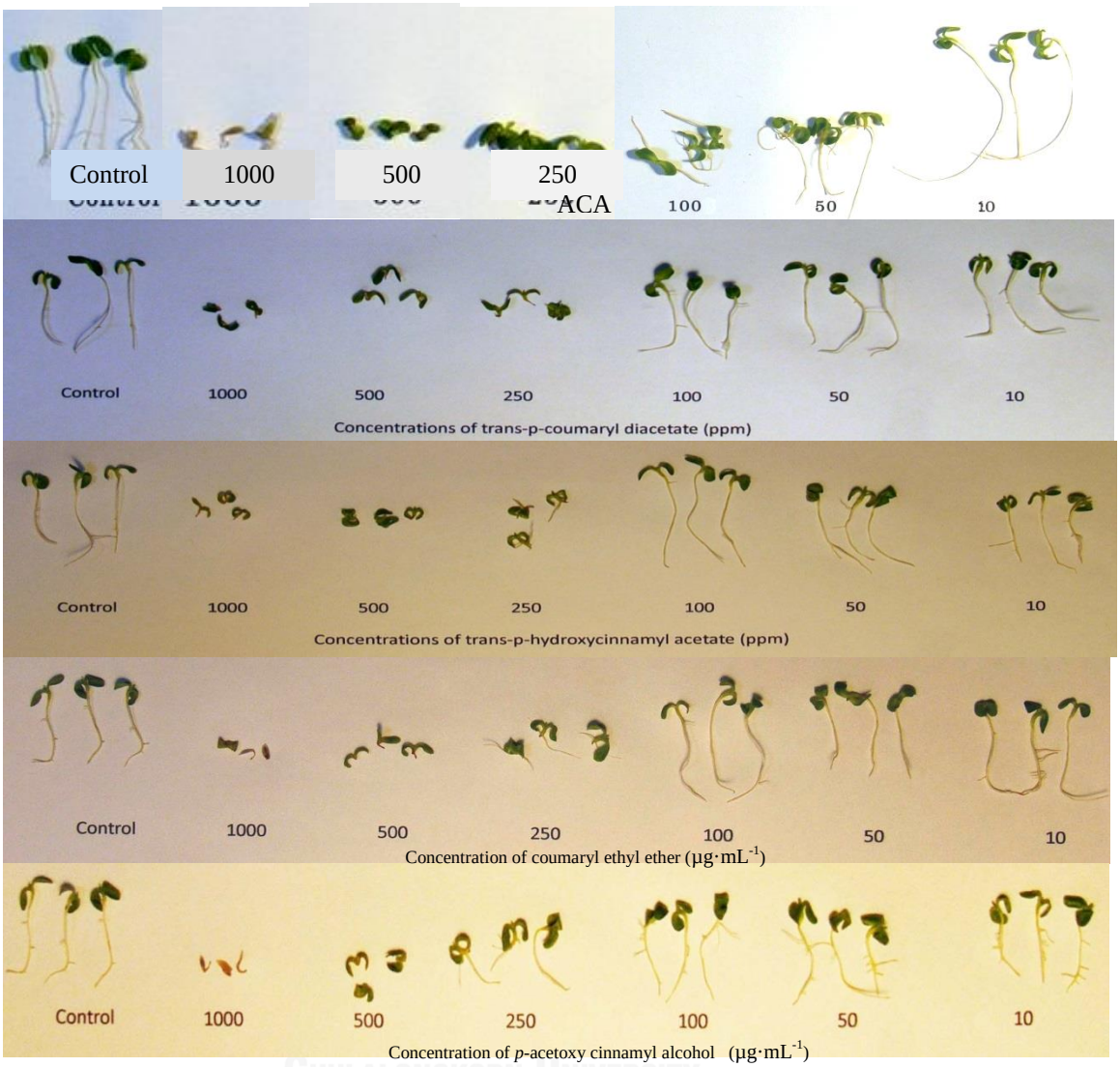


Figure 3.27 The phytotoxicity of isolated compounds and their derivatives against lettuce seedling after incubation for 6 days

Table 3.20 Phytotoxicity of CH_2Cl_2 extract, isolated compounds and their derivatives

Concentration ($\mu g \cdot mL^{-1}$)	Inhibition (%)						
	Lettuce				Italian ryegrass		
	Cotyledon	Hypocotyl	Radicle	IC_{50}* (mM)	Shoot	Root	IC_{50}* (mM)
DCM extracts							
100	12.9±1.7^{ghi}	21.6±0.8^{i}	59.6±9.9^{cd}	79.2	38.8±2.3^{gh}	50.7±3.0^{de}	90.2
250	15.3±1.8^{gh}	23.5±1.1^{i}	100.0±0.0^{a}		48.8±1.7^{de}	60.5±1.8^{c}	
500	41.1±1.5^{b}	54.9±0.7^{cde}	100.0±0^{-0a}		64.4±0.9^{a}	74.8±2.0^{ab}	
ACA (**3-1**)							
100	14.5±1.5^{ghi}	17.6±1.3^{i}	100.0±0.0^{a}	48.7	19.3±10.4^{l}	16.7±6.3^{kl}	363.0
250	21.0±1.3^{efg}	47.1±0.8^{def}	100.0±0.0^{a}	(0.21)	24.6±4.6^{kl}	30.7±10.7^{hi}	(1.55)
500	67.0±0.8^{a}	80.4±0.0^{a}	100.0±0.0^{a}		40.2±2.3^{fgh}	38.9±5.2^{fgh}	
trans-p-coumaryl diacetate (**3-8**)							
100	2.4±0.6^{j}	39.2±0.3^{fgh}	17.0±9.4^{e}	116.1	30.3±6.1^{ijk}	31.**7**±5.7^{ghi}	489.7
250	5.6±1.5^{ij}	43.1±0.7^{efg}	100.0±0.0^{a}	(0.49)	31.7±7.4^{ij}	35.2±4.6^{fghi}	(2.09)
500	16.1±1.6^{gh}	64.3±0.6^{bc}	100.0±0.0^{a}		44.0±8.3^{efg}	38.6±6.3^{fgh}	
trans-p-hydroxycinnamyl acetate (**3-6**)							
100	12.1±0.7^{ghi}	31.4±1.3^{ghi}	13.6±9.7^{fg}	144.9	46.5±3.6^{ef}	28.3±7.6^{ij}	156.3
250	13.7±1.2^{ghi}	43.1±0.8^{efg}	86.4±3.6^{b}	(0.75)	56.0±6.9^{c}	60.1±4.9^{c}	(0.81)
500	29.8±2.2^{cde}	82.4±0.0^{a}	100.0±0.0^{a}		63.1±6.3^{ab}	70.6±3.8^{b}	
trans-p-hydroxycinnamaldehyde (**3-5**)							
100	29.0±1.3^{cde}	31.4±0.7^{ghi}	15.5±5.6^{efg}	329.2	26.8±5.2^{jk}	8.9±5.7^{l}	964.9
250	36.3±0.8^{cd}	39.2±0.3^{fgh}	15.5±7.0^{efg}	(2.22)	27.1±7.3^{jk}	13.0±8.5^{kl}	(6.52)
500	37.9±1.2^{bc}	45.1±0.4^{efg}	63.8±3.5^{c}		35.1±6.6^{hi}	20.5±5.1^{jk}	
trans- p-acetoxycinnamyl alcohol (**3-17**)							
100	11.3±0.8^{hij}	25.5±0.9^{hi}	20.8±3.9ef	227.7	48.0±5.7^{de}	55.6±8.1^{cd}	50.0
250	18.5±1.2^{fgh}	27.5±1.3^{hi}	24.9±4.6^{ef}	(1.19)	54.4±7.8^{cd}	58.0±5.9^{cd}	(0.26)
500	18.5±1.7^{fgh}	78.4±0.3^{ab}	95.8±3.5^{ab}		57.1±2.5^{bc}	80.2±3.3^{a}	
trans-p-coumaryl alcohol ethyl ether (**3-24**)							
100	21.0±1.1^{efg}	56.9±0.4^{cde}	0.8±6.2^{h}	480.7	28.0±4.4^{jk}	40.3±5.7^{fg}	197.2
250	27.4±0.7^{def}	54.9±0.4^{cde}	10.6±7.3^{gh}	(2.73)	38.8±5.6^{gh}	42.7±4.5^{ef}	(1.12)
500	33.1±1.2^{cd}	59.9±0.3^{cd}	50.6±2.3^{d}		40.4±3.8^{fgh}	60.4±4.7^{c}	

* IC_{50} of radicle and root inhibition ($\mu g \cdot mL^{-1}$, (mM)) were determined by Probit analysis

Means ± SD within the same column indicated by different letter are significantly different (Tukey's HSD, *P<0.05*), (n=30)

The CH_2Cl_2 extract showed strong inhibition both against lettuce seedlings and Italian ryegrass (100 and 87.1% inhibition). The IC_{50} of lettuce seedlings from the CH_2Cl_2 extract was 87.5 μg·mL^{-1} which was higher than those isolated compounds and their derivatives except ACA (**3-1**). The separation of this active fraction led to the isolation of ACA (**3-1**) which displayed the highest inhibition against lettuce seedlings, followed by *trans-p*-coumaryl diacetate (**3-8**) and *trans-p*-hydroxycinnamyl acetate (**3-6**) with IC_{50}: 0.11, 0.89 and 0.91 mM, respectively (**Table 3.20**).

ACA (**3-1**) displayed an effect on the cotyledon to deform with roll shape and chrolosis at 500 μg·mL^{-1}. In addition, this compound completely suppressed lettuce seedlings growth at 1000 μg·mL^{-1}, blocked the main root growth even at 50 μg·mL^{-1} and stimulated the growth of lateral root compared with the lettuce seedlings at 10 μg·mL^{-1} (**Figure 3.28**). The other compounds showed completely blocked the main root of lettuce seedling at 250 μg·mL^{-1} and stimulated the lateral root replacing for maintenance their growth.

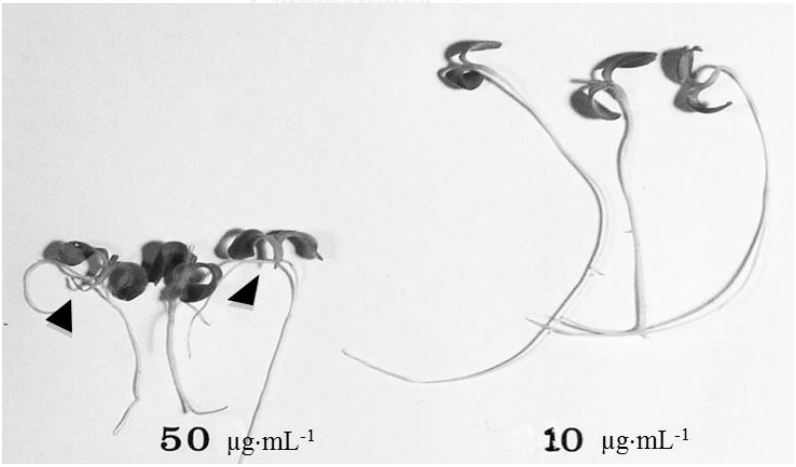


Figure 3.28 The phytotoxicity of ACA against lettuce seedlings at 50 μg·mL^{-1}. The arrow showed the completely destroyed main root and the lettuce had two lateral root.

All studied compounds exhibited strong inhibition to radicle growth of lettuce at 1,000 μg·mL^{-1} (**Figure 3.29**). For Italian ryegrass, *p*-acetoxycinnamyl alcohol (**3-17**) showed the highest suppression on the growth of this weed, followed by *trans-p*-

hydroxycinnamyl acetate (**3**-**6**) (IC_{50}: 0.29 and 0.99 mM, respectively). All tested compounds showed higher %inhibitory against the root (66.8-85.7%) than the shoot (56.7-74.3%) at 1,000 μg·mL^{-1}. The fresh weight of tested plants was decreased when the concentration of tested compounds increased (**Figure 3.30**). This result showed more effective inhibition to dicotyledon than monocotyledon, especially the root part of lettuce seedling.

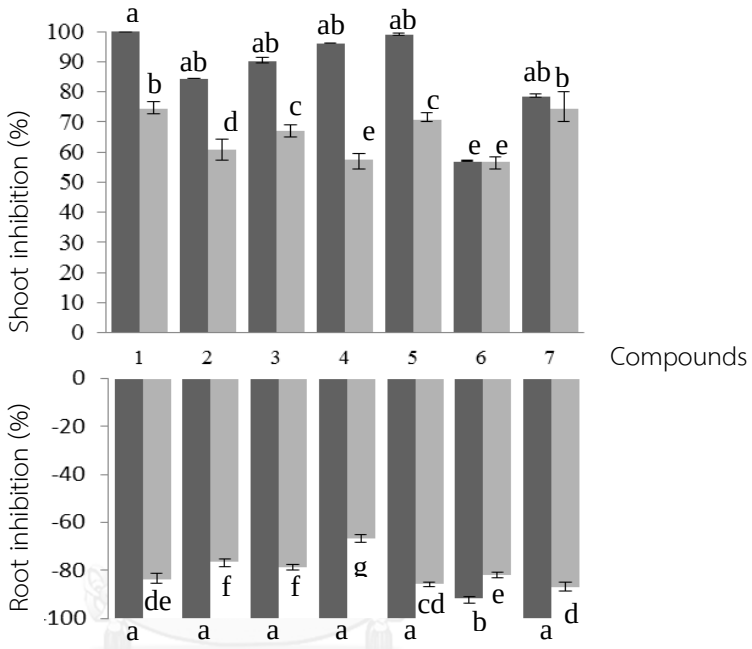


Figure 3.29 The phytotoxic activities of ACA and its derivatives against lettuce seedling (■) and Italian ryegrass (■) at 1000 μg·mL^{-1}.

Compounds **1**: ACA; **2**: *trans-p*-acetoxycinnamyl acetate; **3**: *trans-p*-coumaryl acetate; **4**: *trans-p*-coumaryl aldehyde; **5**: *trans-p*-acetoxycinnamyl alcohol; **6**: *trans-p*-coumaryl alcohol ethyl ether; **7**: DCM extract. Bar graphs are indicated as means ± SD of the mean (n=30). Different alphabetical letters indicate that values compared are significantly different (ANOVA, Tukey's HSD, *P<0.05*).

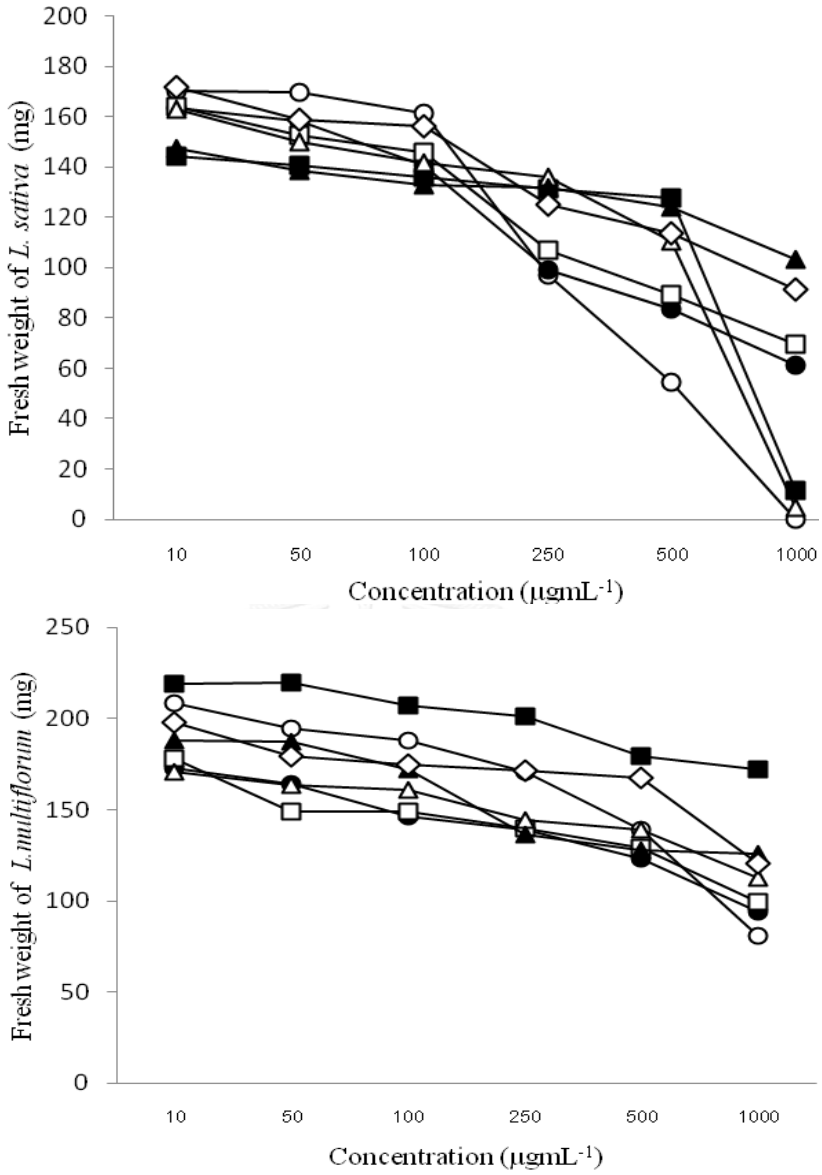


Figure 3.30 Fresh weights (mg) of lettuce seedlings (left) and Italian ryegrass (right) *vs* concentrations of tested compounds their derivatives: ((◇),CH_2Cl_2 extract; (○), ACA (**3-1**); (●), *trans-p*-coumaryl diacetate (**3-8**); (□), *trans-p*-hydroxycinnamyl acetate (**3-6**); (■), *trans-p*-hydroxycinnamaldehyde (**3-5**); (Δ), *p*-acetoxycinnamyl alcohol (**3-17**); (▲), coumaryl ethyl ether (**3-24**).

ACA (**3-1**) contained two acetoxy groups: one at *para* position of the benzene ring while the other at 1′ position on the side chain. Comparing the structures of ACA (**3-1**) and *trans-p*-coumaryl diacetate (**3-8**), one of the acetoxy groups of the latter was located at the end of side chain. Both phytotoxic and antifungal activities were dropped in the case of *trans-p*-coumaryl diacetate (**3-8**), indicating the important site of the acetoxy group at 1′ position on the side chain. The presence of *exo*-methylene in ACA (**3-1**) also exhibited better phytotoxicity against lettuce seedlings than that of the *endo*-methylene group in *trans-p*-coumaryl diacetate (**3-8**). In addition, when replacing *p*-acetoxy group with hydroxyl group, the antiphytopathogen activity was increased against all tested fungi. Moreover, comparing *trans-p*-hydroxycinnamyl acetate (**3-6**) and *trans-p*-acetoxycinnamyl alcohol (**3-17**), where the hydroxyl and acetoxy groups in both compounds were interchanged. The antifungal activity of the former was less than the other compounds. Thus, the phenolic hydroxyl group at *para* position was essential to this biological activity. Oonmetta-aree and co-workers (2006) reported that ACA (**3-1**) had a mechanism of action involving disruption of cytoplasmic membrane and coagulation cell contents [55]. The present study was in good accordance with the previous report; ACA (**3-1**) had a higher activity than other compounds.

According to this study, it was suggested that 1′-acetoxy group in chavicol analogues be required for the phytotoxic and antiphytopathogens activity. The other compounds exhibited slightly activity because they did not contain the *para* phenolic hydroxyl group, which is thought to confer activity because it can initiate free radical attack on membrane lipids of fungi [68]. These findings indicated that acetoxyl and hydroxyl groups showed important inhibitory activity.

3.8 Conclusion

From the screening part, the CH_2Cl_2 extract from the rhizomes of *A. galanga* showed higher antifungal activity against *P. parasitica* than MeOH extract. The isolation of CH_2Cl_2 extract together with exploring bioassay activity of separating fractions led to the isolation of 4 bioactive compounds against phytopathogenic fungi. Those included ACA (**3-1**), *trans-p*-coumaryl diacetate (**3-8**), *trans-p*-hydroxycinnamyl acetate (**3-6**) and *trans-p*-hydroxycinnamaldehyde (**3-5**). Two compounds (*trans-p*-coumaryl alcohol ethyl ether (**3-24**) and *p*-acetoxycinnamyl alcohol (**3-17**)) were obtained from the basic solvolysis of ACA (**3-1**). ACA (**3-1**) showed the highest antifungal activity against *P. parasitica* and strongly inhibited the growth of lettuce seedlings. According to this investigation, ACA (**3-1**) was interesting for further *in vivo* studies on pineapple leaves and suckers.

CHAPTER 4

ANTIPHYTOPATHOGENIC FUNGAL AGENTS FROM THE HEARTWOODS OF *Mansonia gagei* Drumm.

4.1 Introduction of *Mansonia gagei* Drumm.

Mansonia gagei Drumm. (Sterculiaceae) has been used as a traditional medicinal plant. Only one species was found in Thailand [91]. The common names in Thai were *chan-cha-mod, chan-hom* and *chan-pha-ma*. This plant has been used as cardiac stimulant, onilivertigo, antiemetic, antidepressant and refreshment agents [92].

The plants in *Mansonia* genus have general characteristics as follows: “large tree, scattered in dry evergreen forests on the slope of limestone hills, leave simple, toothed, flower several in a short inflorescence”. A natural dry wood scented was used for cremation ceremony [93]. The flower, leave and heartwood of *M. gagei* are presented in **Figure 4.1**.

Figure 4.1 Flower, leave and heartwood of *M. gagei* Drumm.

The chemical constituents of the *Mansonia* genus have been investigated. The separation of the heartwoods of *M. altissima* led to the isolation of primarily 1,2-

naphthoqiunones- the mansonone type, mansonones A-H (**4-1** to **4-8**) [94, 95], mansonone I (**4-9**) [96] and mansonone L (**4-10**) [97]. Four new sesquiterpenoid derivatives isolated from the CH_2Cl_2 extract of the heartwoods of *M. gagei* were identified as mansonones N- Q (**4-11** to **4-14**) [98]. Three more new compounds: mansonones R and S (**4-15** and **4-16**) mansoxetane [4-(3-hydroxy-1,2-oxetanyl)-2,2-dihydroxy-4-(2-formyl-1-ethyl)-6,6-dimethoxy biphenyl] (**4-17**), dehydrooxoperezinone (**4-18**), 3-methoxy-4,5-dihydroxybenzaldehyde (**4-19**) [99, 100] were additionally identified. In addition, three new coumarins: 3,8-dimethyl-5-isopropyl-6-methoxycoumarin (mansonrin A) (**4-20**), 3,8-dimethyl-5-isopropyl-6-hydroxycoumarin (mansorin B) (**4-21**) and 2,3-dihydro-3,6,9-trimethyl naphtho[1,8-bc]pyran-7-oxa-8-one (mansorin C) (**4-22**) were addressed. **Figure 4.2** shows the chemical structures isolated from *Mansonia* spp.

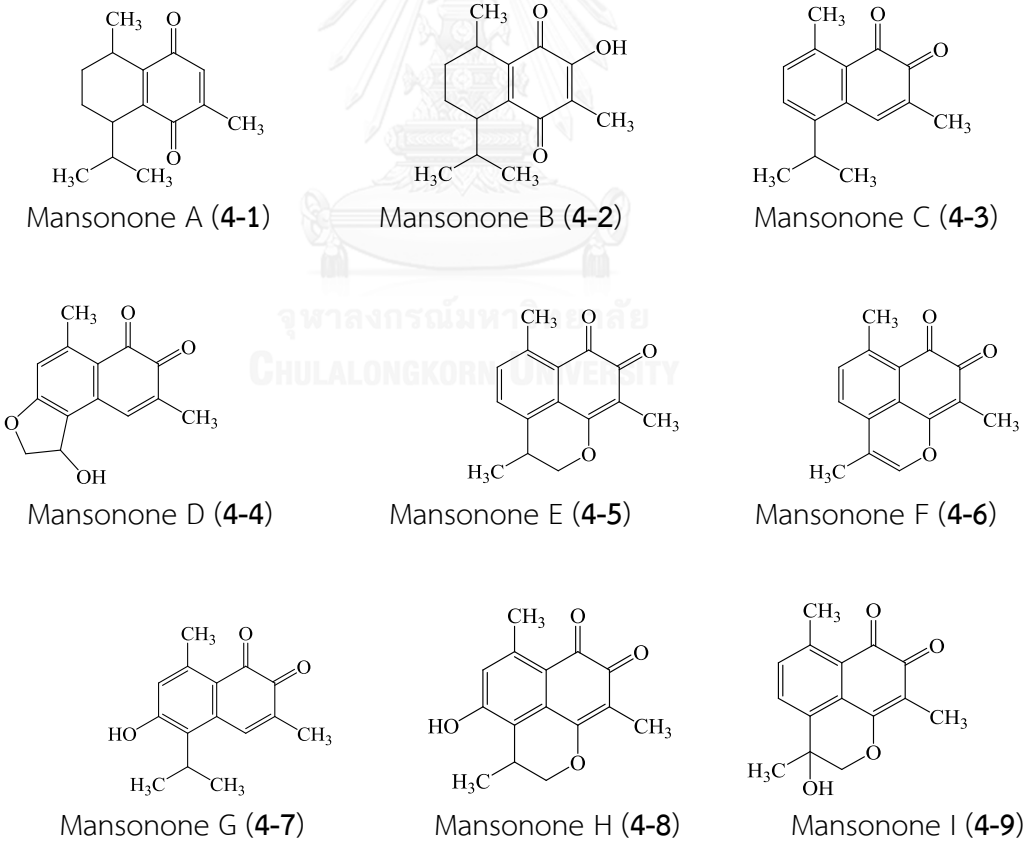
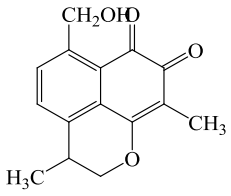
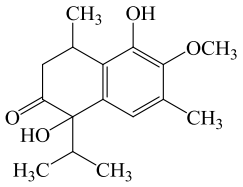


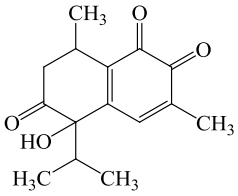
Figure 4.2 The chemical structures of *Mansonia* spp.



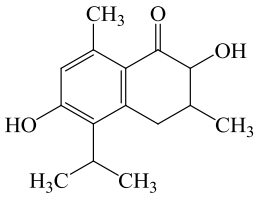
Mansonone L (**4-10**)



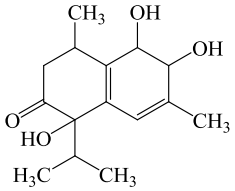
Mansonone N (**4-11**)



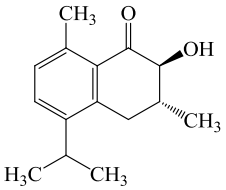
Mansonone O (**4-12**)



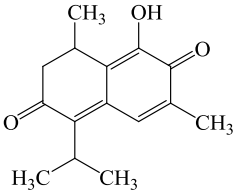
Mansonone P (**4-13**)



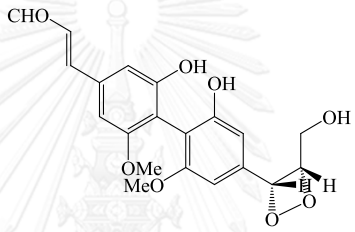
Mansonone Q (**4-14**)



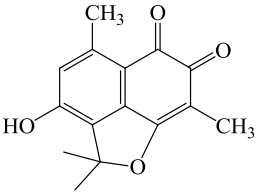
Mansonone R (**4-15**)



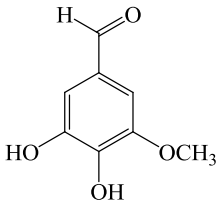
Mansonone S (**4-16**)



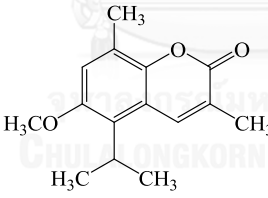
Mansoxetane (**4-17**)



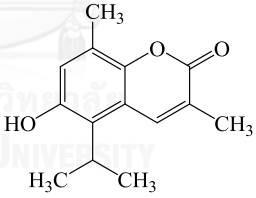
Dehydrooxoperezinone (**4-18**)



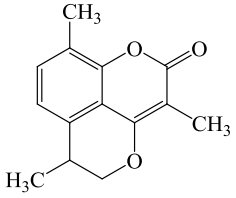
3-methoxy-4,5-di hydroxybenzaldehyde (**4-19**)



Mansorin A (**4-20**)



Mansorin B (**4-21**)



Mansorin C (**4-22**)

Figure 4.2 (cont.)

Only a few studies on biological activity from *Mansonia* spp. have been reported. In 2003b, Tiew and co-workers reported antifungal, antioxidant and larvicidal activities of isolated compounds from the heartwoods of *M. gagei*. Mansonone C (**4-3**) displayed potent antifungal activity against *Cladosporium cucumerinum* and *Andida albicans*, and larvicidal activity against *Aedes aegypti* followed by mansonone E (**4-5**) and mansorin A (**4-20**) [18]. However, only

mansonone N (**4-11**) showed radical scavenging activity. Tiengtham *et al.* (2004) isolated nine compounds from the roots of *M. gagei.* Mansonone C (**4-3**) exhibited high antihistamine activity with 92% at 0.1 mg·mL^{-1} [101]. The cytotoxicity of the hexane and CH_2Cl_2 extracts against brine shrimp *Artemia salina* Linn. exhibited LC_{50} of 23.69 and 22.83 µg·mL^{-1}. Among isolated compounds, mansorin B (**4-21**) and mansonone C (**4-3**) revealed high activity against brine shrimp with LC_{50} of 0.61 and 2.08 µg·mL^{-1} [102]. In addition, the anti-multidrug resistant bacteria from the extract of *M. gagei* cotreatment with chloramphermical and tetracyclines were investigated [103].

The antithrombin and anticancer activities of the extract from the heartwoods of *M. gagei* were examined by Tiew (2002) [99]. The CH_2Cl_2 extract displayed high antithrombin activity of 71% inhibition at 1,000 µg·mL^{-1}. Among the isolated compounds, dehydrooxoperezinone (**4-18**), mansonones H (**4-8**) and G (**4-7**) inhibited strong activity with 99.7, 97.6 and 96.3%, respectively. For the anticancer activity, mansonones C (**4-3**), G (**4-7**) and H (**4-8**) demonstrated potent inhibitors against cancer cell lines. Mansorin A (**4-20**) displayed selective activity against human myeloma with 87.5% inhibition, while mansorin B (**4-21**) inhibited human leukemia and human breast carcinoma with 87.0 and 95.3% inhibition, respectively. The anti-estrogenic activity from this plant using yeast two-hybrid assay was investigated by El-Halawany and coworkers (2007, 2011 and 2013). Mansonones C (**4-3**), G (**4-7**), F (**4-6**) and S (**4-16**) displayed the most potent estrogen binding and estrogen antagonistic effects. Changwong *et al*., (2012) isolated mansorins A-C (**4-20** – **4-22**), mansonones C (**4-3**), E (**4-6**), G (**4-7**) and H (**4-8**) from the heartwoods of *M. gagei*. Mansonone E (**4-5**) revealed the highest both of acetyl cholinesterase (AChE) and butyryl cholinesterase (BChE) with low IC_{50} of 23.5 and 62.4 µM, respectively [104].

To the best of our knowledge, there was no report concerning the antiphytopathogenic activity from the heartwoods of *M. gagei*. The objectives of this study are to isolate the active compounds from *M. gagei* and to test antiphytopathogenic fungi activity against four selected fungi.

4.2 Plant materials and extraction

The heartwoods of *M. gagei* were purchased from the herbal drugstore "Chow Khrom Per", Bangkok, Thailand in October 2010. The voucher specimen no. 43281 was submitted to Herbarium of the Royal Forest Department of Thailand. Dried heartwoods (10 kg) were milled and extracted three times with CH_2Cl_2 at RT. The extracts were filtered and the solvent was removed by vacuum rotatory evaporator to give 315.3 g of the CH_2Cl_2 extract (3.15% yield).

4.3 Separation of the CH_2Cl_2 extract and bioassay test

The CH_2Cl_2 extract (220 g) was fractionated by quick column using silica gel (No. 7729, Merck). A stepwise elution was conducted by 100% hexane and increasing the polarity with EtOAc and finally with 10% MeOH in EtOAc. The fractions were collected and combined according to TLC results and then evaporated to obtain 7 sub fractions: **MGH1**-**MGH7**. The results of the separation and %yield are shown in **Table 4.1**.

Table 4.1 The separation of the CH_2Cl_2 extract by quick column.

Fraction No.	Solvent system (%v/v)	Remarks	Weight (g)
MGH1	100% hexane	yellow wax	3.7
MGH2	10% EtOAc in hexane	dark red liquid	21.8
MGH3	20% EtOAc in hexane	dark red liquid	37.6
MGH4	40% EtOAc in hexane	orange brown solid	35.8
MGH5	60-80% EtOAc in hexane	red brown solid	51.1
MGH6	100% EtOAc	brown solid	17.8
MGH7	10% MeOH in EtOAc	dark brown solid	26.1

MGH5 as red brown solid gave the highest yield as 51.1 g (23.2% yield based on the CH_2Cl_2 extract), followed by **MGH3, MGH4** and **MGH7** with 37.6, 35.8 and 26.1 g (17.1, 16.3 and 11.9% yield), respectively. **MGH1**, **MGH2** and **MGH6** gave slightly

low yield as 3.7, 21.8 and 17.8 g (1.7, 9.9 and 8.1% yield), respectively. All seven fractions were subjected to antifungal bioassay against *P. parasitica* using agar incorporation method at 1000 μg·mL^{-1} as described in Chapter II. The results are accumulated in **Table 4.2** and **Figure 4.3**.

According to the bioassay guide, **MGH4** displayed the highest antifungal activity against *P. parasitica* with 73.3% inhibition, followed by **MGH5** (63.3%), **MGH3** (60.0%), **MGH6** (57.0%) and **MGH7** (47.0%), whereas **MGH1** and **MGH2** did not inhibit the mycelium growth of this fungus. Therefore, **MGH3** to **MGH6** as the active fractions were selected to proceed for further separation.

Table 4.2 %inhibition of mycelial growth from separated sub-fractions against *P. parasitica* at 1,000 μg·mL^{-1}

Fraction No.	Growth (mm)*	%Inhibition*
MGH1	90.0±0.0	0.0±0.0
MGH2	90.0±0.0	0.0±0.0
MGH3	36.0±1.0	60.0±0.6
MGH4	24.0±1.7	73.3±0.4
MGH5	33.0±1.0	63.3±0.2
MGH6	38.7±0.6	57.0±0.6
MGH7	47.7±1.7	47.0±0.4
DMSO	90.0±0.0	0.0±0.0
Metalaxyl (100 μg·mL^{-1})	0.0±0.0	100.0±0.0

*Values, an average ± standard deviation (SD) of 3 replicates of the mean mycelial growth inhibition

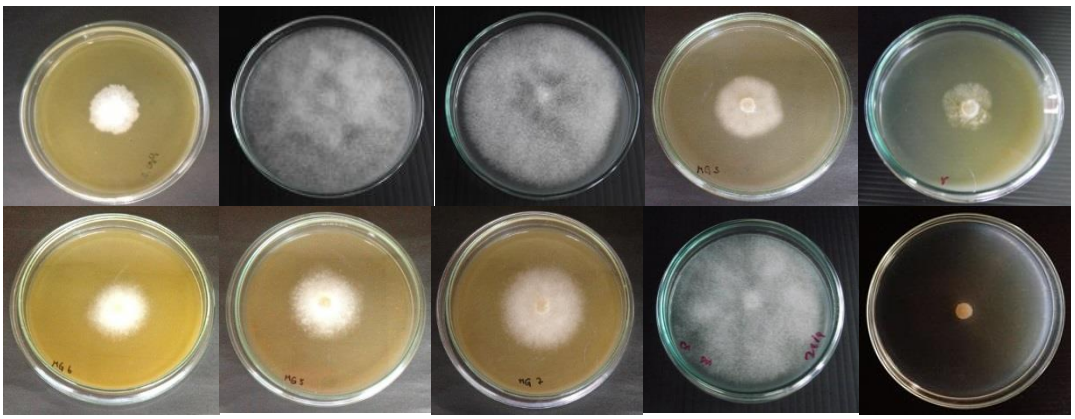
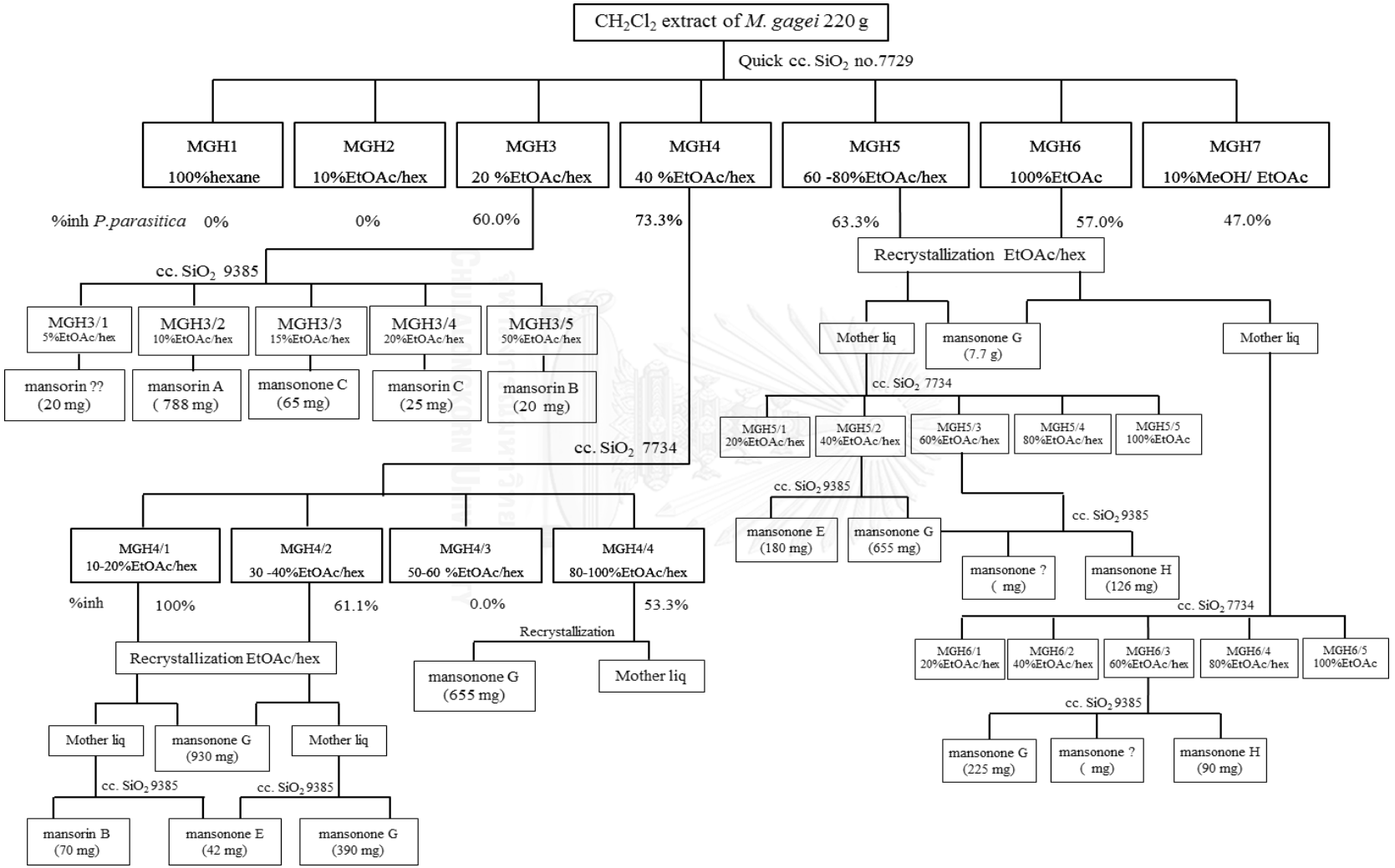


Figure 4.3 The inhibition of mycelium growth from separated sub-fraction of the CH_2Cl_2 extract at 1000 μg·mL^{-1}.
Above (left to right): CH_2Cl_2 extract, **MGH1**, **MGH2**, **MGH3** and **MGH4**
Below (left to right): **MGH5**, **MGH6**, **MGH7**, DMSO and metalaxyl

4.4 Isolation of bioactive compounds

Each fraction, **MGH3** (25.5 g), **MGH4** (35.0 g), **MGH5** (51.0 g), and **MGH6** (17.0 g) was rechromatographed on silica gel (no.9385 or no. 7734) column using stepwise solvent system of hexane–CH_2Cl_2, CH_2Cl_2-EtOAc and EtOAc-MeOH of increasing polarity. Seven pure compounds were obtained after further isolation on silica gel column. Mansorin A (**4-20**, 788 mg), mansorin C (**4-22**, 25 mg) and mansonone C (**4-3**, 65 mg) were obtained from **MGH3**. Mansorin B (**4-21**, 90 mg) was obtained from **MGH4**. Mansonone E (**4-5**, 228 mg) was obtained from both **MGH4** and **MGH5**. Mansonone H (**4-7**, 216 mg) was obtained from both **MGH5** and **MGH6**. The major compound of this plant as mansonone G (**4-6**, 10.9 g) was isolated from **MGH4**-**MGH6**. The isolation of the bioactive compounds was summarized in **Scheme 4.1**.



Scheme 4.1 The separation of the CH_2Cl_2 extract of the heartwoods of *M. gagei* and bioassay guided against *P. parasitica* at 1,000 μg·mL^{-1}

4.5 Structural elucidation

4.5.1 Compound 4-20: mansorin A

Compound **4-20** (788 mg) as light yellow crystal revealed a single spot on TLC with R_f 0.4 (developing solvent: EtOAc:hexane (1:4)). The ^{1}H NMR spectrum of compound **4-20** is shown in **Figure 4.4**.

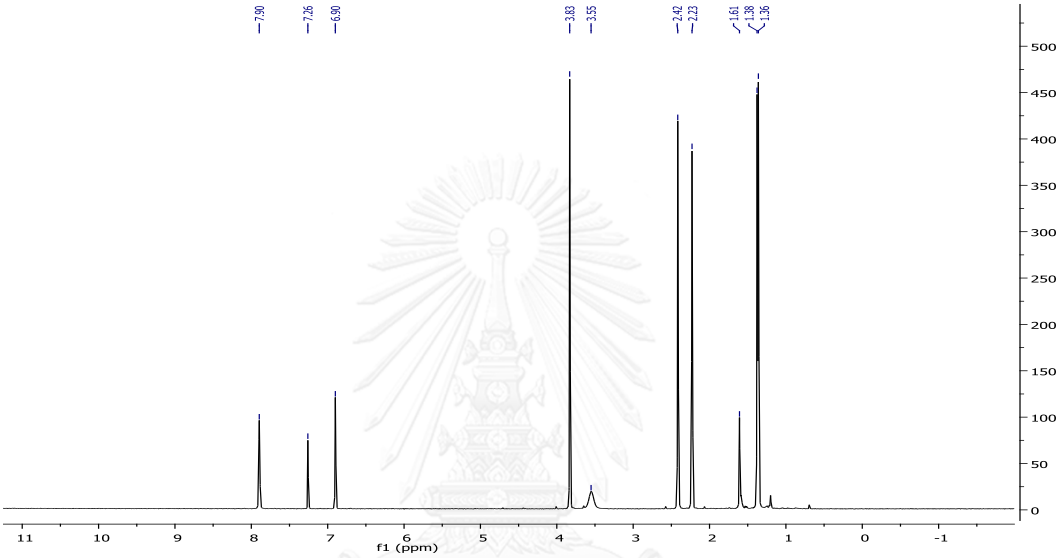
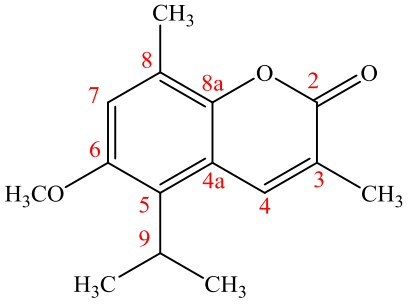


Figure 4.4 The ^{1}H NMR spectrum ($CDCl_3$) of compound **4-20**

By comparison of the ^{1}H NMR spectroscopic data of compound **4-20** with that of reported mansorin **A**, compound **4-20** was designated as mansorin A ($C_{15}H_{18}O_3$). The ^{1}H NMR spectral assignment of mansorin A and compound **4-20** is collected in Table 4.5.



mansorin A (**4-20**)

Table 4.3 The comparison of ^{1}H NMR spectral assignment of mansorin A and compound **4-20 [99]**

Position	Chemical shift (ppm)	
	mansorin A*	Compound 4-20**
4	7.90 (*s*, 1H)	7.90 (*s*, 1H)
7	6.90 (*s*, 1H)	6.90 (*s*, 1H)
9	3.56 (m, 1H)	3.55 (m, 1H)
3-CH_3	2.23 (*s*, 3H)	2.23 (*s*, 3H)
6-OCH_3	3.83 (*s*, 3H)	3.83 (*s*, 3H)
8-CH_3	2.42 (*s*, 3H)	2.42 (*s*, 3H)
9-$(CH_3)_2$	1.38 (*d*, *J* = 7.3 Hz, 6H)	1.37 (*d*, *J* = 7.1 Hz, 6H)

*^{1}H NMR spectra was measured in $CDCl_3$ at 500 MHz, **400 MHz

4.5.2 Compound 4-3: mansonone C

Compound **4-3** as orange needle (65 mg) was obtained after recrystallization with a mixture of EtOAc and hexane. It showed a single spot on TLC with R_f 0.5 (developing solvent: EtOAc:hexane (3:7)). The ^{1}H NMR spectrum is displayed in **Figure 4.5**.

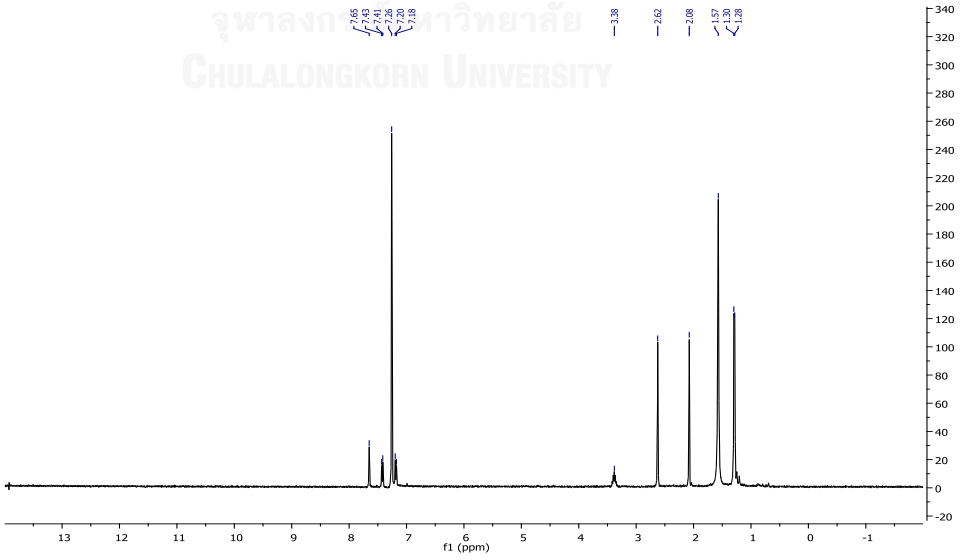
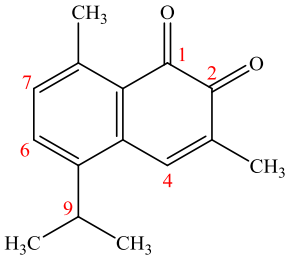


Figure 4.5 The ^{1}H NMR spectrum ($CDCl_3$) of compound **4-3**

The ^{1}H NMR spectral assignments of mansonone C and compound **4-3** are compared as presented in **Table 4.4**. Based on the comparison of physical properties

and the ^{1}H NMR data with authentic sample, compound **4-3** was identified as mansonone **C**.



mansonone **C** (**4-3**)

Table 4.4 The comparison of ^{1}H NMR spectral data of mansonone C and compound **4-3** [99]

Position	Chemical shift (ppm)	
	mansonone C	Compound 4-3
4	7.63 (*d*, *J* = 1.5 Hz, 1H)	7.65 (*s*, 1H)
6	7.16 (*d*, *J* = 8.2 Hz, 1H)	7.19 (*d*, *J* = 8.0 Hz, 1H)
7	7.40 (*d*, *J* = 8.2 Hz, 1H)	7.43 (*d*, *J* = 8.1 Hz, 1H)
9	3.36 (m, 1H)	3.38 (m, 1H)
3-CH_3	2.02 (*d*, *J* = 2.0 Hz, 3H)	2.08 (*d*, *J* = 1.8 Hz, 3H)
8-CH_3	2.60 (*s*, 3H)	2.63 (*s*, 3H)
9-$(CH_3)_2$	1.27 (*d*, *J* = 7.0 Hz, 1H)	1.29 (*d*, *J* = 7.0 Hz, 1H)

4.5.3 Compound 4-22: mansorin C

This compound revealed a single spot on TLC with R_f 0.25 (developing solvent: EtOAc:hexane (3:7)). After recrystallization with a mixture of EtOAc and hexane, compound **4-22** (25 mg) as white crystal was obtained. The molecular formula of this compound was proposed as $C_{14}H_{14}O_3$ based on the ^{1}H NMR spectrum (**Figure 4.6**). The comparative ^{1}H NMR spectral data of mansorin C and compound **4-22** is presented in **Table 4.5**.

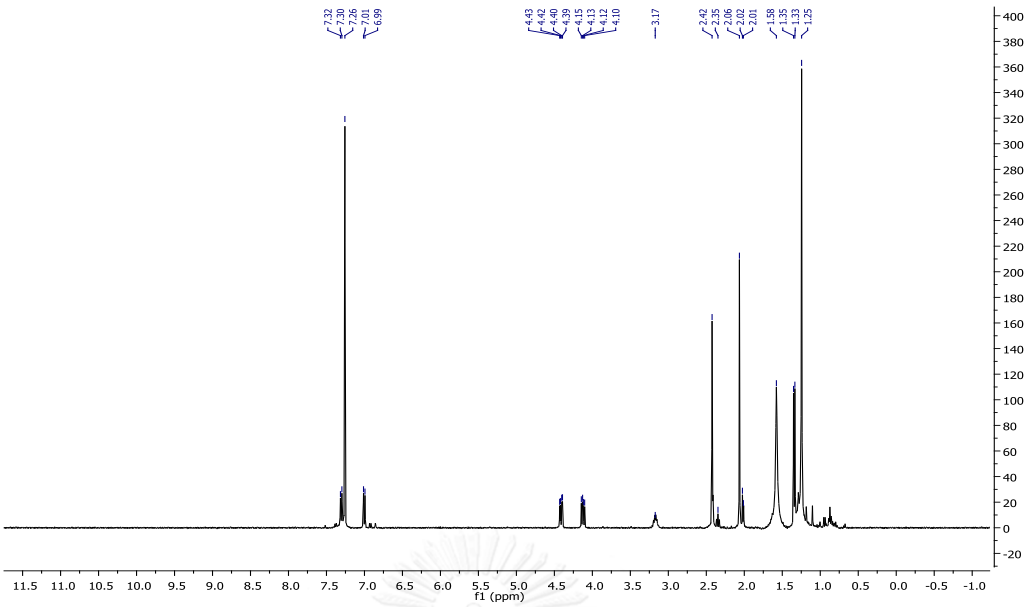
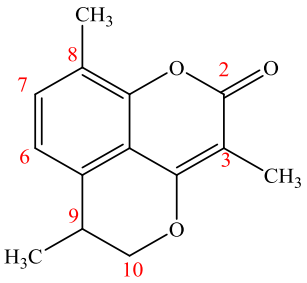


Figure 4.6 The ^{1}H NMR spectrum ($CDCl_3$) of compound **4-22**

Table 4.5 The comparison of ^{1}H NMR spectral data of mansorin C and compound **4-22** [99]

Position	Chemical shift (ppm)	
	mansorin C	Compound 4-22
6	6.98 (*d*, *J* = 7.6 Hz, 1H)	7.00 (*d*, *J* = 7.6 Hz, 1H)
7	7.28 (*d*, *J* = 7.6 Hz, 1H)	7.31 (*d*, *J* = 7.6 Hz, 1H)
9	3.15 (m, 1H)	3.17 (m, 1H)
10	4.10 (*dd*, *J* = 6.7, 11.0 Hz, 1H)	4.13 (*dd*, *J* = 6.7, 10.8 Hz, 1H)
10	4.39 (*dd*, *J* = 4.0, 10.7 Hz, 1H)	4.41 (*dd*, *J* = 4.1, 10.7 Hz, 1H)
3-CH_3	2.04 (s, 3H)	2.07 (*s*, 3H)
8-CH_3	2.39 (*s*, 3H)	2.43 (*s*, 3H)
9-CH_3	1.32 (*d*, *J* = 7.4 Hz, 3H)	1.33 (*d*, *J* = 7.0 Hz, 3H)

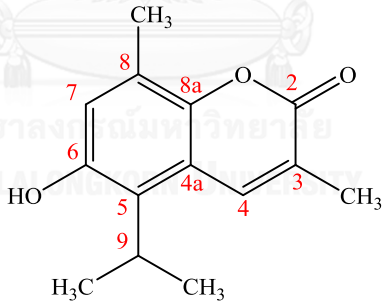
The structure of compound **4-22**, based on physical properties and the ^{1}H NMR data, was elucidated as mansorin **C** (**4-22**).



mansorin **C** (**4-22**)

4.5.4 Compound 4-21: mansorin B

The recrystallization of **MGH4/1** with a mixture of EtOAc-hexane yielded compound **4-21** (65 mg) as pale yellow powder. The TLC showed a single spot with R_f 0.40 (developing solvent: EtOAc:hexane (3:7)). The ^{1}H and ^{13}C NMR spectra of compound **4-21** are shown in **Figures 4.7-4.8**. The comparison of the ^{1}H and ^{13}C NMR spectral data of mansorin B and compound **4-21** are presented in **Table 4.6**. Based on the comparison of physical properties and the NMR data with authentic sample, compound **4-21** was identified as mansorin B (**4-21**).



mansorin B (**4-21**)

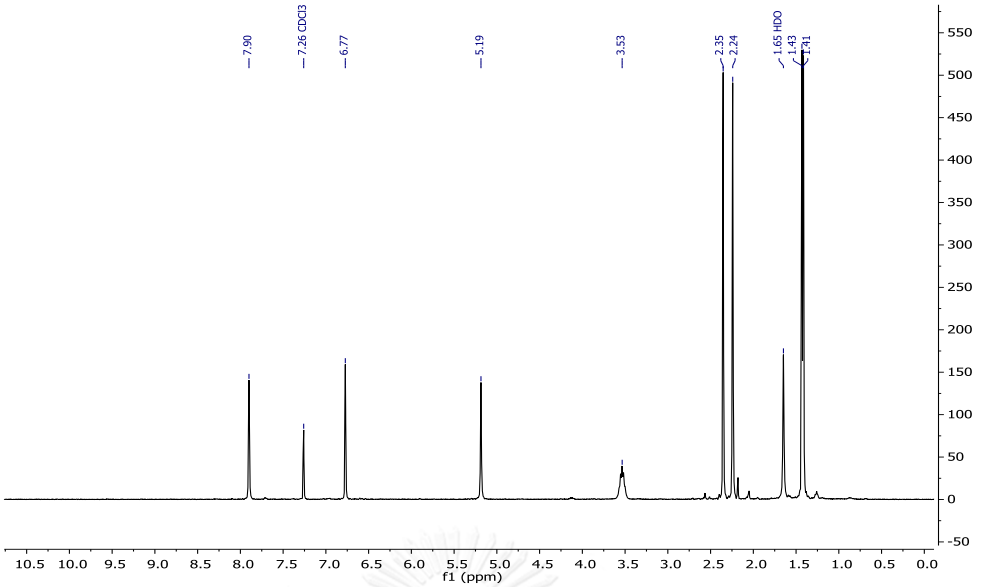


Figure 4.7 The ^{1}H NMR spectrum ($CDCl_3$) of compound **4-21**

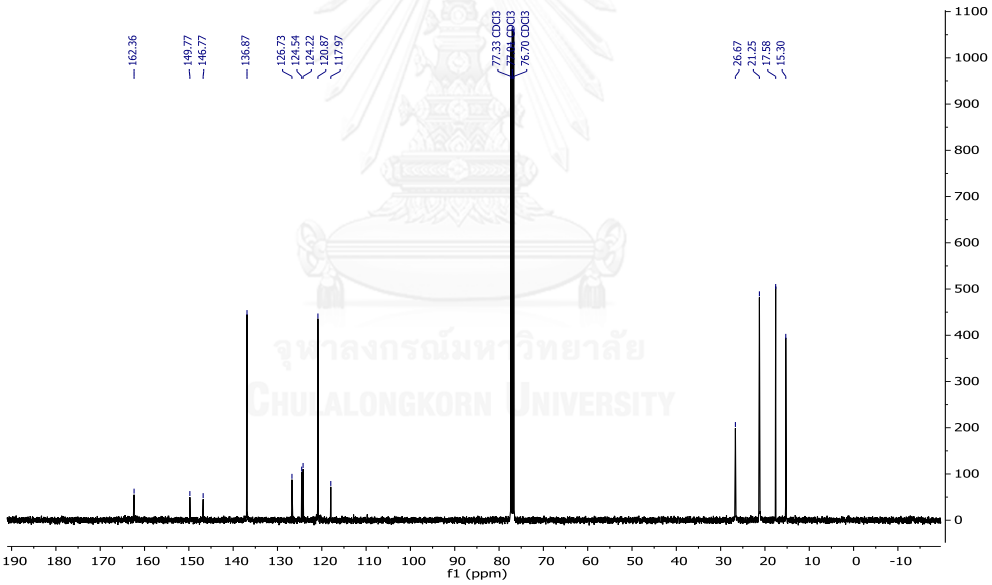


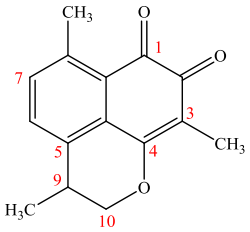
Figure 4.8 The ^{13}C NMR spectrum ($CDCl_3$) of compound **4-21**

Table 4.6 The comparison of ^{1}H and ^{13}C NMR spectral data of mansorin B and compound **4-21** [99]

Position	Chemical shift (ppm)			
	mansorin B		Compound 4-21	
	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
2		162.6		162.3
3		124.3		124.5
4	7.90 (*s*, 1H)	137.1	7.90 (s, 1H)	136.9
4a		117.9		117.9
5		126.7		126.7
6		150.0		149.8
7	6.79 (*s*, 1H)	120.9	6.77 (*s*, 1H)	120.9
8		124.1		124.2
8a		147.6		146.8
9	3.51 (m, 1H)	26.6	3.53 (m, 1H)	26.7
3-CH_3	2.22 (*d*, *J* = 1.2 Hz, 3H)	17.6	2.24 (*s*, 3H)	17.6
6-OH	5.67 (*s*, 1H)		5.19 (*s*, 1H)	
8-CH_3	2.31 (*s*, 3H)	15.3	2.35 (*s*, 3H)	15.3
9-$(CH_3)_2$	1.40 (*d*, *J* = 8.0 Hz, 6H)	21.9	1.42 (*d*, *J* = 7.0 Hz, 6H)	21.3

4.5.5 Compound 4-5: mansonone E

Compound **4-5** (228 mg) as orange powder showed a single spot on TLC with R_f 0.25 (developing solvent: EtOAc:hexane (3:7)). The ^{1}H NMR spectrum is shown in **Figure 4.9**. The comparison of the ^{1}H NMR spectral data of mansonone E and compound **4-5** are collected in **Table 4.7**. According to the NMR data, compound **4-5** was identified as mansonone **E** (**4-5**).



mansonone **E** (**4-5**)

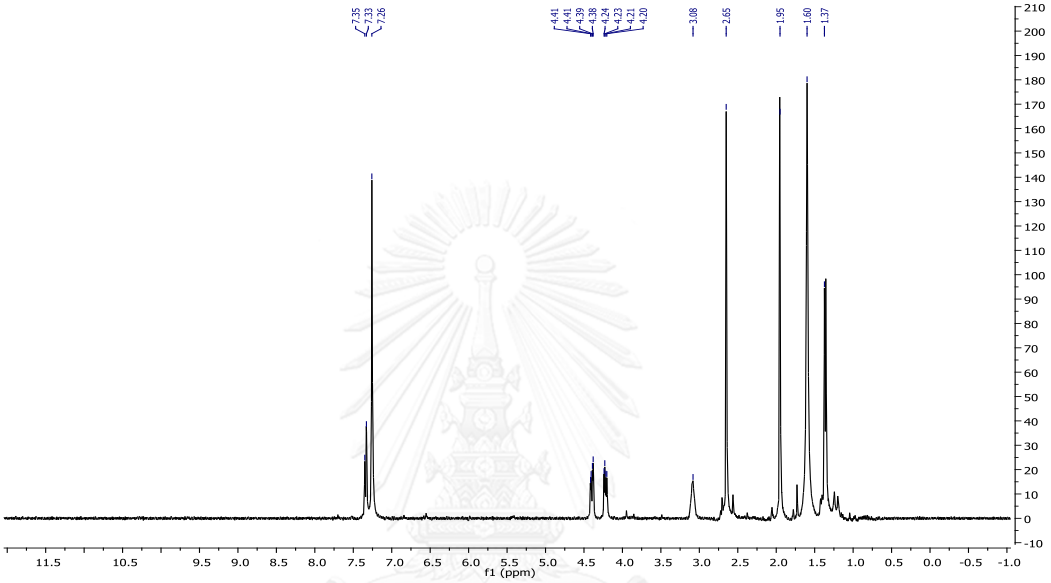


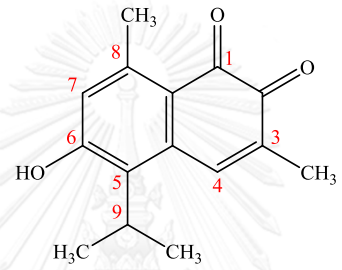
Figure 4.9 The ^{1}H NMR spectrum ($CDCl_3$) of compound **4-5**

Table 4.7 The comparison of ^{1}H NMR spectral data of mansonone E and compound **4-5** [99]

Position	Chemical shift (ppm)	
	mansonone E	Compound 4-5
6	7.35 (*d*, *J* = 7.9 Hz, 1H)	7.34 (*d*, *J* = 8.5 Hz, 1H)
7	7.26 (*d*, *J* = 7.9 Hz, 1H)	7.00 (*d*, *J* = 7.6 Hz, 1H)
9	3.10 (m, 1H)	3.17 (m, 1H)
10	4.23 (*dd*, *J* = 10.7, 4.0 Hz, 1H)	4.22 (*dd*, *J* = 10.1, 4.3 Hz, 1H)
	4.41 (*dd*, *J* = 11.0, 5.2 Hz, 1H)	4.40 (*dd*, *J* = 10.1, 3.4 Hz, 1H)
3-CH_3	1.95 (s, 3H)	1.95 (s, 3H)
8-CH_3	2.65 (s, 3H)	2.65 (*s*, 3H)
9-CH_3	1.37 (*d*, *J* = 7.0 Hz, 3H)	1.36 (*d*, *J* = 7.1 Hz, 3H)

4.5.6 Compound 4-7: mansonone G

The separation of **MGH4** to **MGH6** furnished compound **4-7** (10.9 g) as orange powder. This compound exhibited only one spot on TLC with R_f 0.15 (developing solvent: EtOAc:hexane (3:7)). The ^{1}H and ^{13}C NMR data were similar to compound **4-7** (mansonone **C**), except for the absence of the set of *ortho*-coupled proton signals and the presence of singlet aromatic proton at δ_H 7.87 (**Figures 4.10-4.11**). The comparison of the ^{1}H and ^{13}C NMR data of compound **4-7** and mansonone **G** is presented in **Table 4.8**. Thus, compound **4-7** was identified as mansonone G (**4-7**).



mansonone G (**4-7**)

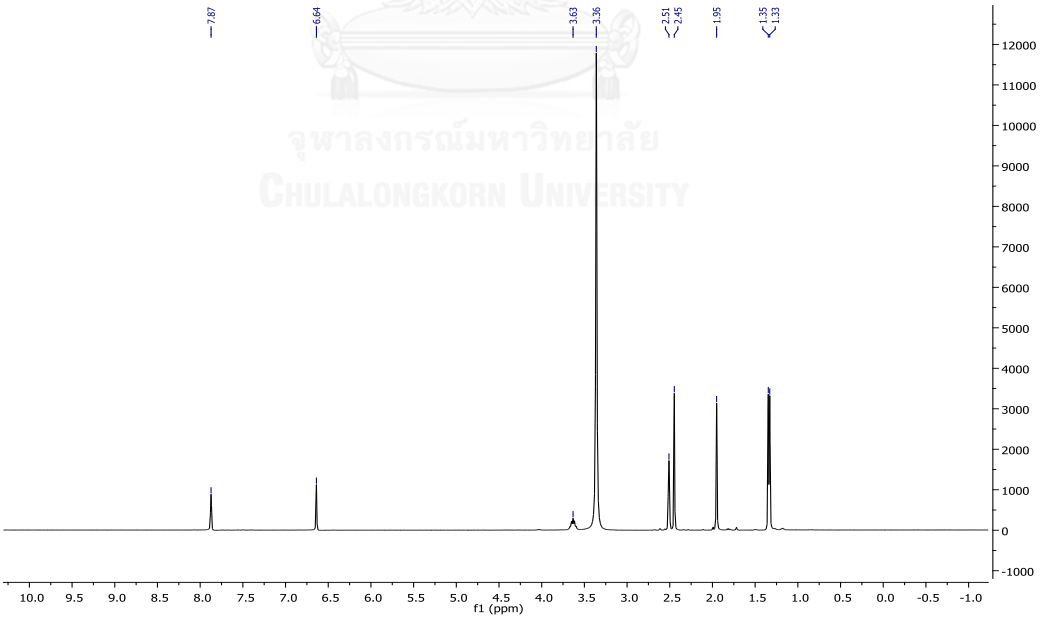


Figure 4.10 The ^{1}H NMR spectrum (DMSO) of compound **4-7**

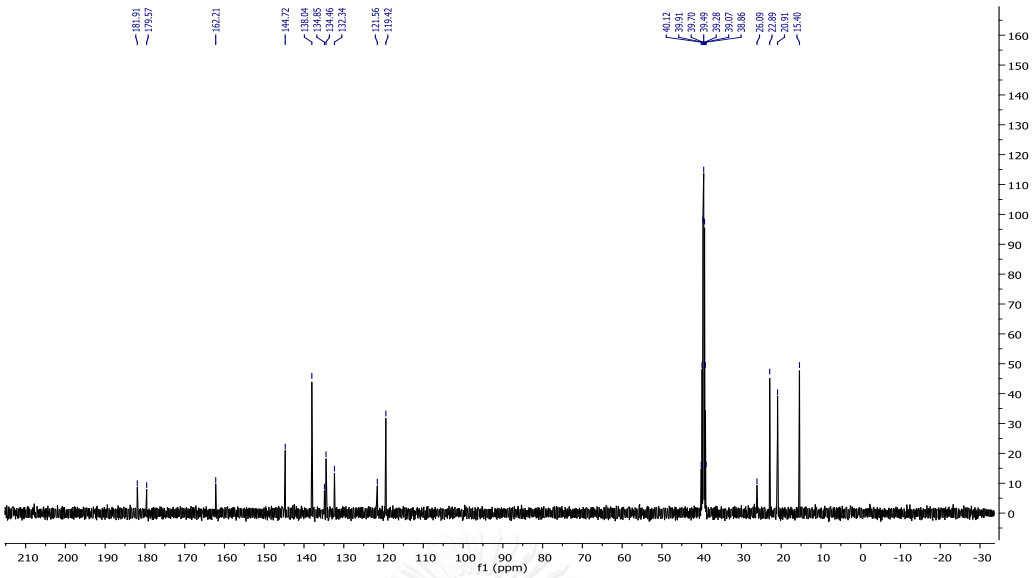


Figure 4.11 The ^{13}C NMR spectrum (DMSO) of compound **4-7**

Table 4.8 The comparison of ^{1}H and ^{13}C NMR spectral data of mansonone G and compound **4-7** [99]

Position	Chemical shift (ppm)			
	Mansonone G[a]		Compound 4-7[b]	
	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
1		181.2		179.6
2		183.8		181.9
3		135.7		134.9
4	7.7 (*s*, 1H)	140.4	7.87 (*s*, 1H)	138.1
4a		122.9		121.6
5		134.7		132.4
6		164.4		162.2
7	6.5 (*s*, 1H)	120.6	6.64 (*s*, 1H)	119.5
8		136.6		134.5
8a		147.8		144.8
9	3.58 (m, 1H)	28.1	3.63 (m, 1H)	26.1
3-CH_3	2.06 (*s*, 3H)	15.7	1.95 (*s*, 3H)	15.4
8-CH_3	2.58 (*s*, 3H)	23.6	2.45 (*s*, 3H)	22.9
9-$(CH_3)_2$	1.42 (*d*, *J* = 7.0 Hz, 6H)	21.4	1.34 (*d*, *J* = 7.0 Hz, 6H)	20.9

[a] ^{1}H and ^{13}C NMR spectra was measured in $CDCl_3$ at 500 and 125 MHz

[b] ^{1}H and ^{13}C NMR spectra was measured in DMSO at 400, 75 MHz

4.5.7 Compound 4-8: manosonone H

Compound **4-8** (216 mg) as red pallet displayed a single spot on TLC with R_f 0.44 (developing solvent: EtOAc:hexane (1:1)). The ^{1}H NMR spectrum is shown in **Figure 4.12**. The comparison of the ^{1}H NMR spectral data of mansonone H and compound **4-8** are presented in **Table 4.9**. From the NMR data, compound **4-8** was identified as mansonone H (**4-8**).

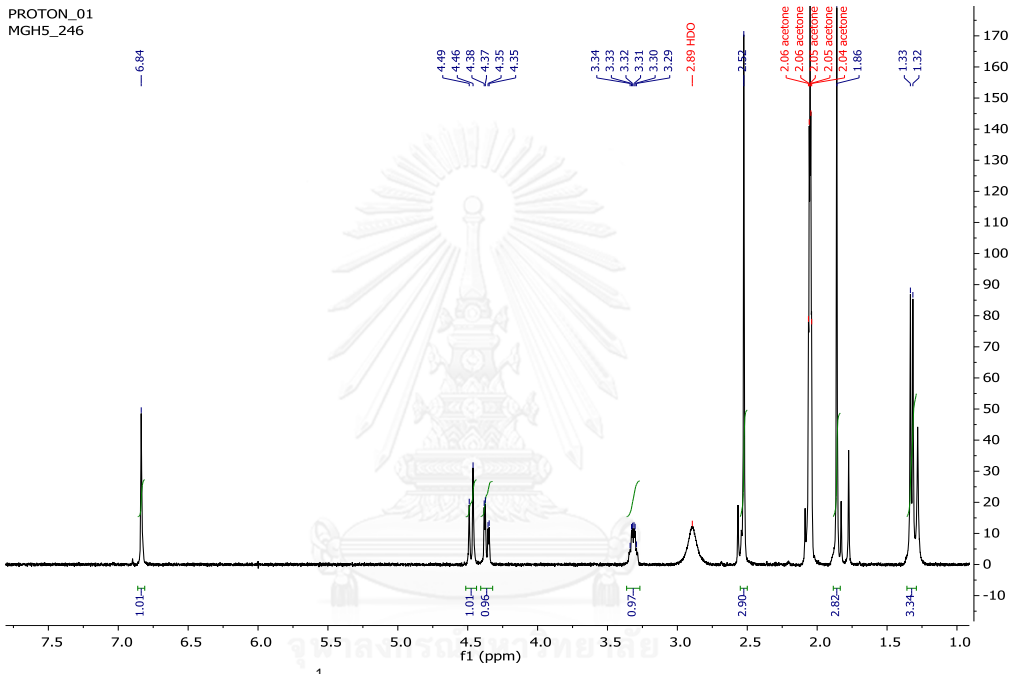
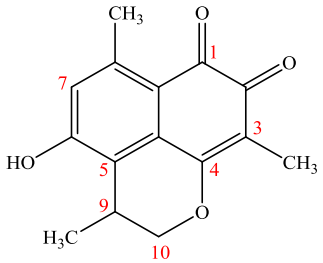


Figure 4.12 The ^{1}H NMR spectrum (acetone d_6) of compound **4-8**

Table 4.9 The comparison of ^{1}H NMR spectral data of mansonone H and compound **4-8** [99]

Position	Chemical shift (ppm)	
	mansonone H	Compound 4-8
6	-	-
7	6.71 (*s*, 1H)	6.84 (*s*, 1H)
9	3.27 (m, 1H)	3.32 (m, 1H)
10	4.46 (*dd*, *J* = 10.7, 0.9 Hz, 1H)	4.48 (*d*, *J* = 10.9 Hz, 1H)
	4.33 (*dd*, *J* = 11.0, 3.9 Hz, 1H)	4.36 (*dd*, *J* = 10.9, 3.3 Hz, 1H)
3-CH_3	1.89 (s, 3H)	1.86 (s, 3H)

8-CH_3	2.55 (s, 3H)	2.52 (*s*, 3H)
9-CH_3	1.31 (*d*, *J* = 7.0 Hz, 3H)	1.32 (*d*, *J* = 7.0 Hz, 3H)



Mansonone H (**4-8**)

4.6 *In vitro* antiphytopathogenic fungi activity of isolated compounds

All isolated compounds form the heartwoods of *M. gagei* were tested for antiphytopathogenic activity against *P. parasitica* by agar incorporation method which described in Chapter II at various concentrations (1, 10, 100 and 1,000 μg·mL^{-1}). The results of antifungal activity of these compounds were presented in **Table 4.10**.

Table 4.10 Percent inhibition and IC_{50} of isolated compounds against *P. parasitica*.

Compounds	%Inhibition**				IC_{50}
	1 μg·mL^{-1}	10 μg·mL^{-1}	100 μg·mL^{-1}	1000 μg·mL^{-1}	
mansorin A (**4-20**)	19.4±1.1^{lmn}	30.0±0.0^{hij}	33.3±2.2^{ghi}	38.9±0.8^{fgh}	>1,000
mansorin B (**4-21**)	15.2±1.9^{mno}	32.2±1.6^{ghi}	56.7±2.4^{d}	61.1±1.6^{c}	87.7
mansorin C (**4-22**)	13.3±0.8^{klm}	16.7±2.2^{mno}	17.7±1.8^{lmn}	17.8±1.2^{lmn}	>1,000
mansonone C (**4-3**)	27.8±2.1^{ijk}	53.3±1.2^{de}	61.1±1.2^{c}	84.4±2.6^{bc}	3.2
mansonone E (**4-5**)	0.0±0.0^{o}	11.1±1.8^{mn}	68.7±0.8^{c}	94.2±2.2^{ab}	62.5
mansonone G (**4-7**)	13.9±2.2^{mno}	41.7±0.6^{fg}	44.4±0.6^{ef}	81.2±1.8^{b}	110.0
mansonone H (**4-8**)	3.1±2.1^{no}	18.4±0.6^{lmn}	22.7±1.2^{jkl}	33.3±1.5^{fgh}	>1,000
Metalaxyl	45.9±1.4^{ef}	90.0±0.0^{ab}	100.0±0.0^{a}	100.0±0.0^{a}	2.2

*%yield based on CH_2Cl_2 extract, **Values, an average ± standard deviation (SD) of 3 replicates of the mean mycelial growth inhibition, [a]the different letter are significantly different (Tukey's HSD, *P<0.05*), (n=3)

The results showed that mansonone C (**4-3**) displayed the highest antiphytopathogenic activity with IC_{50} 3.2 μg·mL^{-1} against *P. parasitica*; followed by

mansonone E (**4-5**), mansorin B (**4-21**) and mansonone G (**4-7**) with IC_{50} 62.5, 87.7 and 110.0 μg·mL^{-1}, respectively. In case of inhibition, mansonone E (**4-5**) displayed the highest activity with 94% and followed by mansonone C (**4-3**) and G (**4-7**). From the results in Table 4.12, mansonone C (**4-3**) presented the antifungal activity higher than mansonone E at the lowest concentration (1 μg·mL^{-1}). Even though, mansorin B (**4-21**) and mansonone C (**4-3**) showed the IC_{50} value at low concentration. However, when compared the activity at high concentration (1000 μg·mL^{-1}), their inhibition lower than mansonone E (**4-5**) and G (**4-7**). That means when increasing the concentration higher than the maximum inhibitory, the compound did not increase to the effect of antifungal activity. Whereas mansorins A (**4-20**) and C (**4-22**) showed IC_{50} more than 1,000 μg·mL^{-1} and the inhibition as 39 and 18%, respectively.

4.6.1 Minimum inhibitory concentration (MIC) of isolated compounds

The MIC of isolated compounds was determined by double-broth microdilution method involving 96-well microtitre-plates as described in Chapter III. The results are displayed in **Table 4.11**.

Table 4.11 MIC of isolated compounds against phytopathogenic fungi

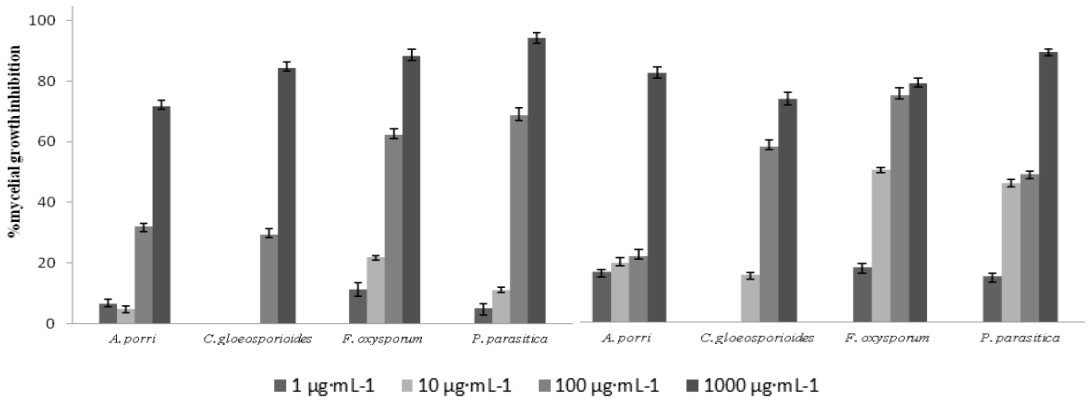
Compounds	MICs (μg·mL^{-1})			
	A. por	*C. glo*	*F. oxy*	*P. par*
Mansorin A (**4-20**)	1,000	>2,000	>2000	2000
Mansorin B (**4-21**)	500	>2,000	1000	2000
Mansorin C (**4-22**)	>2000	>2000	>2000	>2000
Mansonone C (**4-3**)	112	112	56	112
Mansonone E (**4-5**)	>2000	2,000	1000	2000
Mansonone G (**4-7**)	1000	2000	500	2000
Benomyl	ND	ND	<9.7	ND

Mansonone C (**4-3**) displayed the highest antiphytopathogenic activity with MIC 56.0 μg·mL^{-1} for *F. oxysporum* and 112.0 μg·mL^{-1} for the other tested fungi; followed by mansorin B (**4-21**), mansonones G (**4-7**), E (**4-5**) and mansorin A (**4-20**), respectively. Nevertheless, considering the yield of isolated compounds, little yield of mansonone C (**4-3**) was gained whereas mansonones E (**4-5**) and G (**4-7**) were obtained in much higher amount. Therefore, mansonones E (**4-5**) and G (**4-7**) were thought to be responsible as antifungal agents of this plant and were further investigated against other phytopathogenic fungi (*A. porri*, *C. gloeosporioides* and *F. oxysporum*) by agar incorporation method at various concentrations (0, 1, 10, 100 and 1,000 μg·mL^{-1}). After incubated at 27°C for 7 days, the mycelium growth of fungi was measured. The results as IC_{50} exhibited in **Table 4.12** and %inhibition of mansonone E (**4-5**) and G (**4-7**) at various concentrations showed in **Figure 4.13-4.15.**

Table 4.12 The IC_{50} of mansonones E and G against the mycelium growth of phytopathogenic fungi

	IC_{50}[a]		
Fungi	mansonone E	mansonone G	metalaxyl
A. porri	425.0	525.0	NT
C. gloeosporioides	375.0	87.5	NT
F. oxysporum	67.5	11.5	NT
P. parasitica	62.5	110.0	2.2

[a] IC_{50} using graphically, NT: Not Test



Mansonone E (**4-5**) Mansonone G (**4-7**)

Figure 4.13 Percent inhibition of mansonones E and G against phytopathogenic fungi

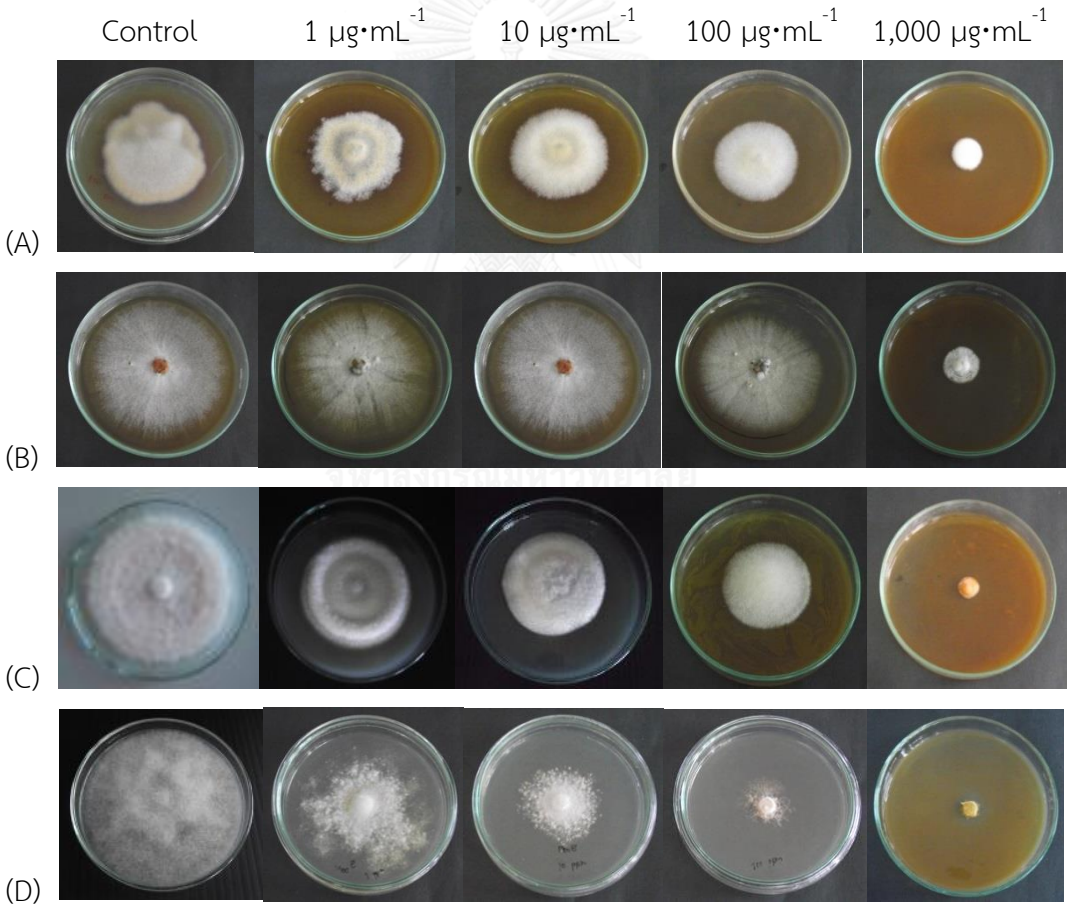


Figure 4.14 The mycelial growth inhibition zone of mansonone E (**4-5**) at various concentrations against (A): *A. porri*, (B): *C. gloeosporioides*, (C): *F. oxysporum*, (D): *P. parasitica.*

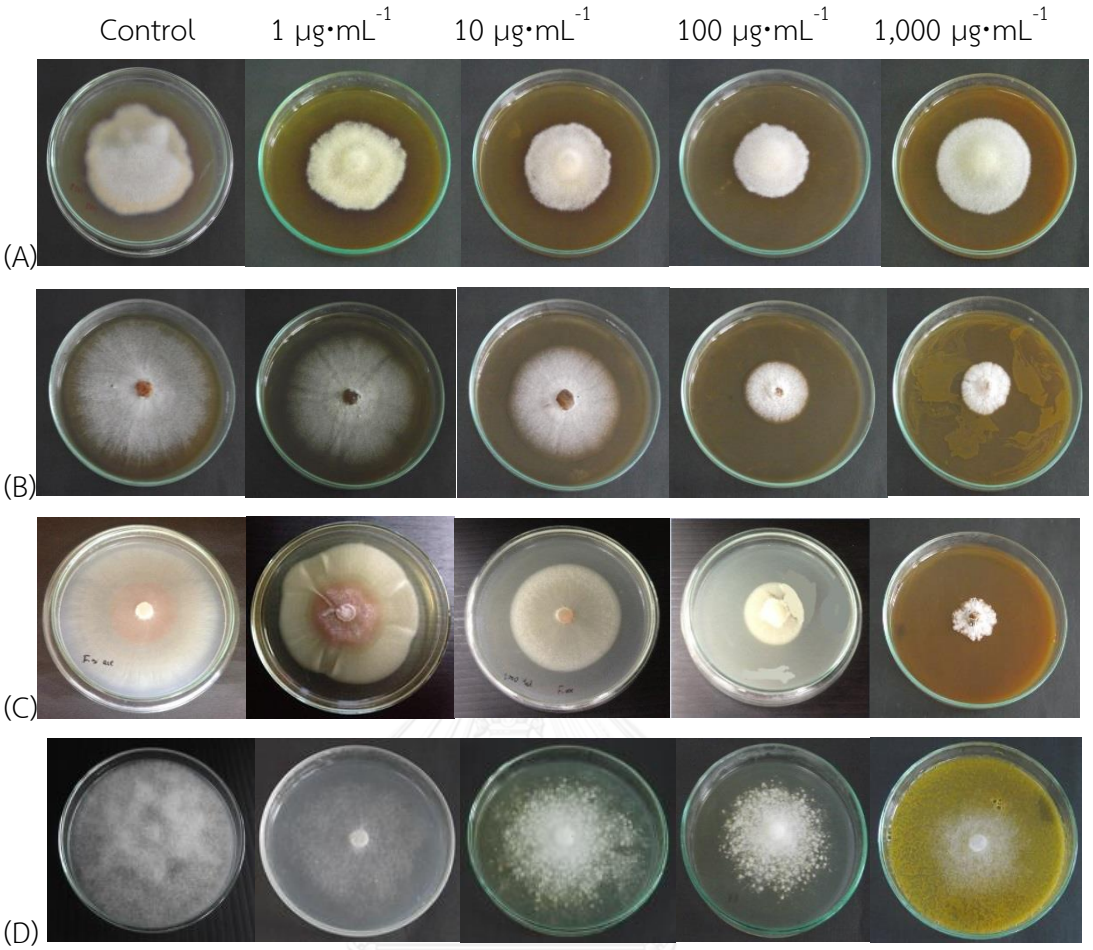


Figure 4.15 The mycelial growth inhibition zone of mansonone G (**4-7**) at various concentrations against (A): *A. porri*, (B): *C. gloeosporioides*, (C): *F. oxysporum*, (D): *P.parasitica*

From the antifungal activity of isolated compounds, it was observed the essence of substituent of 1,2-naphthoquinones: mansonones C (**4-3**), E (**4-5**) and G (**4-7**). To illustrate this, these compounds had the same main structure; however, they exhibited the antifungal activity with different extent. Mansonones C (**4-3**) and G (**4-7**) possessed almost the same structure, except for mansonone C (**4-3**) bearing OH at 6-position. This may be the reason why mansonone C (**4-3**) can be effective to inhibiting the mycelial growth of fungi higher than mansonone G (**4-7**). When mansonone E (**4-5**) was compared with mansonone G (**4-7**), the former composed of a pyran ring in its structure. This moiety may possess lower antifungal activity. In

addition, it was disclosed that coumarins as mansorins A (**4-20**), B (**4-21**) and C (**4-22**) displayed lower activity than 1,2-naphthoquinones (mansonones).

Nevertheless the yield of mansonone C (**4-3**) in nature was quite low when compared with those of mansonones E (**4-5**) and G (**4-7**). Therefore, the latter two mansonones were used for further investigation. Mansonone G (**4-7**) displayed strong antifungal activity against *F. oxysporum*, *C. gloeosporioides* and *A. porri* with IC_{50} 11.5, 87.5 and 525.0 μg·mL^{-1}, respectively, whereas mansonone E (**4-5**) inhibited *F. oxysporum, C. gloeosporioides* and *A. porri* with IC_{50} 67.5, 375.0 and 435.0 μg·mL^{-1}, respectively. From these results, it has demonstrated the specific correlation between compound and fungi species.

4.7.2 Antifungal activity of active compounds against spore germination

Spore suspensions were prepared under aseptic conditions according to a modified procedure described by Chang *et al.* (2007) [105]. The spores of *F. oxysporum* were grown on Petri plate containing PDA. Following 7-10 days of incubation at RT, fungal spores were removed using sterilized water. The resulting suspension was filtered aseptically through a sterilized muslin cloth. The filtrate which was adjusted using sterilized water to a concentration of 10^4 spore·mL^{-1} was estimated with haemacytometer slide (depth 0.1 mm, 1/400 mm^2) under microscope. One mL (10^4 spore·mL^{-1}) suspension was added to each tube containing 9 mL of active compound at various final concentrations (10, 100, 500 and 1000 spore·mL^{-1}), which the final concentration of spore suspension was 10^3 spore·mL^{-1}. These tested tubes were incubated at RT for 1 h and then 100 μL of the suspensions was streaked aseptically on fresh PDA plates. The plates were incubated at RT for 48 h. The fungal colonies originated from germinated spores were recorded compared with 0.5%DMSO plate and evaluated the %inhibition of spore germination (**Table 4.13**). The percentages of spore germination were calculated as follows:

Percentage inhibition of spore germination = [(C-T) × 100/C]

C: mean number of fungal colonies of control plate

T: mean number of fungal colonies of treatment

Table 4.13 Percent inhibition of mansonones E (**4-5**) and G (**4-7**) on spore germination of *F. oxysporum*

Compounds	Inhibition spore germination of *F. oxysporum* (%)			
	10 μg·mL^{-1}	100 μg·mL^{-1}	500 μg·mL^{-1}	1,000 μg·mL^{-1}
mansonone E	16.7±1.6	24.3±1.9	57.5±2.2	60.0±1.6
mansonone G	8.4±2.1	29.9±2.8	39.3±1.9	39.8±1.8

*Values, an average ± standard deviation (SD)

Mansonone E (**4-5**) displayed better inhibition against spore germination of *F. oxysporum* than mansonone G (**4-7**) with 60.0 and 39.8% at 1,000 μg·mL^{-1}, respectively. According to previous reports concerning the biological activities of mansonones, the antifungal activity of mansonones as phytoalexins, particularly the accumulation of mansonones E (**4-5**) and F (**4-6**) in *Ulmus hollandica* infected with *Ceratocystis ulmi* [106-108]. Six mansonones were isolated from *U. americana* which infected with *C. ulmi* [109, 110]. Another work involving the accumulation of phytoalexins in *U. americana* in response to infection by *Ophiostoma ulmi* was addressed [111].

4.8 Conclusions

The search for antifungal agents from the heartwoods of *M. gagei* against *P. parasitica* was conducted. According to the screening part (Chapter II), the CH_2Cl_2 extract displayed the antifungal activity higher than MeOH extract. The separation of the active fractions of the CH_2Cl_2 yielded six compounds. Based on spectroscopic data and their physical properties, the isolated compounds were characterized as mansorin A (**4-20**), mansonone C (**4-3**), mansorin C (**4-22**), mansorin B (**4-21**), mansonone E (**4-5**), mansonone G (**4-7**) and mansonone H (**4-8**). Mansonone C (**4-3**) displayed the highest antifungal activity with IC_{50} 3.23 μg·mL^{-1} against *P. parasitica*. However, mansonone C (**4-3**) was present in low yield. Mansonones E (**4-5**) and G (**4-7**) which were constituted in higher yield exhibited strong activity and were selected

for further antifungal examination against *A. porri* (71.0 and 75.0% inhibition), *C. gloeosporioides* (84.3 and 67.2% inhibition), *F. oxysporum* (88.2 and 71.0% inhibition) and *P. parasitica* (94.2 and 81.2% inhibition). The anti-spore germination of *F. oxysporum* of mansonones E (**4-5**) and G (**4-7**) was conducted (60.0and 39.8 %). For further study these compounds were tested *in vivo* against pineapple heart rot disease caused by *P. parasitica* as presented in Chapter 6.

CHAPTER 5
ANTIPHYTOPATHOGENIC FUNGI AGENTS FROM THE FLOWERS OF *Melodorum fruticosum* Lour

5.1 Introduction of *Melodorum fruticosum* Lour

M. fruticosum is the plant of Annonaceae family; the common names are known as devil tree, white cheesewood and Lamduan in Thai. This small shrub tree reaches a height between 8 to 12 m. The bark with dark brown shallow longitudinal groove smooth or broken stems. The leaves are single alternate leave elliptic or oblong-elliptic, 5-12 cm in long size, 2-3 cm in width, apex acute or pointed leave distortion with smooth edges smooth top surface is shiny and dark green. There are solitary, axillary of terminal flowers with cream or pale yellow color, small 3 outer sepal flowers and 3 inner petals are thick soft petal, downy outer petals and almost circular. Upon full bloom, a width range 2-2.3 cm, solitary and scented, the flower gives out a pleasant fragrance, especially in the evening (**Figure 5.1**). The flowers bloom in April for approximately 2 months. Fruits violet, slightly apiculate, ripe carpels ovoid, glabrous, 8 mm in long and 7 mm in diameter, stalks slender [112]. *Melodorum* sp. is a rare genus and contains only two species in Thailand including *M. fruticosum* and *M. siamensis* [113].

These plants are widely distributed throughout Indo-China, the north and northeast of Thailand containing mixed deciduous forests and dry evergreen forests in central and eastern part of the region. This plant was used as medical for tonic, mild cardiac stimulant, fever and hematinic resolve dizziness [114].

Figure 5.1 Fresh and dried flowers of *M. fruticosum*

The chemical constituents of *M. fruticosum* have been studied. Jung and coworkers (1990a) reported the presence of the heptene derivatives (heptadienes) with benzoyl moiety conjugated with C7 dienone or lactone terminal which appeared to arise from heptose or the equivalents. These compounds named melodienone (**5-1**), isomelodienone (**5-2**) and acetylmelodorinol (**5-3**) (**Figure 5.2**). Jung *et al*. (1990) isolated several bioactive compounds from the stem barks [115]. The active compounds were identified as dichamanetin (**5-4**), pinocembrin (**5-5**), polycarpol (**5-6**), benzyl benzoate (**5-7**) and a mixture of stigmasterol (**5-8**) and *β*-sitosterol (**5-9**). In 1990, Jung and colleagues reported four addition new bioactive heptenes including melodorinol (**5-10**), homomelo-dienone (**5-11**), 7-hydroxy-6-hydromelodinone (**5-12**), and homoisomelodienone (**5-13**) [116].

In addition, Tuchinda *et al*. (1991) studied the bioactive butenolides from the leaves and branches, five butenolides were isolated and identified as (4*Z*)-6-acetoxy-7-benzoyloxy-2,4-heptadien-4-olide (**5-14**), (4*E*)-6-acetoxy-7-benzoyloxy-2,4-heptadien-4-olide (**5-15**), (4*Z*)-7-benzoyloxy-6-hydroxy-2,4-heptadien-4-olide (melodorinol), (4*E*)-7-benzoyloxy-6-hydroxy-2,4-heptadien-4-olide (**5-16**) and (4*Z*)-6-benzoyloxy-7-hydroxy-2,4-heptadien-4-olide (**5-17**) [117]. Moreover, two flavonoids (chrysin (**5-18**) and pinocembrin (**5-5**)) and benzoic acid (**5-19**) were isolated. The chemical constituents from the flowers were investigated and assigned as 5,7-dimethoxyflavone (**5-20**), (4*Z*)-7-benzoyloxy-2,4-heptadien-6-one-4-olide (melodorinone A) (**5-21**), (4*E*)-7-benzoyloxy-2,4-heptadien-6-one-4-olide

(melodorinone B) (**5-22**), (*E*)-7-benzoyloxy-4-hydroxy-1-methoxy-2,4-heptadien-1,6-dione (tautomelodorinone) (**5-23**) [118, 119]. Juengwatanatrakul (2000) explored the chemical constituents from the seeds and roots. A novel triterpene was identified from the roots as lanosta-7,9(11),24-trien-3**β**-acetoxy-15**α**-ol (acetylpolycarpol (**5-24**)) [120].

Moreover, the odor constituents from the flowers were analyzed by solid-phase microextraction-GC-MS. The major volatile components were identified as ***β***-phelladrene (8.98%), *p*-methylanisole (7.02%), δ-cadinene (4.18%), germacrene B (3.18%) and bicyclogermacrene (3.62%) [80]. The chemical structures of *M. fruticosum* are shown in **Figure 5.2**.

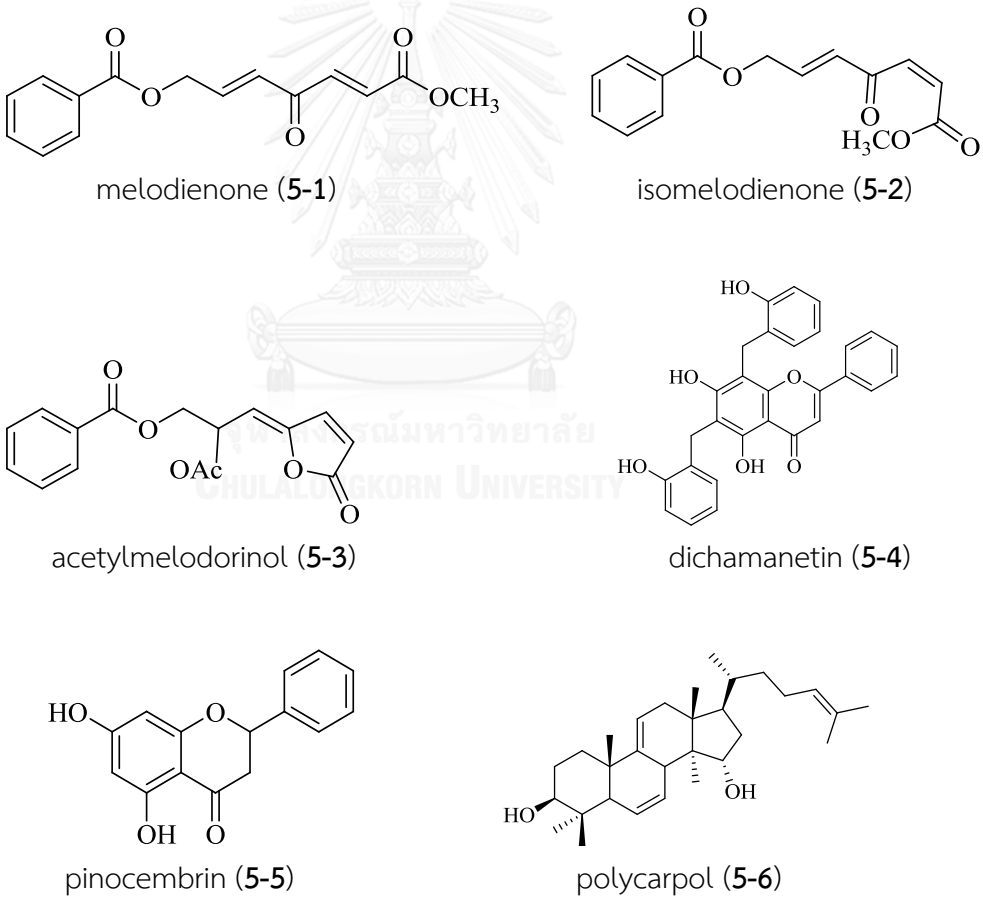
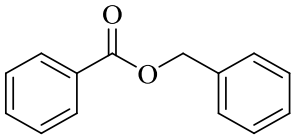
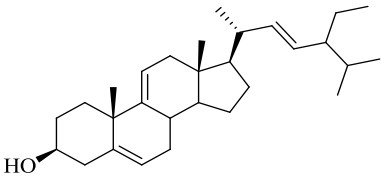


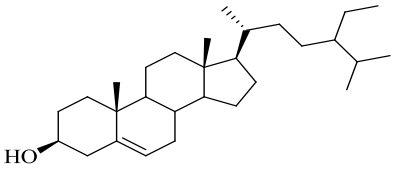
Figure 5.2 The chemical structures of *M. fruticosum*



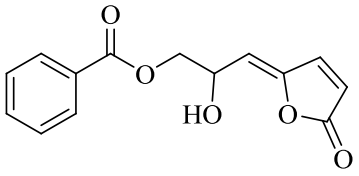
benzylbenzoate (**5-7**)



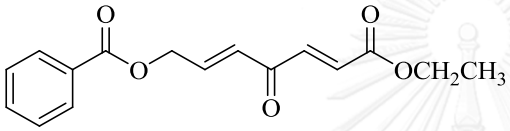
stigmasterol (**5-8**)



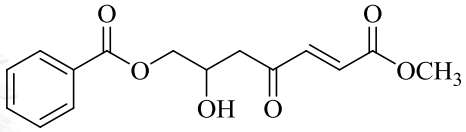
β-sitosterol (**5-9**)



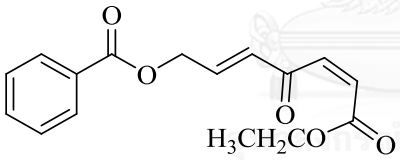
melodorinol (**5-10**)



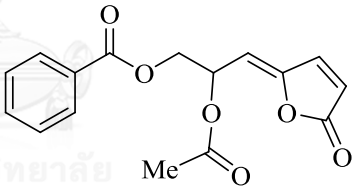
homomelodienone (**5-11**)



7-hydroxy-6-hydromelodinone (**5-12**)



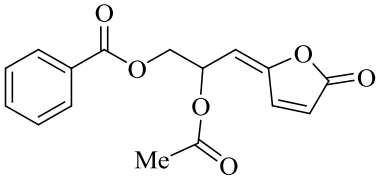
homoisomelodienone (**5-13**)



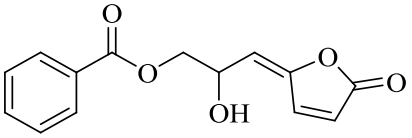
(4*Z*)-6-acetoxy-7-benzoyloxy-

(4*E*)-6-acetoxy-7-benzoyloxy-

2,4-heptadien-4-olide (**5-14**)



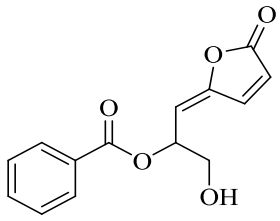
2,4-heptadien-4-olide (**5-15**)



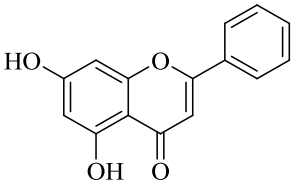
(4*E*)-7-benzoyloxy-6- hydroxy-

2,4-heptadien-4-olide (**5-16**)

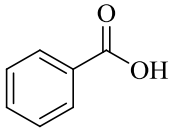
Figure 5.2 (cont.)



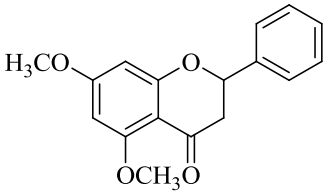
(4*Z*)-6-benzoyloxy-7-hydroxy-2,4-heptadien-4-olide (**5-17**)



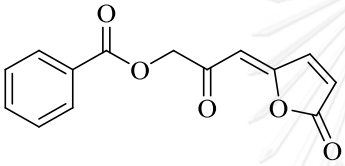
chrysin (**5-18**)



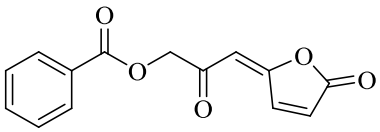
benzoic acid (**5-19**)



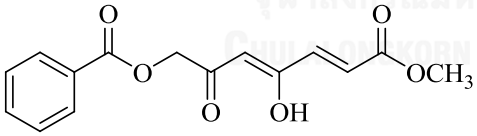
5,7-dimethoxyflavone (**5-20**)



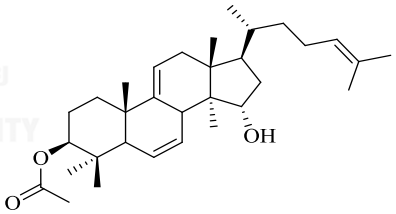
7-benzoyloxy-6-oxo-2,4*Z*-heptadien-1,4-olide (melodorinone A) (**5-21**)



7-benzoyloxy-4-hydroxyl-1-methoxy-2*E*,4*Z*-heptadien-1,6-dione (melodorinone B) (**5-22**)



tautomelodorinone (**5-23**)



acetylpolycarpol (**5-24**)

Figure 5.2 (cont.)

Table 5.1 The biological activity of isolated compounds from *M. fruticosum*

Compounds	Cytotoxicities* (μg·mL^{-1})											Brine shrimp LC_{50} (ppm)	Potato disc
	MCF-7	KB	HT-29	9-PS	A-549	SK-MEL-5	Melme-3M	Mel2	P-388	HT	Vero		
(5-1)	4.43b	-	3.85b	-	8.92b	-	-	-	-	-	-	24 (15/37)b	-
(5-2)	0.17b	-	0.51b	-	1.69b	-	-	-	-	-	-	10 (3/19)b	-
(5-3)	2.38c	1.2d	1.96c	-	2.89c	4.21c	2.74c	-	0.08d	1.8d		246 (163/383)b	-
	0.4b	-	0.3b	-	2.74b	-	-	-	-	-	-	-	-
	2.2d	-	1.3d	-	2.2d	-	-	-	-	-	-	-	-
(5-4)	-	5.38a	5.1a	-	>10a	-	-	-	-	-	-	3 (1/6)a	50/70a
(5-5)	-	>10a	>10a	7.53a	>10a	-	-	-	-	-	-	12 (8/20)a	20/90a
(5-6)	0.22a	-	0.12a	-	0.54a	-	-	-	-	-	-	254 (153/448)a	180/70
(5-7)	-	>10a	>10a	>10a	>10a	-	-	-	-	-	-	2 (1/3)a	110/30
(5-8),(5-9)	14.84	-	8.1a	-	15.13a	-	-	-	-	-	-	110 (51/181)a	10a
(5-10)	1.99c	-	2.87c	-	5.89c	3.75c	3.32c	-	-	-	-	-	-
	2.4d	2.3d	2.6d	-	7/9d	-	-	0.75d	0.35d	2.2d	-	-	-
(5-11)	37.88c	-	25.61c	-	36.91c	-	-	-	-	-	-	-	-
(5-12)	0.25c	-	1.92c	-	3.28c	1.04c	1.1c	-	-	-	-	-	-
(5-13)	36.32c	-	4.26c	-	6.7c	-	-	-	-	-	-	-	-
(5-15)	2.2d	0.49d	2.0d	-	1.1d	-	-	1.6d	0.14d	1.7d	-	-	-
(5-16)	2.7d	2.0d	1.7d	-	2.6d	-	-	0.92d	0.36d	3.7d	-	-	-
(5-17)	3.9d	2.6d	2.4d	-	2.5d	-	-	0.58d	0.33d	2.0d	-	-	-
(5-21)	5.0e	>20e	-	-	-	-	-	-	4.54e	-	15e	-	-
(5-22)	>20e	>20e	-	-	-	-	-	-	-	-	5e	-	-
(5-23)	7.5e	>20e	-	-	-	-	-	-	0.6e	-	7.5e	-	-
(5-24)	-	-	-	-	-	-	-	-	-	-	-	-	-

[a][115], [b][116], [c][121], [d][117], [e][118].

*Tumor cell lines: MCF-7 = Human breast carcinoma. KB = Human nasopharyngeal carcinoma, HT-29 = Human colon adrenocarcinoma, 9-PS = A chemical-induced murine lymphatic leukemia, A-549 = Human lung carcinoma, SK-MEL-5 = Human melanoma, metastasis of axillary node, Melme-3M = Human melanoma, metastasis of lung, Mel2 = Human melanoma, not specific, P-388 = Murine lymphatic leukemia, HT = Human fibrosacoma (HT-1080), Vero = African green monkey kidney cell line

The biological activities including cytotoxicity, antitumor and plant growth regulation of the components from *M. fruticosum* were examined as presented in **Table 5.1**. In addition, antifungal and antioxidant activities of the essential oil and various extracts from the flowers were addressed. The CH_2Cl_2 extract displayed the highest antifungal activity to all tested pathogens (*Collectotrichum* sp. (46.7%inh.), *C. asianum* (42.6%), *C. siamense* (38.4%), *C. gloeosporioides* (40.9%), *C. fruiticola* (49.7%), *C. acutatum* (36.4%), *Trichoderma reesei* (22.2%) and *Lasiodipplodia theobromae* (52.9%) [50].

However, there have been no reports concerning the antiphytopathogenic activity from the flowers of this plant. Therefore, the objectives of this research are to isolate the active compounds from the flowers of *M. fruticosum* and to test for antiphytopathogenic fungi activity against four selected fungi.

5.2 Plant material and extraction

Dried flowers of *M. fruticosum* were purchased from the herbal drugstore "Chow Khom Per", Bangkok, Thailand in June 2013. According to the results of antifungal activity screening in Chapter II, the CH_2Cl_2 extract showed antifungal activity higher than the MeOH part. Thus, further investigation would focus on the CH_2Cl_2 extract. Dried flowers (10 kg) were milled and extracted by maceration for three times with CH_2Cl_2 at RT. After that, the extracts were filtered and evaporated under reduce pressure to gain the CH_2Cl_2 extract as dark brown solid 325.5 g (3.26% yield of dry weight) and kept in the dark until used.

5.3 Separation of the CH_2Cl_2 extract from the flowers and bioassay guide

After extraction, the CH_2Cl_2 extract (150 g) was fractionated by quick column using silica gel (Merck, Kieselgel 60 Art 7729). A stepwise elution was conducted by 100% hexane and increasing the polarity with EtOAc and finally with 10% MeOH in EtOAc. All 26 fractions were collected and combined according to TLC behavior and then evaporated to obtain 10 subfractions (**MFF1**-**MFF10**). **Table 5.2** showed the separation results of the CH_2Cl_2 extract and percent yield.

MFF8 provided the highest yield 53.92 g (35.9% yield based on the CH_2Cl_2 extract) followed by **MFF3**, **MFF7** and **MFF9** (19.2, 14.2 and 11.6% yield). The other fractions gave low yield (0.3-7.6%). For further study, all fractions were tested for antifungal activity against *P. parasitica* by agar incorporation method as described in Chapter II for bioassay guide at 1,000 $\mu g \cdot mL^{-1}$ to search for active fractions. The results of antifungal activity of these fractions are displayed in **Table 5.2** and **Figure 5.3**.

Table 5.2 The separation of the CH_2Cl_2 extract by quick column.

Fraction No.	Solvent system (%v/v)	Remarks	Weight (g)	Yield (%)
MFF1	100% hexane	yellow oily wax	2.77	1.84
MFF2	5% EtOAc in hexane	orange oily wax	0.43	0.27
MFF3	5-10% EtOAc in hexane	orange brown oily wax	28.8	19.20
MFF4	10% EtOAc in hexane	dark orange oily wax	4.25	2.83
MFF5	10-20% EtOAc in hexane	dark brown semi solid	3.94	2.62
MFF6	20% EtOAc in hexane	dark yellow semi solid	6.26	4.17
MFF7	40% EtOAc in hexane	brown semi solid	21.29	14.19
MFF8	60% EtOAc in hexane	brown semi solid	53.92	35.95
MFF9	80% EtOAc in hexane	dark brown solid	17.39	11.59
MFF10	100% EtOAc- 5% MeOH in EtOAc	dark solid	11.37	7.58

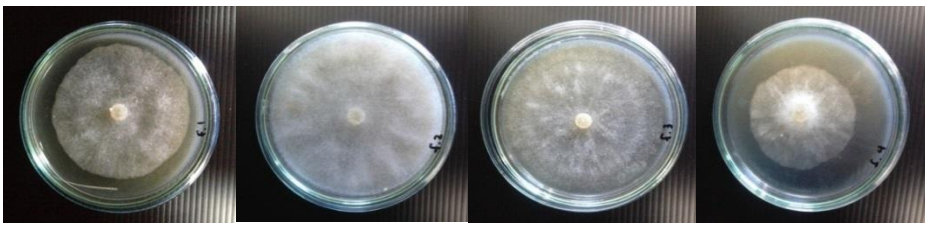
Table 5.3 The percentage inhibition of mycelial growth of subfractions against *P. parasitica* at 1,000 μg·mL^{-1}

Fraction no.	Growth (mm)[a]	%inhibition[a]
MFF1	75.3±2.5	16.3±2.8
MFF2	90.0±0.0	0.0±0.0
MFF3	82.3±1.5	8.6±1.7
MFF4	65.0±6.1	27.8±6.8
MFF5	0.0±0.0	100.0±0.0
MFF6	0.0±0.0	100.0±0.0
MFF7	27.7±2.1	69.2±2.3
MFF8	13.3±2.1	85.2±2.3
MFF9	37.0±0.0	58.9±0.0
MFF10	80.0±0.0	11.1±0.0
DMSO	100.0±0.0	0.0±0.0
Metalaxyl (100 μg·mL^{-1})	0.0±0.0	100.0±0.0

[a]Values, an average ± standard deviation of 3 replicates, of the mean diameter of mycelial growth and inhibition

From **Table 5.2** and **Figure 5.3**, **MFF5-6** completely inhibited *P. parasitica* at concentration of 1,000 μg·mL^{-1}, followed by **MFF8** (85.2%), **MFF7** (69.2%) and **MFF9** (58.9%). The mycelial growth of fungi from **MFF2** was found to grow faster than that of the control plate. This suggested that some active compounds be present. **MFF1**, **3**, **4** and **10** slightly inhibited the mycelial growth of *P. parasitica* (16.3, 8.6, 27.8 and 11.1%, respectively).

(A)



(B)

(C)

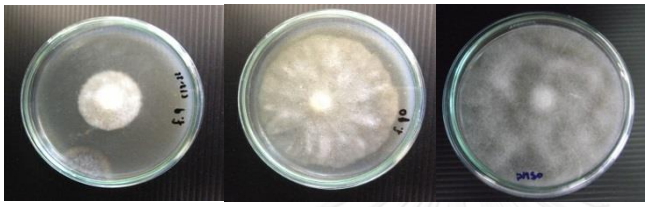


Figure 5.3 The inhibition of separated fractions of *M. fruticosum* flowers against *P. parasitica* at 1,000 μg·mL^{-1}

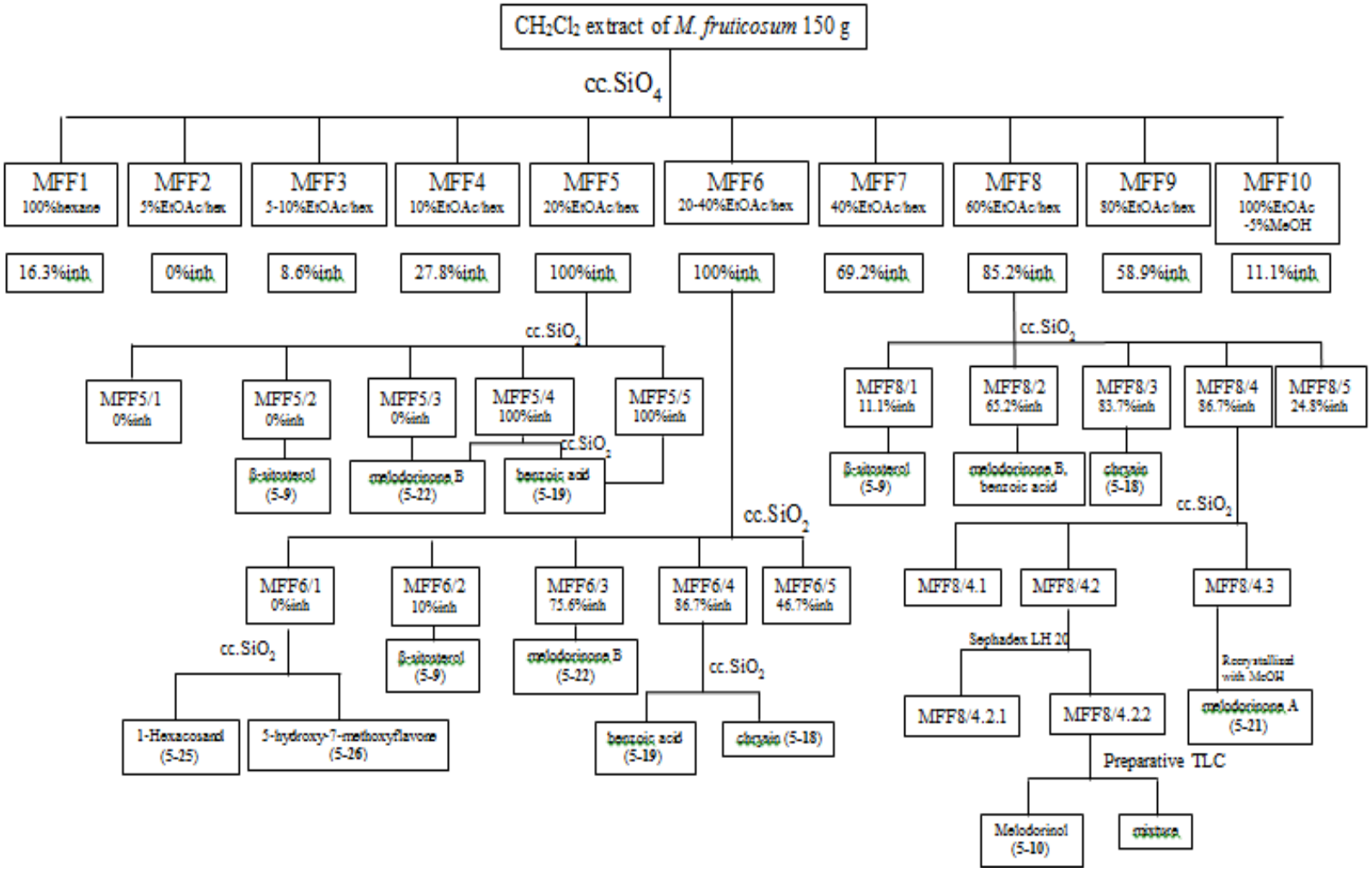
A: **MFF1**, **MFF2**, **MFF3**, **MFF4**

B: **MFF5**, **MFF6**, **MFF7**, **MFF8**

C: **MFF9**, **MFF10**, DMSO

5.4 Isolation and identification of bioactive compounds

According to the results above, bring to the further study to isolate the pure compounds from **MFF5**, **6** and **8** which displayed excellent antifungal activity. Each active fraction **MFF5** (3.90 g), **MFF6** (6.2 g) and **MFF8** (50.0 g) were subjected to column chromatography using silica gel 60 (Merck, art 7734, 63-200 mesh; art 9385, 40-63 mesh) and Sephadex LH-20, Chromatotron. Thin layer chromatography (TLC) was prepared on pre coated silica gel 60 F254 plates. The elution system as EtOAc in hexane, gradually increased the polarity with EtOAc was used for this isolation. Finally, 8 compounds were isolated from these active fractions including 1-hexacosanol (**5-25**), 5-hydroxy-7-methoxyflavone (**5-26**), ***β***-sitosterol (**5-8**), melodorinone B (**5-22**), benzoic acid (**5-19**), chrysin (**5-8**), melodorinol (**5-10**), melodorinone A (**5-21**). The **scheme 5.1** showed the fractionation, isolation and bioassay guide of antifungal activity against *P. parasitica* at 1000 μg·mL^{-1}.

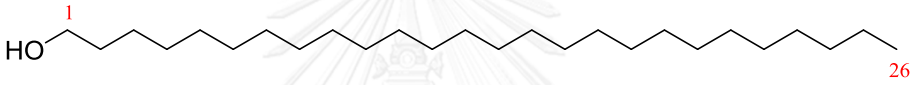


Scheme 5.1 The isolation of DCM extract of *M. fruticosum* flower

5.5 Structural elucidation

5.5.1 Compound 5-25: 1-Hexacosanol

Fraction MFF6/1 gave compound **5-25** with white powder (210.0 mg). This compound showed a single spot on TLC with R_f value of 0.63 (elution system 1:3 of EtOAc:hexane). The ^{1}H and ^{13}C NMR spectrum of compound **5-25** is shown in **Figure 5.4-5.5**. The comparison of ^{1}H NMR spectroscopic data of compound **5-25** and reported 1-hexacosanol is collected in **Table 5.4**. From these NMR data analyses, the compound **5-25** was designated as long chain alcohol, 1-hexacosanol (**5-25**). From our best knowledge of literature survey, this compound is isolated for the first time in *M. fruticosum*.



1-Hexacosanol (**5-25**)

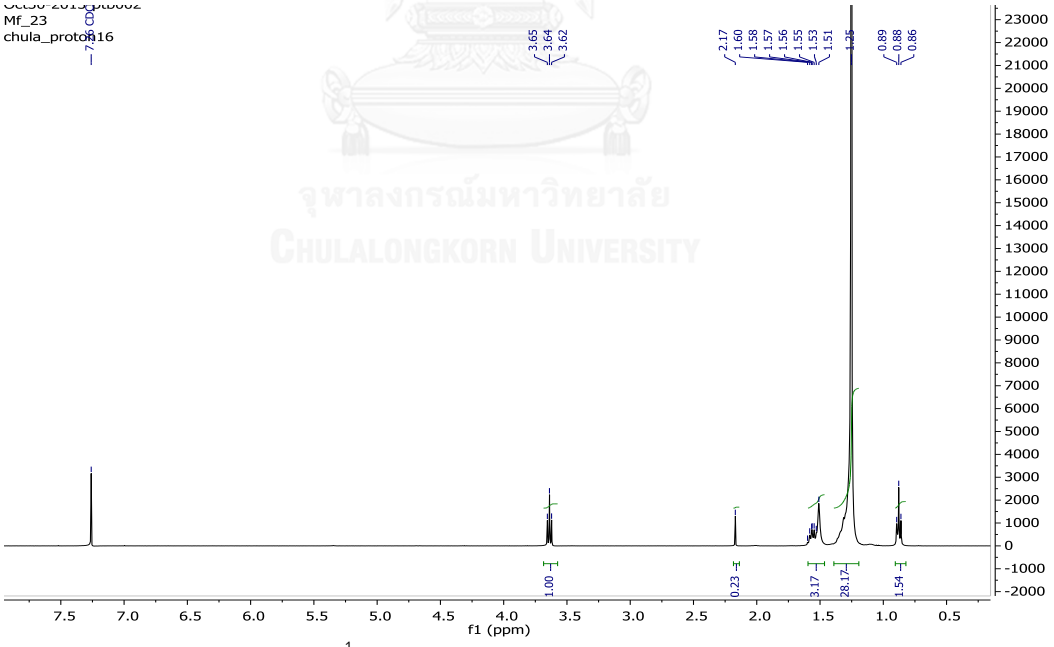


Figure 5.4 The ^{1}H NMR spectrum ($CDCl_3$) of compound **5-25**

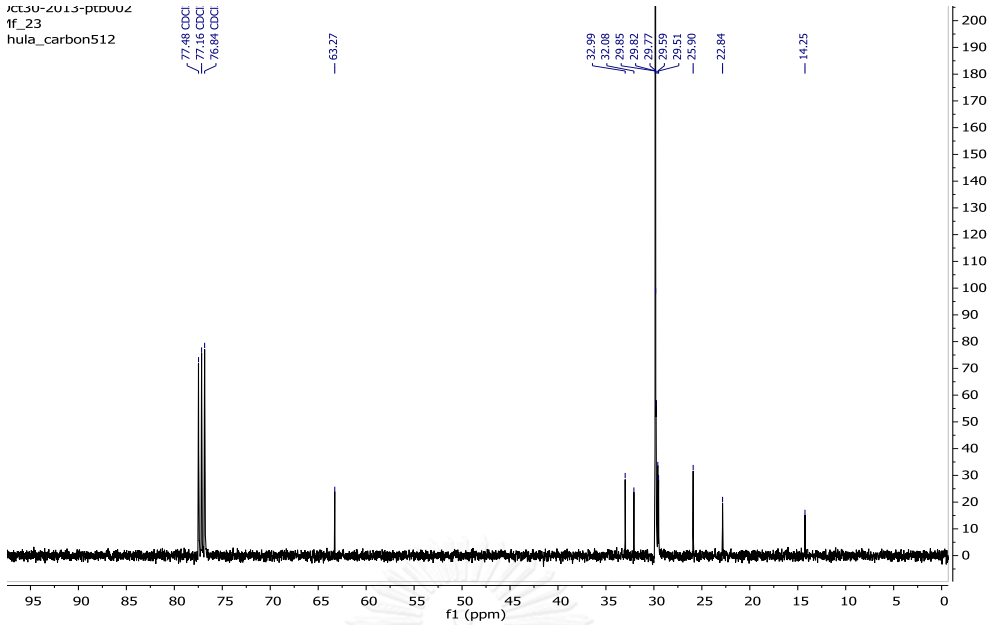


Figure 5.5 The ^{13}C NMR spectrum ($CDCl_3$) of compound **5-25**

Table 5.4 The comparison of ^{1}H and ^{13}C NMR spectral data of 1-Hexacosanol and compound **5-25** **[122]**

	Chemical shift (ppm)			
	1-Hexacosanol [a]		Compound 5-25 [b]	
Position	δ_H	δ_C	δ_H	δ_C
1		63.01		63.27
2		32.90		32.99
24		32.04		32.08
4-23	3.65	29.69		29.77
3	1.25	25.83	3.64	25.90
25	0.90	21.3	1.25	22.84
26		13.8	0.88	14.25

[a] ^{1}H-NMR and ^{13}C-NMR analysis in $CDCl_3$ at 500 MHz

[b] ^{1}H-NMR and ^{13}C-NMR analysis in $CDCl_3$ at 400 MHz

5.5.2 Compound 5-26: 5-hydroxy-7-methoxyflavone

Compound **5-26** was obtained from **MFF6/1** with yellow crystal (19 mg). It showed a single spot on TLC, R_f value as 0.57 (elution system: 2.5:7.5 of EtOAc:hexane). The molecular formula was assigned to be $C_{16}H_{12}O_4$. The ^{1}H and ^{13}C NMR spectrum of compound **5-26** were shown in **Figure 5.6-5.7** and the tentative NMR chemical shift assignment were compared with the previous report [123] in **Table 5.5**.

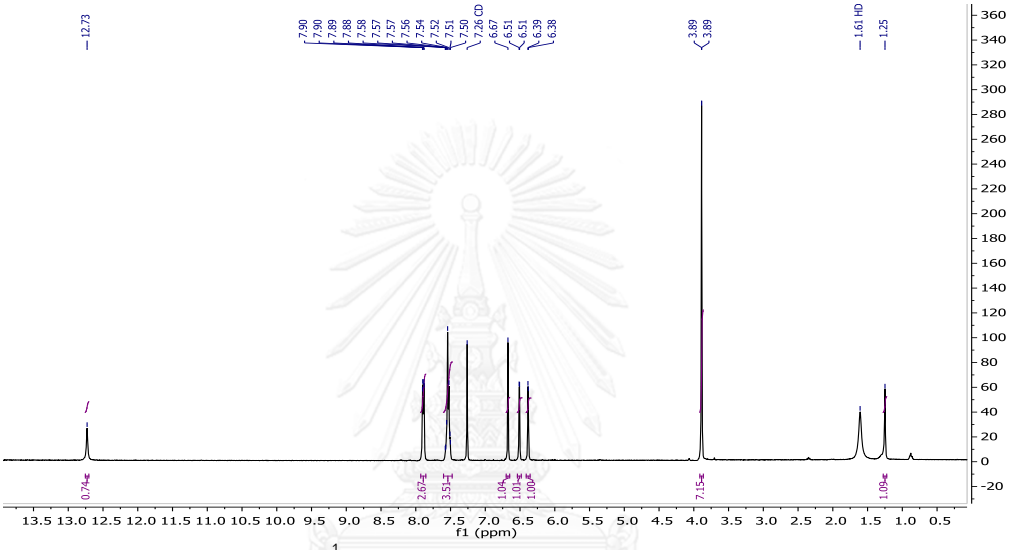


Figure 5.6 The ^{1}H NMR spectrum ($CDCl_3$) of compound **5-26**

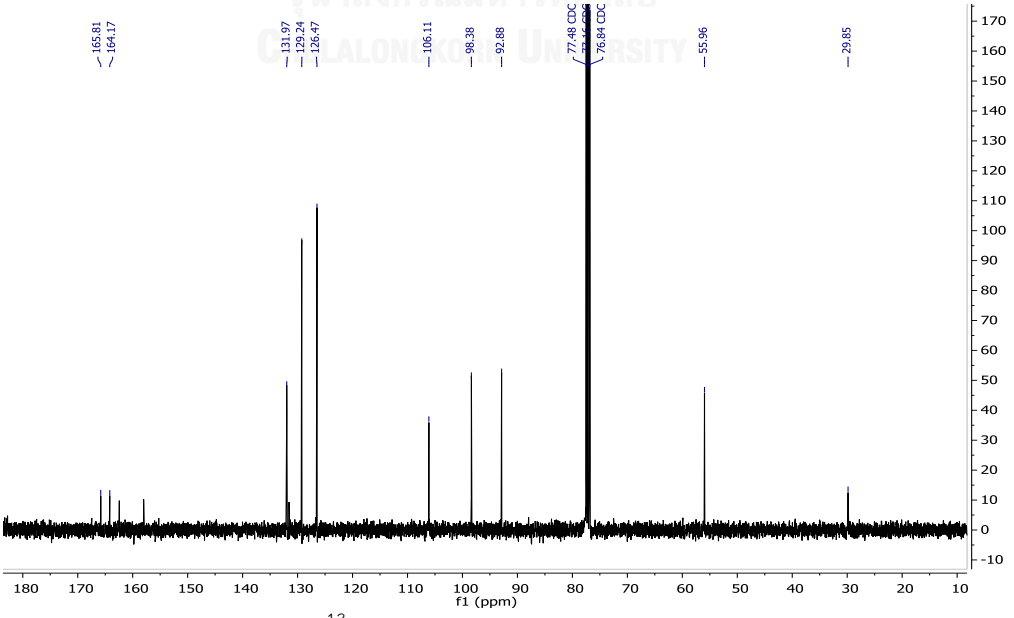


Figure 5.7 The ^{13}C NMR spectrum ($CDCl_3$) of compound **5-26**

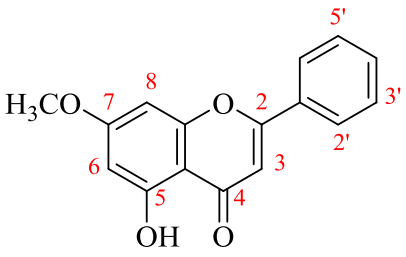
Table 5.5 The comparison of ^{1}H and ^{13}C NMR spectral data of techtochrysin and compound **5-26** [123]

Position	Chemical shift (ppm)			
	5-hydroxy-7-methoxyflavone [a]		Compound 5-26 [b]	
	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
2		163.98		164.17
3	6.65 (*s*)	105.86	6.67 (*s*, 1H)	106.11
4		182.50		-
4a		105.71		-
5		162.18		162.43
6	6.37 (br *d*, *J* = 1.5 Hz)	98.21	6.38 (*d*, *J* = 2.3 Hz, 1H)	98.38
7		165.62		165.81
8	6.47 (br *d*, *J* = 1.5 Hz)	92.68	6.51 (*d*, *J* = 2.2 Hz, 1H)	92.88
8a		157.80		158.01
1'		131.31		131.55
2', 6'	7.89 (m)	126.29	7.89 (m)	126.47
3', 5'	7.54 (m)	128.76	7.53 (m)	129.24
4'	7.54 (m)	131.85	7.53 (m)	131.97
OH	12.73 (*s*)		12.73 (*s*, 1H)	
OCH_3	3.87 (*s*)	55.83	3.89 (*s*, 1H)	55.96

[a] ^{1}H-NMR and ^{13}C-NMR analysis in $CDCl_3$ at 200 and 50 MHz, respectively

[b] ^{1}H-NMR and ^{13}C-NMR analysis in $CDCl_3$ at 400 and 100 MHz, respectively

As the results of ^{1}H and ^{13}C NMR of compound 15 was identified as 5-hydroxy-7-methoxy-2-phenyl-4H-chromen-4-one or 5-hydroxy-7-methoxyflavone or 5-hydroxy-7-methoxyflavone or techtochrysin (**5-26**). From our best knowledge of literature survey, this compound is isolated for the first time in *M. fruticosum*.



5-hydroxy-7-methoxy-2-phenyl-4H-chromen-4-one (**5-26**)

5.5.3 Compound 5-9: *β*-sitosterol

Compound **5-9** was purified by silica gel column of the CH_2Cl_2 extract and recrystallized with EtOAc in hexane, colorless needle (430 mg) with R_f value 0.47 (elution system: 2.5:7.5 of EtOAc:hexane) was obtained and it could not be detected under UV, but could be detected with $KMnO_4$ solution and heat. Molecular formula was determined to be $C_{29}H_{50}O$ according to ^{1}H and ^{13}C NMR spectrum (**Figure 5.8-5.9**). The structure was compared with previous report in **Table 5.6** [124].

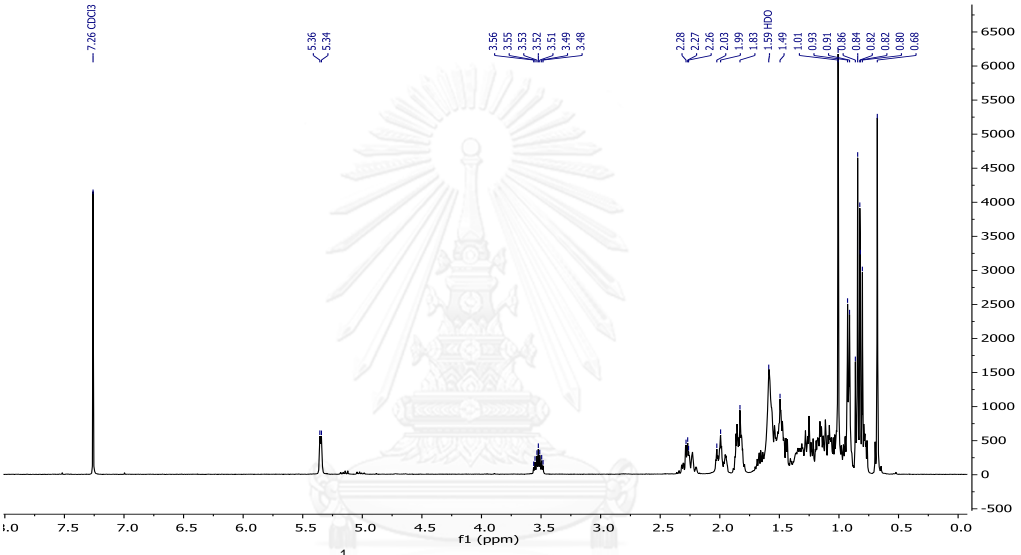


Figure 5.8 The ^{1}H NMR spectrum ($CDCl_3$) of compound **5-9**

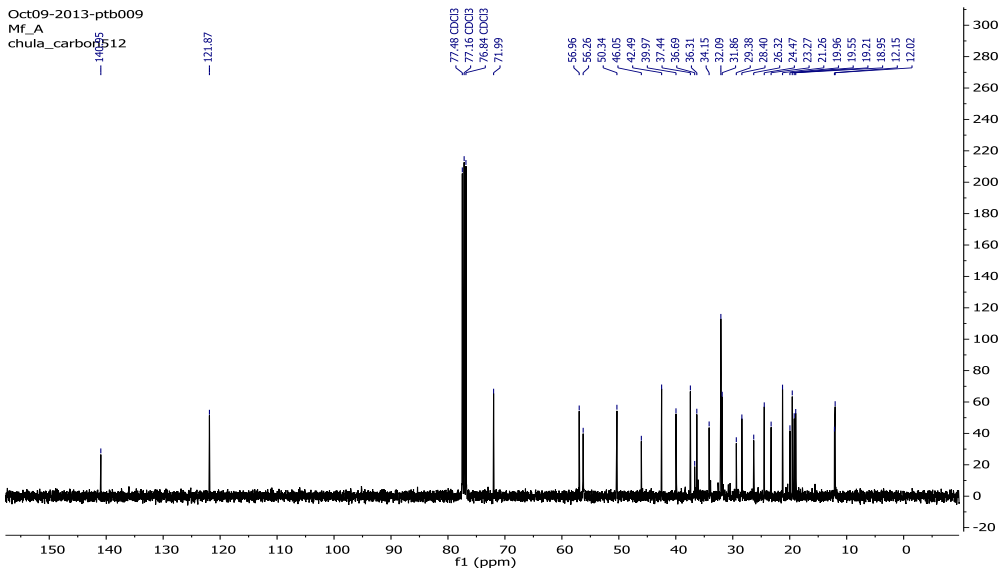
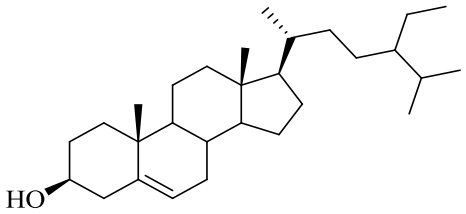


Figure 5.9 The ^{13}C NMR spectrum ($CDCl_3$) of compound **5-9**

Table 5.6 The comparison of ^{1}H and ^{13}C NMR spectral data of ***β*-sitosterol** and compound **5-9** [124]

Position	Chemical shift (ppm)[a]			
	***β*-sitosterol**		**Compound 5-9**	
	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
1	3.53 (m, 1H)	37.5	3.52 (m, 1H)	37.4
2		31.9		31.9
3		72.0		71.8
4		42.6		42.3
5		141.0		140.8
6	5.36 (*d*, *J* = 7.3, 5.1 Hz, 1H)	121.9	5.35 (*d*, *J* = 5.3 Hz, 1H)	121.7
7		32.1		31.7
8		32.2		32.4
9		50.4		50.1
10		36.7		36.5
11		21.3		21.1
12		40.0		39.8
13		42.6		42.3
14		57.0		56.8
15		24.5		24.3
16		28.5		28.4
17		56.3		56.1
18	0.69 (*s*, 3H)	12.1	0.68 (*s*, 3H)	12.0
19	1.02 (*s*, 3H)	19.6	1.01 (*s*, 3H)	19.4
20		36.4		36.1
21	0.93 (*d*, *J* = 6.6, 6.2 Hz, 3H)	19.0	0.92 (*d*, *J* = 6.7Hz, 3H)	18.9
22		34.2		34.0
23		26.3		26.1
24		46.1		45.9
25		29.4		29.2
26	0.85 (*d*, *J* = 7.3, 7.0 Hz, 3H)	19.3	0.85 (*d*, *J* = 7.6 Hz, 3H)	19.0
27	0.83 (*d*, *J* = 7.0 Hz, 3H)	20.1	0.81 (*d*, *J* = 8.7 Hz, 3H)	19.9
28		23.3		23.1
29	0.87 (*t*, *J* = 7.7, 7.0 Hz, 3H)	12.2	0.87 (*d*, *J* = 8.1 Hz, 3H)	1.9
	1.05-2.32 (other)		1.52-2.30 (other)	

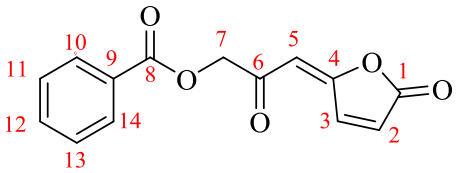
As the NMR spectral data, compound 17 was assigned as ***β***-sitosterol (**5-9**). β-sitosterol has been isolated in various plants such as in *Ocimum sanctum* leaves, *Stylochiton lancifoliun* rhizomes, *Corylus colurna* fruits, aerial parts of *Ageratum conyzoides*, *Etlingera sphaerocephala* rhizomes, *Momordica charantia* leaves and *Rubus suavissimus* leaves [125-129].



β-sitosterol (**5-9**)

5.5.4 Compound 5-20: melodorinone B

Compound **5-20** was isolated as colorless bulky crystal (350 mg) and recrystallization with EtOAc. It was showed one spot on TLC with R_f value as 0.46 (elution system: 2.5:7.5 of EtOAc: hexane) and detected under UV lamp 254 nm. The molecular formula was established from NMR data to be $C_{14}H_{10}O_5$. The ^{1}H NMR spectroscopic data were showed in **Figure 5.10** and **Table 5.7**. Compound 17 could be proposed as in transoid configulation as (4*E*)- 7-benzoyloxy-2,4- heptadien-6-one-4-olide or melodorinone B (**5-20**). This compound has been reported as a new derivative of heptenes group which isolated from flower of *M. fruticosum* [118, 119].



(4*E*)- 7-benzoyloxy-2,4- heptadien-6-one-4-olide (melodorinone B) (**5-20**)

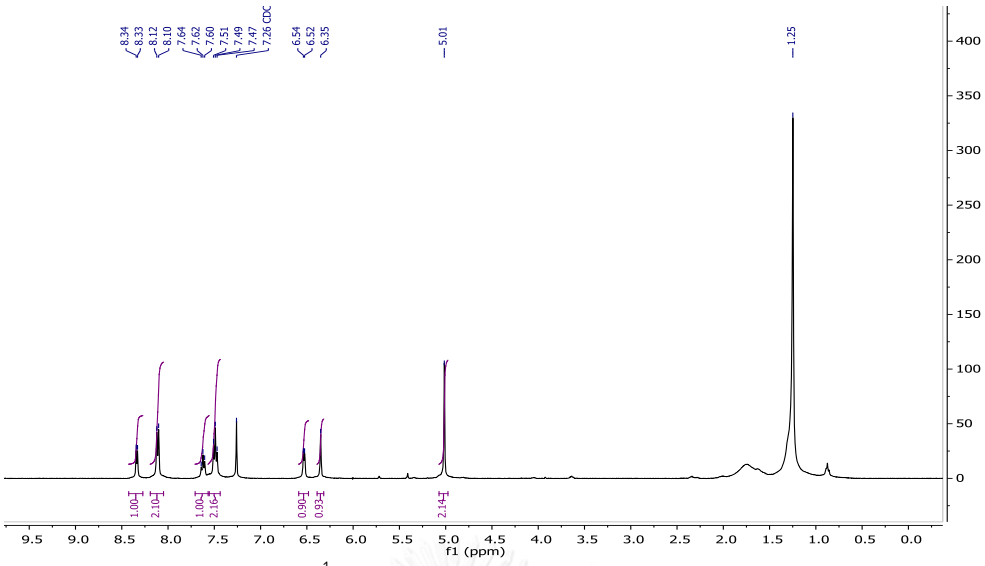


Figure 5.10 The ^{1}H NMR spectrum ($CDCl_3$) of compound **5-20**

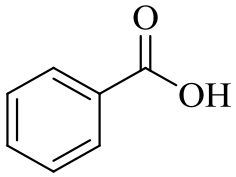
Table 5.7 The comparison of ^{1}H NMR data of Melodorinone-B and compound **5-20** [118]

Position	Chemical shift (ppm)	
	Melodorinone-B	Compound 5-20
2	6.51 (*d*, *J* = 5.7, 1.3 Hz, 1H)	6.53 (*d*, *J* = 4.9 Hz, 1H)
3	8.32 (*d*, *J* = 5.7 Hz, 1H)	8.33 (*d*, *J* = 5.7 Hz, 1H)
4	-	-
5	6.33 (*d*, *J* = 1.3 Hz, 1H)	6.35 (*s*, 1H)
6	-	-
7	4.99 (*s*, 2H)	5.01 (*s*, 2H)
10, 14	8.09 (*m*, 2H)	8.11 (*d*, *J* = 7.7 Hz, 2H)
11, 13	7.47 (*m*, 2H)	7.49 (*m*, 2H)
12	7.60 (*m*, 1H)	7.62 (*m*, 2H)

5.5.5 Compound 5-19: Benzoic acid

Compound **5-19** was isolated as colorless crystal (270 mg). It showed single spot on TLC, R_f 0.40 (elution system: 2.5:7.5 of EtOAc: hexane), detected with UV 254 nm and the molecular formula as $C_7H_6O_2$. As the NMR data (**Figure 5.11-5.12**), the compound 18 was identified as benzoic acid (**Table 5.8**). Benzoic acid is a white solid which slightly soluble in water and used as the intermediate in the synthesis to

derivative of this compound (Goodwin, 1976). Benzoic acid is produced by naturally plants and in animals and used as preservative in foods, fruit juices, soft drink, beverages, toothpastes, mouthwashes, cosmetics, pharmaceuticals and coloring agent [130]. Moreover, benzoic acid was found in Chinese Balsam tree which called gum benzoin [131].



Benzoic acid (**5-19**)

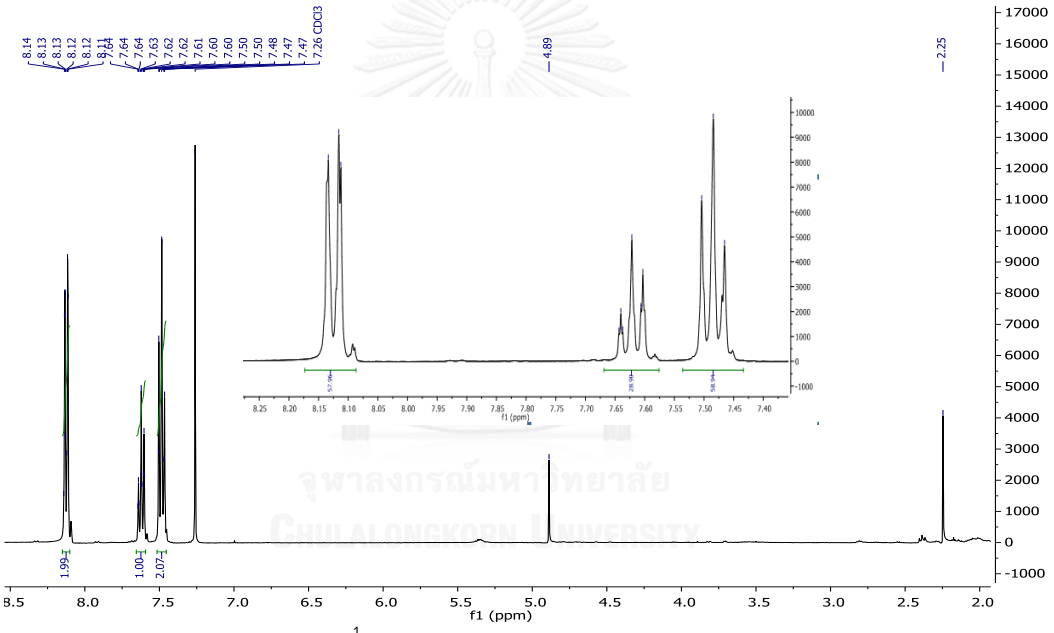


Figure 5.11 The ^{1}H NMR spectrum ($CDCl_3$) of compound **5-19**

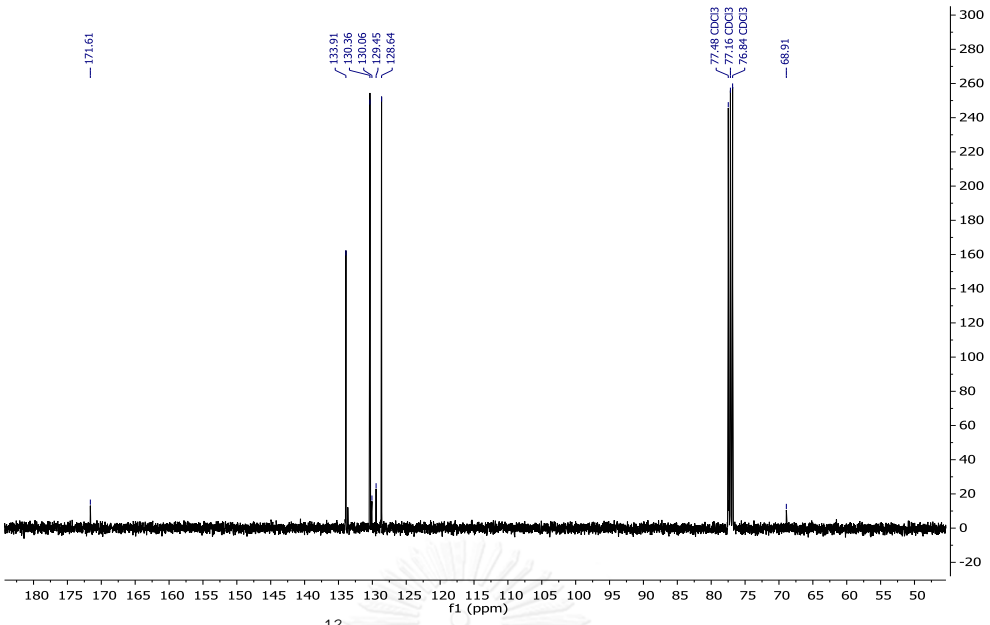


Figure 5.12 The ^{13}C NMR spectrum ($CDCl_3$) of compound **5-19**

Table 5.8 The comparison of ^{1}H NMR spectral data of benzoic acid and compound **5-19** [132]

Position	Chemical shift (ppm)[a]			
	Benzoic acid		Compound 15-19	
	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
1		129.6		129.5
2	8.13 (*dd*, *J* = 8.8, 1.6 Hz)	130.4	8.12 (*dd*, *J* = 8.3, 1.4 Hz, 1H)	130.4
3	7.47 (*t*, *J* = 7.6 Hz)	128.7	7.48 (*t*, *J* = 7.7 Hz, 1H)	128.7
4	7.61 (*tt*, *J* = 7.6, 1.6 Hz)	134.1	7.62 (*m*, 1H)	133.9
5	7.47 (*t*, *J* = 7.6 Hz)	128.7	7.48 (*t*, *J* = 7.7 Hz, 1H)	128.7
6	8.13 (*dd*, *J* = 8.8, 1.6 Hz)	130.4	8.12 (*dd*, *J* = 8.3, 1.4 Hz, 1H)	130.4
7		172.8		171.6

5.5.6 Compound 5-18: 5,7-dihydroxyflavone

However, MFF6 and MFF8 gave compound **5-18** as yellow powder and exhibited a single spot on TLC with R_f value as 0.66 (elution system: 2:3 of EtOAc: hexane). The molecular formula of compound **5-18** was indicated as $C_{15}H_{10}O_4$. **Figure 5.13-14** showed the pattern of ^{1}H NMR spectrum, which similar to flavones

compound. As the comparison of this compound with the previous report (**Table 5.9**), compound **5-18** was assigned as chrysin or 5,7-dihydroxyflavone (**5-18**).

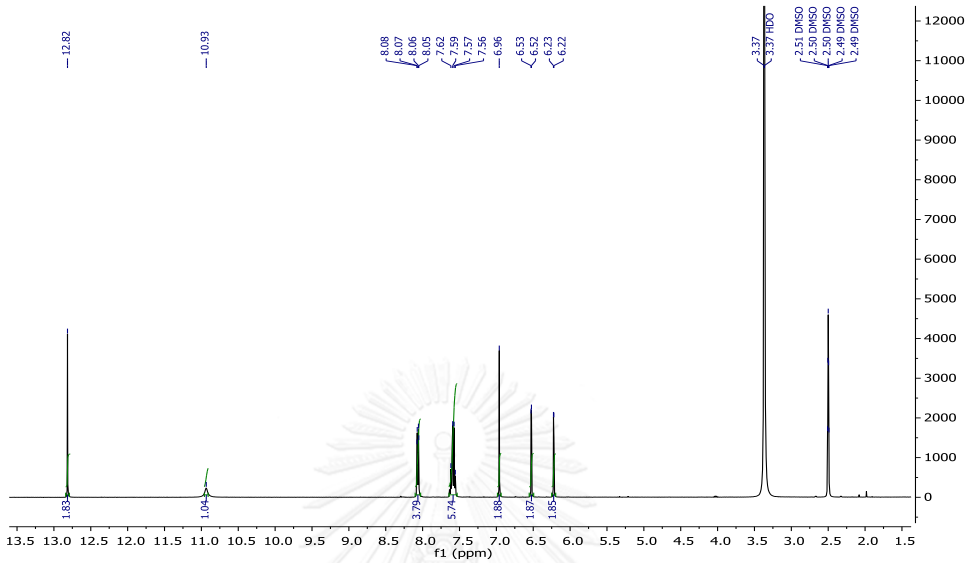


Figure 5.13 The ^{1}H NMR spectrum (DMSO) of compound **5-18**

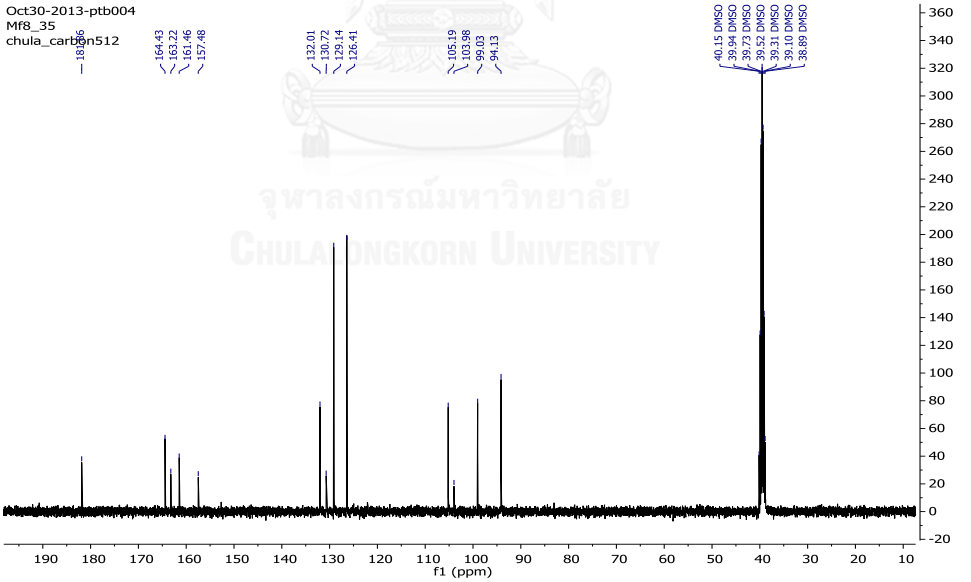
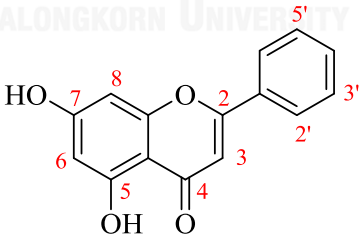


Figure 5.14 The ^{13}C NMR spectrum (DMSO) of compound **5-18**

Table 5.9 The comparison of ^{1}H and ^{13}C NMR spectral data of chrysin and compound **5-18** [118]

Position	Chemical shift (ppm)[a]			
	Chrysin		Compound 5-18	
	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
2	-	163.4	-	163.2
3	6.94 (*s*, 1H)	105.4	6.96 (*s*, 1H)	105.2
4	-	182.0	-	181.9
5	-	161.6	-	161.5
6	6.21 (*d*, *J* = 1.6 Hz, 1H)	99.2	6.22 (*d*, *J* = 2.0 Hz, 1H)	99.0
7	-	164.6	-	164.4
8	6.50 (*d*, *J* = 1.6 Hz, 1H)	94.3	6.53 (*d*, *J* = 2.1 Hz, 1H)	94.1
9	-	157.6	-	157.5
10	-	104.1	-	104.0
1'	-	130.9	-	130.7
2', 6'	8.04 (*d*, *J* = 7.0, 1.4 Hz, 2H)	126.6	8.06 (*dd*, *J* = 8.2, 1.6 Hz, 2H)	126.4
3', 5'	7.58 (*m*, 2H)	129.3	7.59 (*m*, 2H)	129.1
4'	7.58 (*m*, 1H)	132.2	7.59 (*m*, 1H)	132.0
5-OH	12.80 (*s*, 1H)	129.3	12.82 (*s*, 1H)	129.1
7-OH	10.88 (*s*, 1H)	126.6	10.94 (*s*, 1H)	126.4



chrysin or 5,7-dihydroxyflavone (**5-18**)

5.5.7 Compound 5-10: melodorinol

The fraction **MFF8** yielded compound **5-10** as yellow liquid (940 mg). The TLC showed a single spot with R_f value of 0.38 (elution system 2:3 of EtOAc:hexane). The molecular formula of this compound was proposed as $C_{14}H_{12}O_5$. The ^{1}H and ^{13}C NMR spectra of compound **5-10** are shown in **Figure 5.15-16**. As the comparison of

compound 20 and reference NMR data which reported by Tuchinda *et al*., 1991, Jung *et al*., 1991 and Juengwatanatrakul, 2000 [117, 120, 121]. Thus compound 21 was assigned as melodorinol or (4*Z*)-7-benzoyloxy-6- hydroxy-2,4-heptadien-4-olide (**5-10**) (**Table 5.10**).

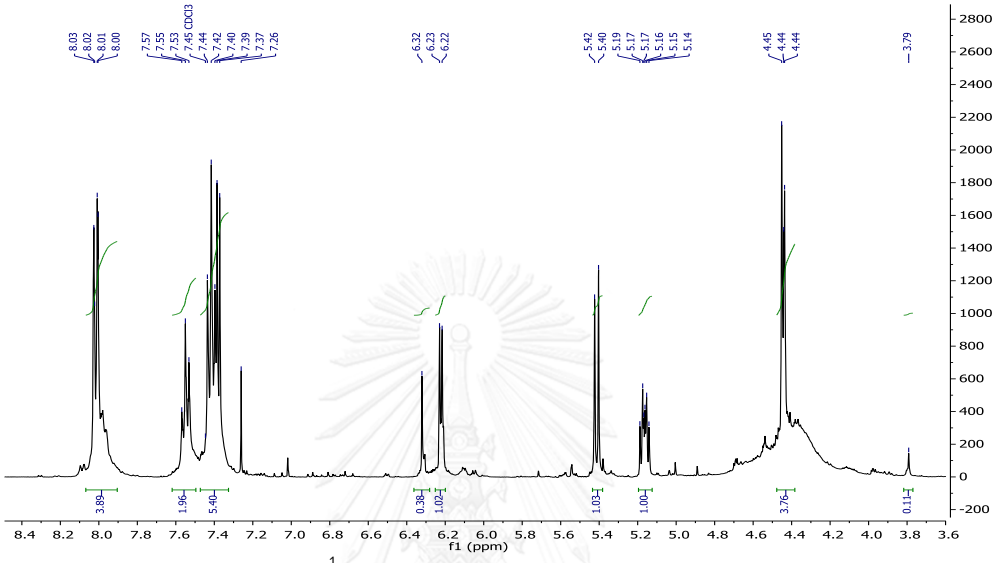


Figure 5.15 The ^{1}H NMR spectrum ($CDCl_3$) of compound **5-10**

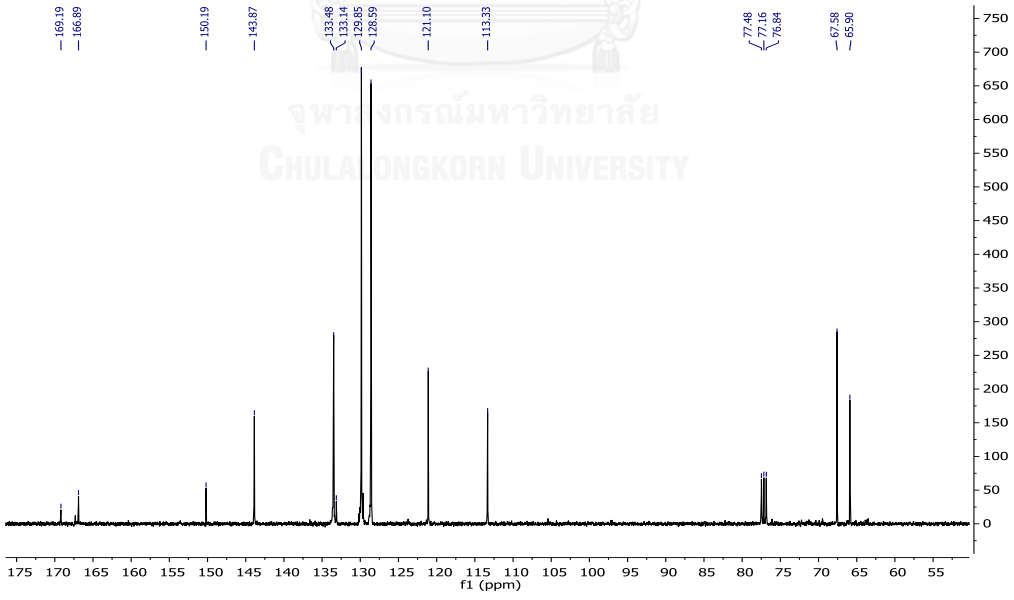
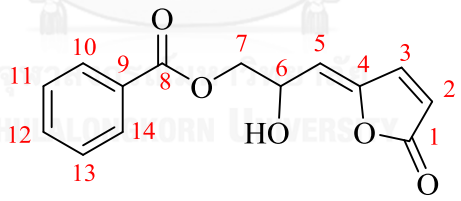


Figure 5.16 The ^{13}C NMR spectrum ($CDCl_3$) of compound **5-10**

Table 5.10 The comparison of ^{1}H and ^{13}C NMR spectral data of melodorinol and compound **5-10** [117]

Position	Chemical shift (ppm)[a]			
	melodorinol		compound 5-10	
	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
1	-	169.09	-	169.19
2	6.23 (*dd*, *J* = 5.4, 0.8 Hz)	120.98	6.22 (*d*, *J* = 5.4Hz, 1H)	121.10
3	7.39 (*d*, *J* = 5.4 Hz)	143.73	7.38 (*d*, *J* = 5.5 Hz, 1H)	143.87
4	-	150.03	-	150.19
5	5.42 (*d*, *J* = 8.2, 0.8 Hz)	113.04	5.43 (*d*, *J* = 8.2 Hz, 1H)	113.33
6	5.18 (*ddd*, *J* = 8.2, 5.7, 4.7 Hz)	65.78	5.17 (*dt*, *J* = 8.2, 5.3 Hz, 1H)	65.90
7	4.44 (*dd*, *J* = 11.4, 5.7 Hz)	67.40	4.44 (*m*, 2H)	67.58
8	4.47 (*dd*, *J* = 11.4, 4.7 Hz)	166.75	-	166.89
9	-	129.39	-	129.63
10,14	7.42 (m)	129.69	7.42 (*m*, 2H)	129.85
11,13	7.56 (m)	128.44	7.55 (*m*, 2H)	128.59
12	8.03 (m)	133.36	8.02 (*m*, 2H)	133.48
OH	2.96 (br)	-	-	-



(4*Z*)-7-benzoyloxy-6- hydroxy-2,4-heptadien-4-olide or melodorinol (**5-10**)

5.5.6 Compound 5-21: melodorinone A

The recrystallization of fraction **MfF8** with MeOH and hexane yielded compound **5-21** as white needle (870 mg). The TLC showed a single spot with R_f value of 0.27 (elution system 2:3 of EtOAc:hexane). The molecular formula of this compound was proposed as $C_{14}H_{10}O_5$. The ^{1}H and ^{13}C NMR spectrum of compound **5-21** are shown in **Figure 5.17-5.18** and **Table 5.11**.

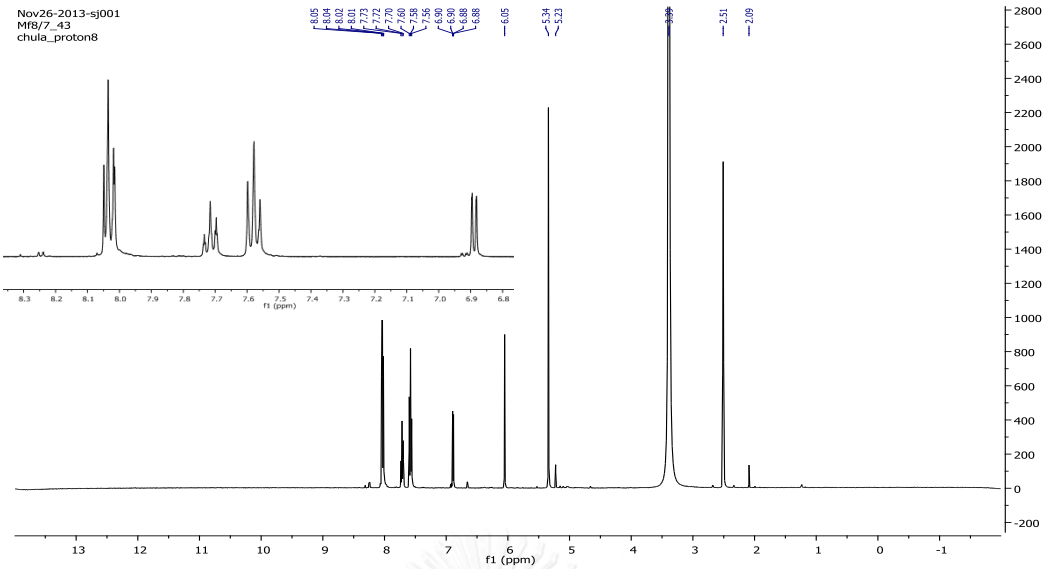


Figure 5.17 The ^{1}H NMR spectrum (DMSO) of compound **5-21**

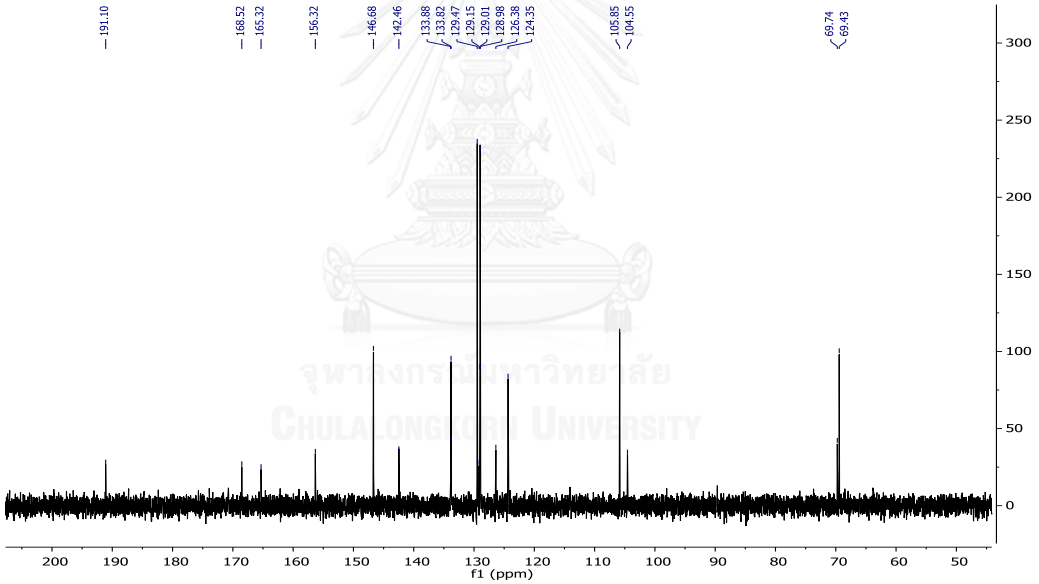


Figure 5.18 The ^{13}C NMR spectrum (DMSO) of compound **5-21**

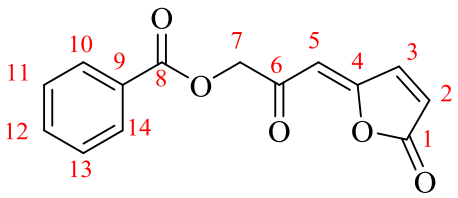
Table 5.11 The comparison of ^{1}H and ^{13}C NMR spectral data of melodorinone A and compound **5-21** [118]

Position	Chemical shift (ppm)[a]			
	melodorinone A		Compound 5-21	
	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
1	-	166.9	-	168.5
2	6.49 (*d*, *J* = 5.5 Hz, 1H)	124.2	6.88 (*d*, *J* = 5.4 Hz, 1H)	124.3
3	7.54 (*d*, *J* = 5.5 Hz, 1H)	145.0	6.04 (s, 1H)	146.7
4	-	155.9	-	156.3
5	5.69 (*s*, 1H)	107.3	5.33 (*s*, 2H)	105.9
6	-	190.8		191.1
7	5.38 (*s*, 2H)	69.3	5.22 (s, 1H)	69.4
8	-	165.8	-	165.3
9	-	129.3	-	129.5
10,14	8.09 (*m*, 2H)	129.3	8.03 (*s*, 3H)	129.5
11,13	7.44 (*m*, 2H)	128.4	7.57 (*t*, *J* = 7.7 Hz, 1H)	128.9
12	7.57 (*m*, 1H)	133.3	7.71 (*s*, 1H)	133.8

[a] ^{1}H-NMR and ^{13}C-NMR analysis in $CDCl_3$ at 300 and 75 MHz, respectively

[b] ^{1}H-NMR and ^{13}C-NMR analysis in DMSO at 400 and 100 MHz, respectively

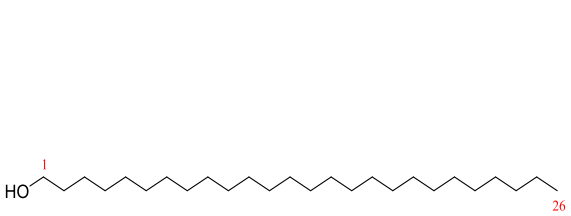
As the NMR data, compound **5-21**was assigned the structure as melodorinone-A or (4Z)-7-benzoyloxy-2,4-heptadiene-6-one-4-olide (**5-21**).



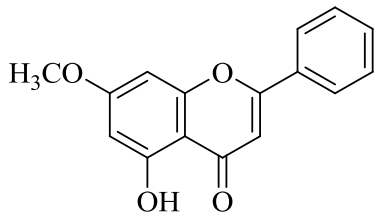
(4Z)-7-benzoyloxy-2,4-heptadiene-6-one-4-olide or melodorinone-A (**5-21**)

To summarize, nine pure compounds including 1-hexacosanol (**5-25**), 5-hydroxy-7-methoxyflavone (**5-26**), ***β***-sitosterol (**5-9**), melodorinone-B (**5-22**), benzoic

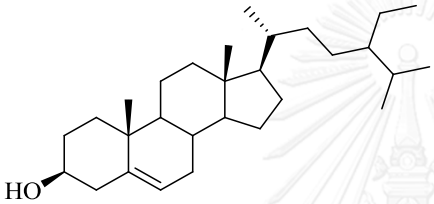
acid (**5-19**), chrysin (**5-18**), melodorinol (**5-10**) and melodorinone-A (**5-21**) were isolated from the active fraction of *M. fruticosum* flower. All structures of isolated compounds were presented in **Figure 5.19**. All isolated compounds were subjected to antifungal activity test except for 5-hydroxy-7-methoxyflavone (**5-26**) because it was obtained only in small amount.



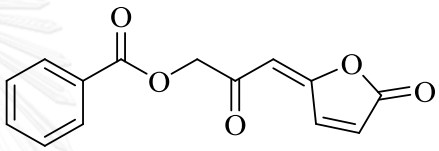
1-hexacosanol (**5-25**)



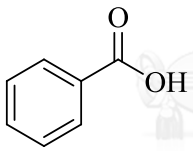
5-hydroxy-7-methoxyflavone (**5-26**)



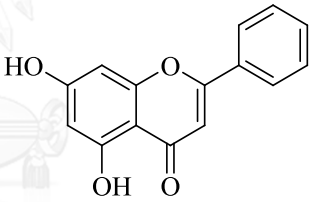
β-sitosterol (**5-9**)



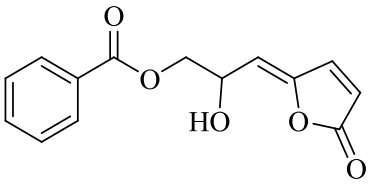
melodorinone-B (**5-22**)



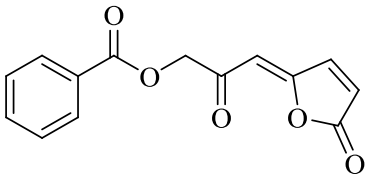
benzoic acid (**5-19**)



chrysin (**5-18**)



melodorinol (**5-10**)



melodorinone-A (**5-21**)

Figure 5.19 The structure of isolated compounds from flowers of *M. fruticosum*

5.6 Antiphytopathogenic fungi of isolated compounds *in vitro*

The isolated compounds form flowers of *M. fruticosum* were tested antiphytopathogenic activity against *P. parasitica* at various concentrations (1, 10, 100

and 1,000 μg•mL^{-1}) by agar incorporation method which described in Chapter 2 for bioassay guide to select the active compounds. The results of antifungal activity of these compounds are present in **Figure 5.20-5.21.**

From the results, benzoic acid (**18**) displayed completely against mycelial growth of *P. parasitica* at 1000 μg·mL^{-1}, followed by melodorinol (**5-10**) with 93.3% inhibition. Whereas melodorinone-A (**5-21**) and chrysin (**5-18**) displayed slight inhibition as 20 and 14.8%, respectively. On the other hand, 1- hexacosanol (**5-25**), **β**-sitosterol (**5-9**) and melodorinone-B (**5-22**) cannot inhibit the mycelial growth of *P. parasitica*. Moreover, **β**-sitosterol stimulated the mycelial growth of *P. parasitica* faster than the control group. In case of **β**-sitosterol, the previous study this compound serves as a nutrient source of *Phytophthora* species, sitosterol promoted significant mycelial growth and spore production when the medium was supplemented with sitosterol [133, 134]. Therefore this is the reason why this compound cannot inhibit *P. parasitica* in this study. Although, **β**-sitosterol does not display against the mycelial growth of *P. parasitica*, however, the biological activities of this compound showed antimicrobial, anticancer, anti-diabetic and several other medicinal activities [135].

Furthermore the structure activity relationship, benzoic acid derivative such as melodorinones A, B (**5-21, 5-22**) and melodorinol from this plant presented the antifungal activity less than benzoic acid (**5-19**). Therefore, the core structure as benzoic acid displayed the essential for antifungal activity and when it derived to other compounds in this plant the antifungal activity will decrease.

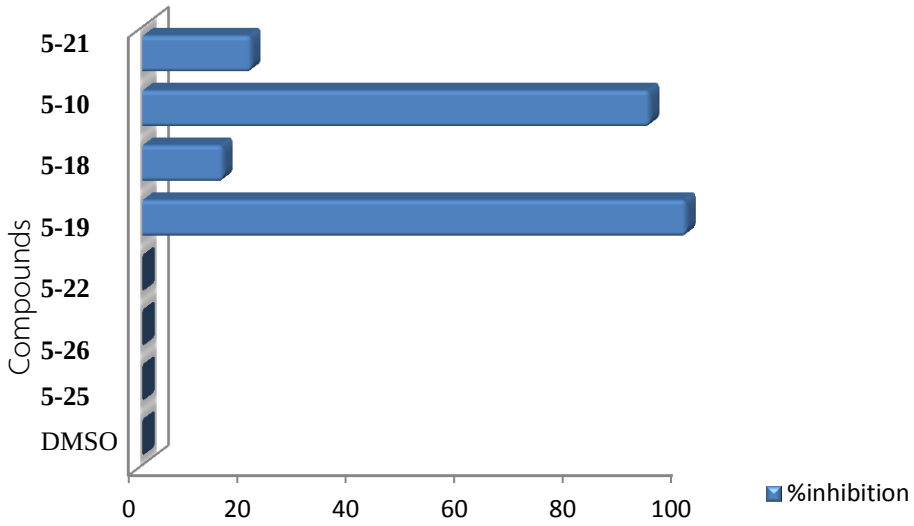


Figure 5.21 Antifungal activity of isolated compounds at 1000 µg·mL^{-1}.

(A)

(B)

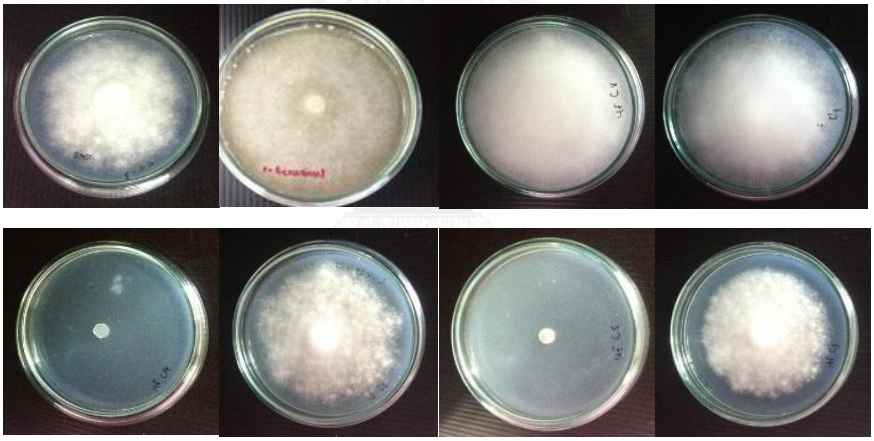


Figure 5.22 Antifungal activity of isolated compounds from flower of *M. fruticosum* at 1000 µg·mL^{-1}. A) DMSO, **5-25**, **5-26** and **5-22**; B) **5-19**, **5-18** and **5-21**

Base on the above results, *P. parasitica* was completely inhibited by benzoic acid (**5-19**) and melodorinol (**5-10**). Therefore, these compounds were tested at various concentrations against four fungi to evaluate and compare the IC_{50} value of each fungus and each compound. The percent inhibition growth was calculated and IC_{50} value as presented in **Table 5.12** and **Figures 5.22-5.23**.

From this antifungal activity indicated that benzoic acid was possessed the strongest inhibition compared with the other compounds. The data in **Tables 5.12-**

5.13, *P. parasitica* as the sensitive strain when compared with other tested fungi, benzoic acid revealed IC_{50} value higher than melodorinol as 107.5 and 130.0 μg·mL^{-1}, respectively. This compound showed strongly against *P. parasitica* with 90.6% inhibition and completely inhibited mycelial growth at 500 μg·mL^{-1}. However, benzoic acid and melodorinol showed the IC_{50} value more than 1000 μg·mL^{-1} in case of *C. gloeosporioides* and *F. oxysporum*, respectively. For the other fungi, benzoic acid against *A. porri* and *F. oxysporum* with IC_{50} as 325 and 450 μg·mL^{-1}, respectively and melodorinol inhibited *A. porri* and *C. gloeosporioides* (350 and 900 μg·mL^{-1}).

Table 5.12 Antifungal activity of benzoic acid (**5-19**) from *M. fruticosum* flower against phytopathogenic fungi at various concentrations and IC_{50}

Fungi	Concentration of benzoic acid (μg·mL^{-1})					
	%inhibition*					IC_{50}
	10	100	250	500	1000	
A. porri	7.1±0.6	8.2±1.5	25.1±1.2	100.0±0.0	100.0±0.0	325
C. gloeosporioides	0.0±0.0	0.0±0.0	5.6±0.0	10.7±0.3	13.7±0.3	>1000
F. oxysporum	9.3±2.9	19.6±1.2	40.4±1.5	51.1±1.7	100.0±0.0	450
P. parasitica	6.3±0.6	46.0±1.0	90.6±0.0	100.0±0.0	100.0±0.0	107.5

*Values, an average ± standard deviation (SD)

Table 5.13 Antifungal activity of melodorinol (**5-10**) from *M. fruticosum* flower against phytopathogenic fungi at various concentration and IC_{50}

Fungi	Concentration of melodorinol (μg·mL^{-1})					
	%inhibition*					IC_{50}
	10	100	250	500	1000	
A. porri	16.1±0.0	31.8±0.0	42.3±1.0	58.1±0.0	68.5±1.0	350
C. gloeosporioides	8.0±0.6	23.9±0.6	26.7±1.2	33.3±1.2	52.4±0.6	900
F. oxysporum	17.8±1.5	21.9±0.6	28.1±0.6	34.1±2.5	44.3±1.7	>1000
P. parasitica	11.1±1.2	45.2±1.0	65.9±0.3	76.7±1.0	93.3±0.6	130.0

*Values, an average ± standard deviation (SD)

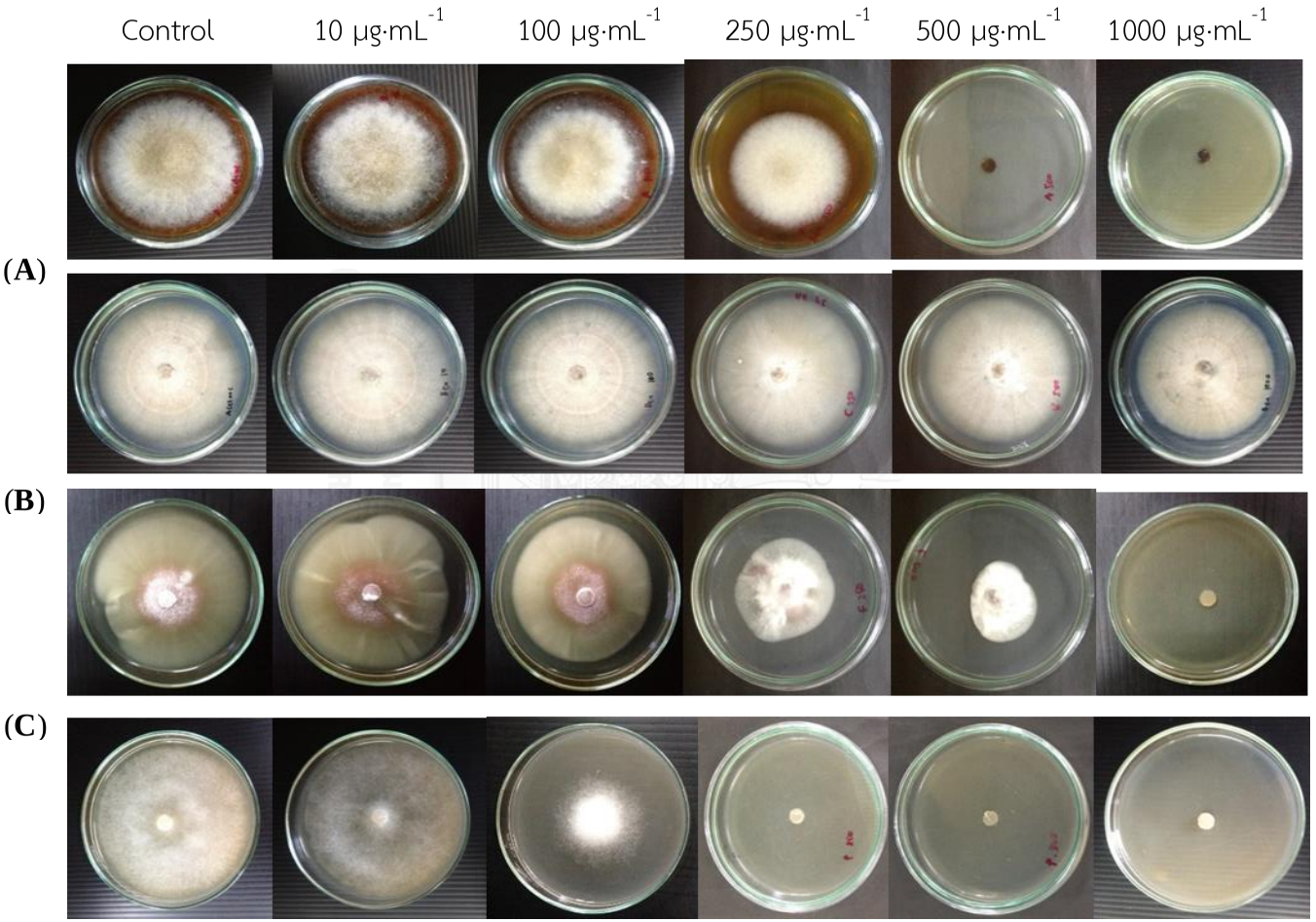


Figure 5.23 Antiphytopathogenic fungi of benzoic acid isolated from flower of *M. fruticosum* at different concentration (A): *A. porri*, (B): *C. gloeosporioides*, (C): *F. oxysporum*, (D): *P. parasitica*

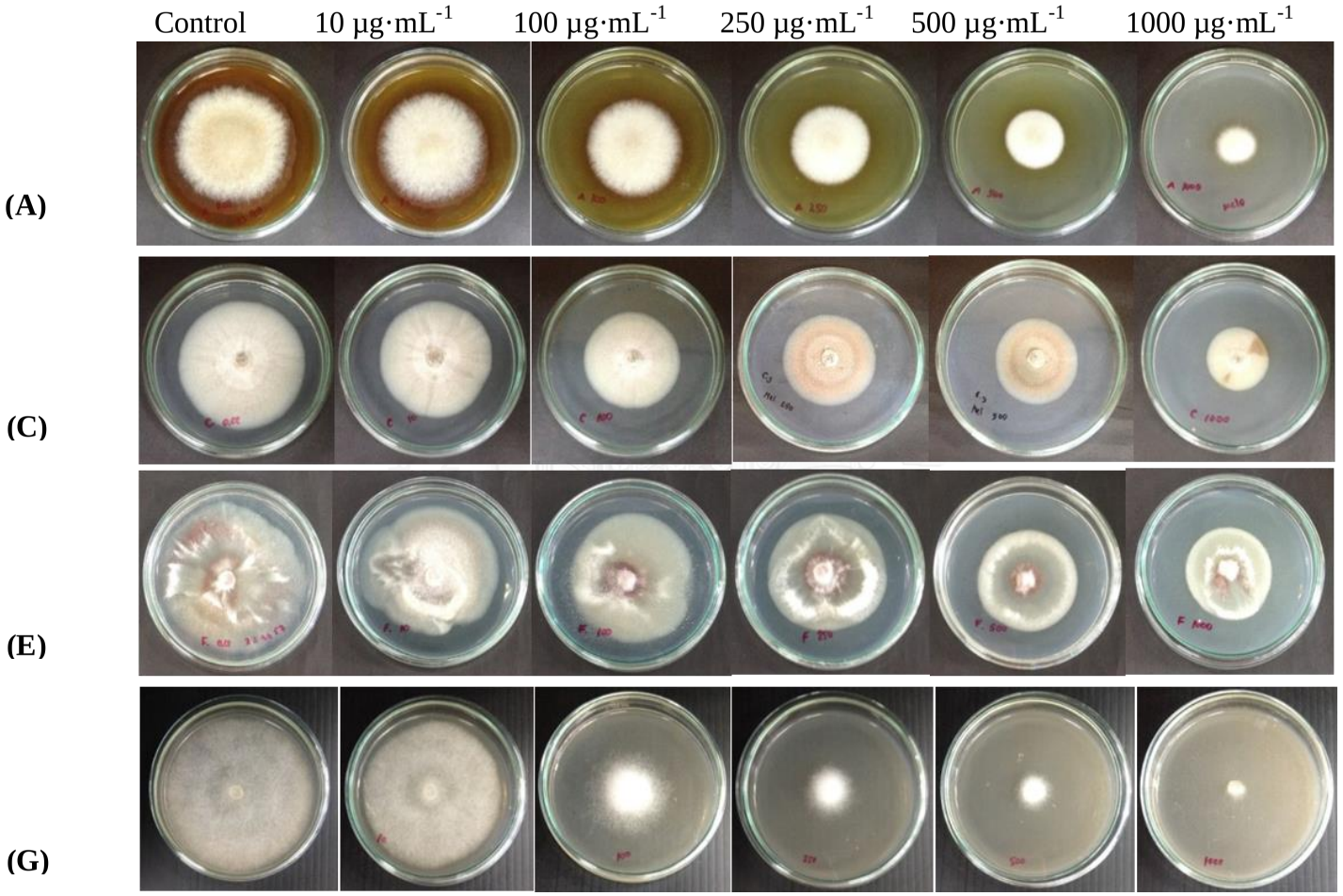


Figure 5.24 Antiphytopathogenic fungi of melodorinol isolated from flower of *M. fruticosum* at different concentration (A): *A. porri*, (B): *C. gloeosporioides*, (C): *F. oxysporum*, (D): *P. parasitica*

5.7 Conclusions

According to the separation of the CH_2Cl_2 extract from the flowers of *M. fruticosum* and followed the active fractions by antifungal activity against *P. parasitica*. Eight pure compounds were obtained including 1-hexacosanol (**5-25**), 5-hydroxy-7-methoxyflavone (**5-26**), ***β***-sitosterol (**5-9**), melodorinone-B (**5-22**), benzoic acid (**5-19**), chrysin (**5-18**), melodorinol (**5-10**), melodorinone-A (**5-21**). Benzoic acid exhibited antifungal activity the highest when compared among the tested compounds and followed by melodorinol with IC_{50} values as 107.5 and 130 $\mu g \cdot mL^{-1}$, respectively. Therefore, both benzoic acid and melodorinol are effective against the growth of fungi *in vivo* test in next chapter.

CHAPTER 6

IN VIVO ANTIPHYTOPATHOGENIC FUNGAL OF BIOACTIVE COMPOUNDS

6.1 Isolation of *P. parasitica* and pathogenicity

In this research, pineapple was chosen as the representative tested organism because Thailand has been number one in exporting pineapple. In addition, pineapple heart rot disease was interesting since it was a main crop loss in pineapple field especially in the largest harvested field as Prachuabkhirikhan and Petchaburi.

P. parasitica was directly isolated from pineapple heart rot disease because the old culture could not show disease symptom after inoculation. That was because the culture kept long time to subculture or the host specific [136], thus it lost the pathogenic activity. The fresh infected pineapple leaves were clean with sterilized towels paper and placed on RNV selective media (**Figure 6.1**). After the plates were incubated for 2 days, the hypha tips from the leaf section were cut and placed on carrot agar plates [137]. The unique characteristics of *P. parasitica* were that the mycelium had shape like chrysanthemums flower and sticky like spider, white hyphae and coenocytes [19]. When it was grown on carrot agar at RT for 7 days and water flooded above the mycelial growth for 3 days. After that the sporangia were produced (**Figure 6.2**) and then moved the plate to 4°C for 1 h and removed to RT. Abundant zoospores were released from sporangiospore after temperature was shifted [138, 139].

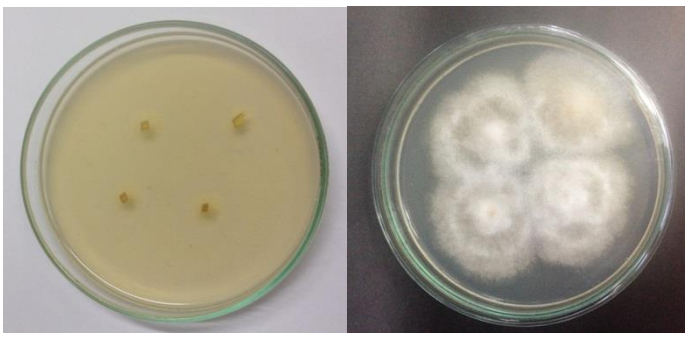


Figure 6.1 Isolation of *P. parasitica*; infected pineapple leave on RNV media (left), 3 days after incubated (right)

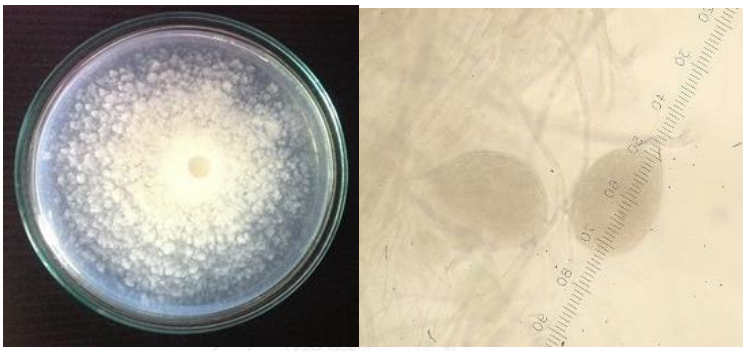


Figure 6.2 The growth of *P. parasitica* on PDA like chrysanthemum flower (right), sporangiospore of *P. parasitica* contained many zoospores (left).

The pathogenicity of disease was determined on pineapple leaf under laboratory using Koch's postulation methods [3]. Briefly, the artificial inoculation was commenced with cleaning the pineapple leaf by 1.5% NaOCl for 5 min and rinsing with sterilized water. After air drying, leaf was wounded by sterilized cork borer at the basal white portion of leaves. The mycelial disc was placed down side on wound, covered with wet cotton wool and incubated in plastic bag for 5 days. The symptom of heart rot disease incidence presented in **Figure 6.3,** the mycelial were re-isolated to pure culture and checked the morphology characteristics same as the culture before inoculation.

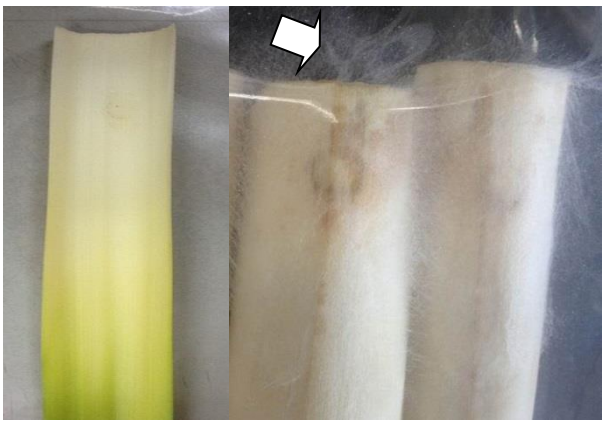


Figure 6.3 Fresh pineapple leaf (left) and the mycelium of *P. parasitica* (white arrow) on pineapple leaf showed symptom of heart rot disease (right).

6.2 Detached leave methods

The methodology was previously described with some modification [21, 140]. The healthy pineapple leave 7-15th leaves from inside of the plants were selected for inoculation and five leaflets of each treatment. The selected leaves were surface sterilized with 1.5% NaOCl and re-rinsed with sterilized water, air dried for 30 min. Following by washing and drying, plant leaves were wounded on the upper side using cork borer No. 2. After that, applying 20 **µ**L of active compounds including mansonones E and G from *M. gagei*, ACA from *A. galanga*, benzoic acid and melodorinol from *M. fruticosum* on wound at different concentrations (250, 500, 1000, 2500 and 5000 µg·mL^{-1}) and air dried. A standard fungicide, metalaxyl and DMSO were served as positive and negative controls, respectively. The zoospore suspension 20 µL with 5 x10^{3} spore·mL^{-1} were measured by haemacytometer and dropped on wound and covered with wet cotton for induce disease. After that all tested leave were contained in plastic bags and incubated at RT, after incubated for 3 days, the lesion area of disease were measured, and calculated the average between length and width of lesion area. The lesion was calculated in percent inhibition compared with negative control: %inhibition = (lesion of control –lesion of treatment)/ lesion of control x 100. The results of the percentage of lesion inhibition was show in **Figure 6.4**.

After 3 day-incubation, the uninoculate control, the leave still turgid and green and not show the disease symptom whereas the inoculation control (0.5%DMSO+0.2%Tween in water) showed the symptom of rot disease invasive around the wound, soaked water and soft tissue (the results not shown). Meanwhile, inoculation and test with positive control as metalaxyl at 250 μg·mL^{-1} and strong inhibited the lesion as 0.8 cm. According to **Figure 6.4**, the antifungal activity *in vivo* when tested with detached method, ACA displayed significantly inhibited the disease symptom at 2500 and 5000 μg·mL^{-1} (P<0.05), followed by melodorinol at the highest concentration (5000 μg·mL^{-1}). In case of melodorinol, the tissue showed the brown color but the mycelium was absent. Mansonones E and G showed poor results against the lesion of disease. ACA displayed higher activity than those compounds from *M. gagei*.

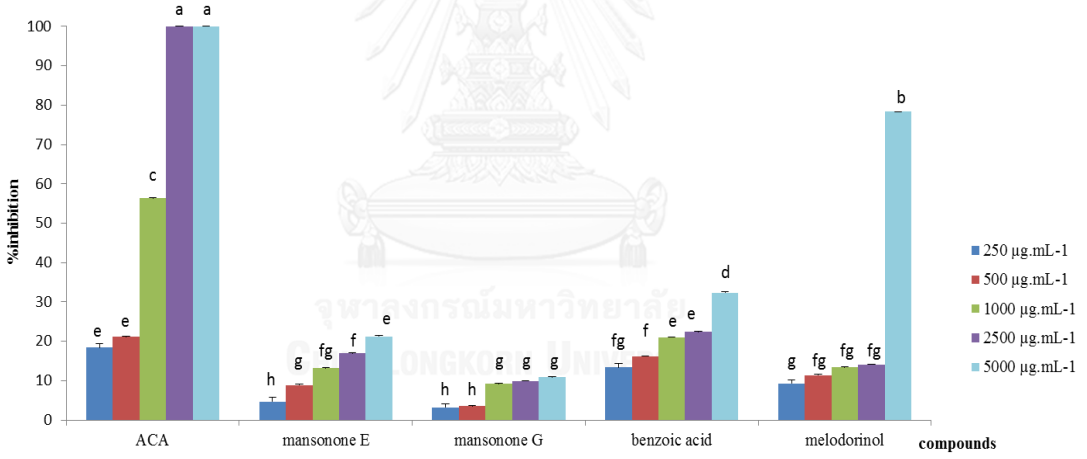


Figure 6.4 Percent lesion inhibition of tested compounds.
The bar represent standard deviation of the mean (n=5), the values designated with different letters differed significantly (P<0.05) according to Tukey's HSD.

For the artificial inoculation results, it depended on the density of zoospore if a higher inoculation density resulted in more rapid and severed lesion formation. In many previous studies, the high density of zoospore from *Phytophthora parasitica* was used; more than 10^6 zoospores/mL for inoculation to test pathogenicity in fruit,

leaves or stems [141, 142]. Takabashi and coworkers studied on the effects of inoculation density of *Pyricularia oryzae* and found the densities of $5x10^4$ and $5x10^5$ conidia mL^{-1} were no marked differences in disease severity [143]. The relationship between inoculum level of *P. capsici* at 20 zoospores per plant caused significant mortality of pepper [144]. Cartwright and colleagues prepared the suspension of *P. parasitica* var. *nicotianae* at 5000 zoospore·mL^{-1} for artificial inoculum to tobacco seedling [145]. Therefore, the concentration of zoospore suspension of $5x10^3$ zoospore·mL^{-1} was prepared and displayed the symptom of disease on leaves (**Figure 6.5**).

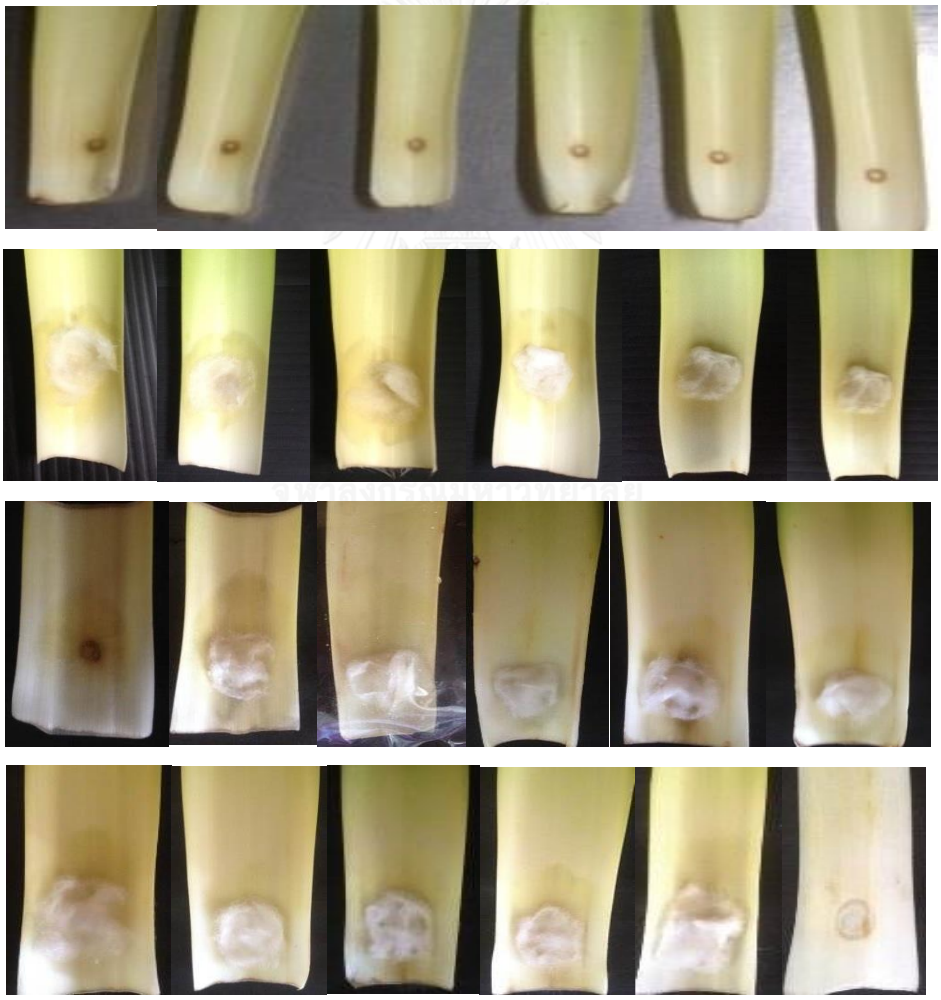


Figure 6.5 The lesion of rot disease caused by zoospore of *P. parasitica* and the antifungal activity of ACA (**A**), mansonone E (**B**), benzoic acid (**C**) and melodorinol (**D**) at various concentrations; (left-right): control, 250, 500, 1000, 2500 and 5000 μg·mL^{-1}.

6.3 Antifungal activity of ACA (3-1) against *P. parasitica in vivo* (pineapple suckers)

From the detached leave method, ACA (**3-1**) exhibited the highest activity against the lesion disease caused by *P. parasitica* on pineapple leaves. The antiphytopathogenic fungi *in vivo* in pineapple suckers was conducted according to Takaya (1980) with some modification [138]. The healthy small suckers of pineapple (size 5-10 cm and age 2 months) were clean and dried. The next steps, the clean pineapple suckers were dipped in the ACA (**3-1**) solution (0.2% Tween 80) at various concentrations (2500, 5000 and 10000 μg·mL^{-1}), air dried and put on cotton in the plastic bag which soaked with 10 mL of the zoospore suspension and sealed. Five suckers per treatment and three replicates were performed. The plastic bags of treatment were incubated at RT for 5 days. After incubated, heart rot disease was detected and recorded the number of sucker and some symptom or sign of disease in pineapple sucker. The results of antifungal activity of ACA (**3-1**) against pineapple heart rot disease caused by *P. parasitica* in pineapple suckers were recorded in **Table 6.1**.

Table 6.1 The antifungal activity of ACA (**3-1**) against pineapple heart rot disease caused by *P. parasitica in vivo*

Treatment	Heart rot*	Sign and Symptom
control (no inoc)	-	healthy green color, new root
control (inoc)	+++++	soaked with water, abundant mycelial, bad smell, leaf
2,500	+++++	easily pull out
5,000	++++	
10,000	++	some disease and some healthy but no new root
metalaxyl**	-	green and healthy

*symptom of heart rot disease; - no symptom, ++40% disease, ++++ 80% disease, +++++ 100% disease.

** metalaxyl 250 μg·mL-1

As the results after incubated the tested plants for 5 days, ACA (**3-1**) showed moderate activity against pineapple heart rot disease caused by *P. parasitica,* even at high concentration (10,000 μg·mL^{-1}), followed by 5000 μg·mL^{-1}. Whereas at lower concentration at 2500 μg·mL^{-1}, all plants showed heart rot symptom with chrolosis, soaked with water, soft tissue at the basal of suckers and when slightly pull on the young symptomatic leaves will easily remove from the suckers, it confirm the presence of the heart rot disease and this pathogen (*P. parasitica*) infections are limited to the basal white of the leaves [146]. On the other hand, the control group with sterilized water and non-inoculation the suckers still showed healthy green leaves and emerge many new roots. In contrast, an inoculated control group (0.2% Tween 80) showed the symptom of heart rot disease in all tested plants and the mycelial of *P. parasitica* covered the suckers, the symptom as 2500 μg·mL^{-1}. This result clearly confirmed the solvent did not have any effect against the growth of *P. parasitica* in pineapple suckers (**Figure 6.6**).

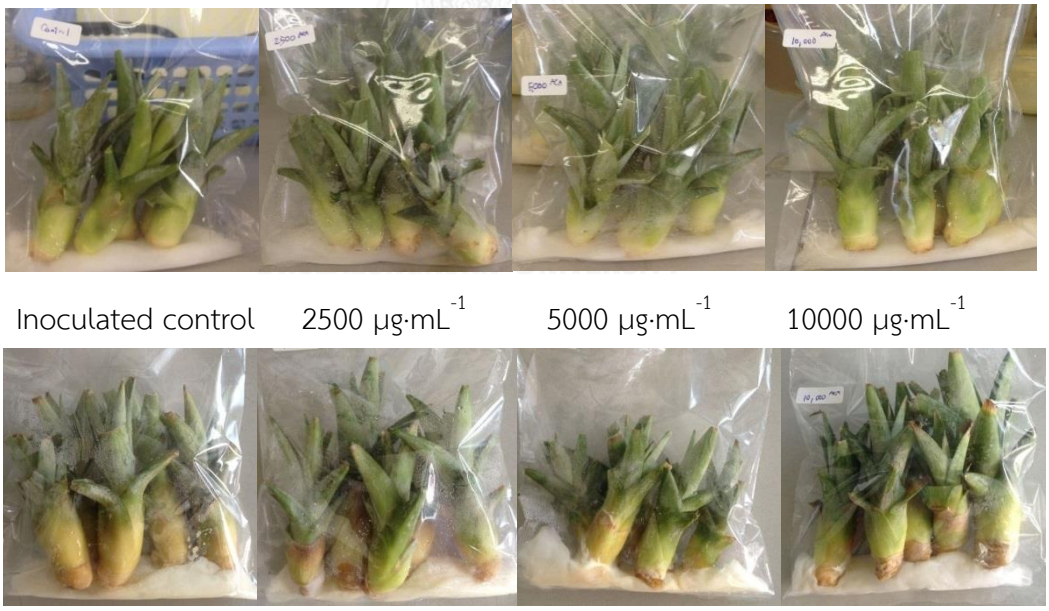


Figure 6.6 The pineapple suckers were tested with ACA (**3-1**) against pineapple heart rot disease caused by *P. parasitica* before inoculation (**A**) and after incubated 5 days (**B**).

6.4 Synergistic effects of bioactive compounds from *A. galanga, M. gagei* and *M. fruticosum*

This study was performed to evaluate the interaction between ACA (**3-1**), mansonone E (**4-5**) and melodorinol (**5-10**) isolated from *A. galanga, M. gagei* and *M. fruticosum*, respectively. The synergy test used agar incorporation method at 1000 $\mu g \cdot mL^{-1}$ final concentration against *P. parasitica.* The results are shown in **Table 6.2** and **Figure 6.7**.

Table 6.2 Percent inhibitory of antifungal activity of synergistic with ACA (**3-1**), mansonone E (**4-5**) and melodorinol (**5-10**)

ratio (v/v)	ACA:man E*		ACA:melo		man E:melo		ACA:man E:melo	
	growth (mm)	%inh	growth (mm)	%inh	growth (mm)	%inh	growth (mm)	%inh
9:1	0.0±0.0	100.0±0.0	0.0±0.0	100.0±0.0	30.0±0.0	66.7±0.0		
3:1	11.0±0.0	87.8±0.0	16.7±14.4	81.5±12.1	27.7±0.6	69.3±0.5		
1:1	24.7±21.3	72.6±19.4	18.0±0.0	80.0±0.0	22.3±0.6	75.2±0.6		
1:3	42.0±0.0	53.3±0.0	26.3±0.6	70.7±0.5	9.3±0.6	89.6±0.5		
1:1:1							15.0±0.0	83.3±0.0
7:2:1							5.3±4.6	94.1±4.2
1:7:2							29.7±0.6	67.1±0.5
2:1:7							23.3±1.5	74.1±1.4

*±SD of mean

Combining these compounds with different ratios, the interaction of these treatments displayed complete inhibition against *P. parasitica* when they combined with ACA (**3-1**) at high concentration (9:1). When reduced the volume of ACA (**3-1**) and increased the volume of other compounds, the percent inhibition was reduced. These compounds may have different mode of antifungal action and the results of combinations showed antagonist more than addition. However, when ACA (**3-1**) and melodorinol or mansonone E was combined at the suitable ratios, the complete inhibition of the mycelial growth of *P. parasitica* was observed. When there are more than two compounds acting together as antifungal, they may be exerting multiple

modes of inhibition on fungi, resulting in a reduced potential for the development of resistance to the antimicrobials [147].

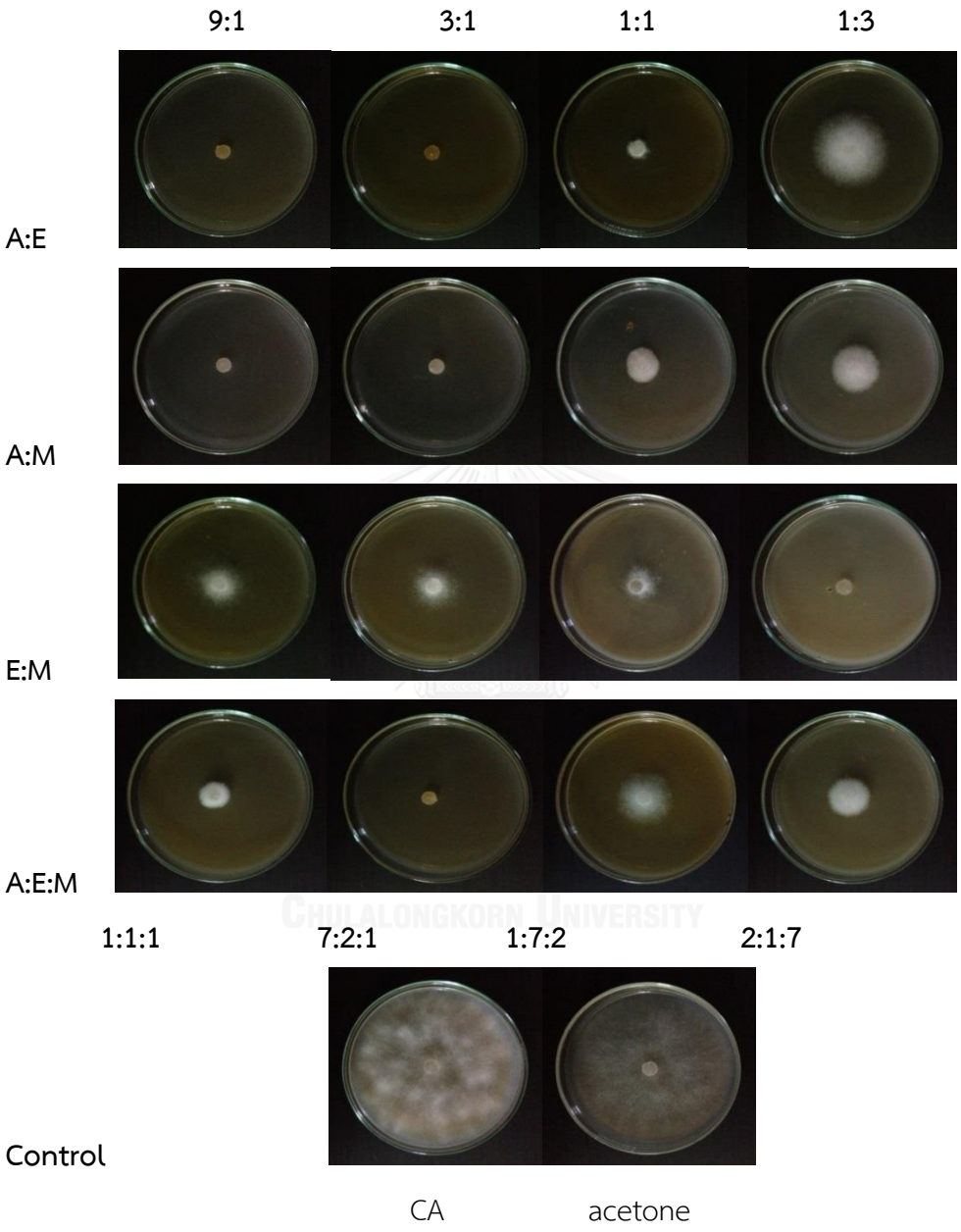


Figure 6.7 Synergist of active compounds against *P. parasitica* at 1000 μg·mL^{-1}. (A:ACA (**3-1**), E: mansonones E (**4-5**) and M: melodorinol (**5-10**))

CHAPTER 7
CONCLUSION

The screening of 46 Thai plants for antiphytopathogenic fungal activity was conducted, the potent plants are *Alpinia galanga*, *Mansonia gagei* and *Melodorum fruticosum*. The CH_2Cl_2 extracts displayed antifungal activity against four selected fungi higher than the MeOH extracts at 1000 μg·mL^{-1}. Each selected plant was macerated with CH_2Cl_2 to get the extract for further separation. According to bioassay guided by antifungal activity against *P. parasitica*, the active fractions were chosen for investigation on their biological active substances. Based on physical properties and spectroscopic data, the isolated compounds were identified.

Phenylpropanoids could be isolated from *A. galangal* rhizomes including 1'-acetoxychavicol acetate (ACA) (**3-1**), *trans-p*-coumaryl diacetate (**3-8**), *trans-p*-hydroxycinnamyl acetate (**3-6**), *trans-p*-hydroxycinnamaldehyde (**3-5**) and two compounds from ACA solvolysis, *i.e.*, *trans-p*-coumaryl alcohol ethyl ether (**3-24**) and *trans-p*-acetoxycinnamyl alcohol (**3-17**). Mansonones (1,2-naphthoquinones) and coumarins were isolated from the heartwoods of *M. gagei*, namely mansorins A, B, C, (**4-20-22**) mansonones C (**4-3**), E (**4-5**), G (**4-7**) and H (**4-8**). For *M. fruticosum* flowers, 8 compounds could be isolated including 1-hexacosanol (**5-25**), 5-hydroxy-7-methoxyflavone (**5-26**), ***β***-sitosterol (**5-9**), benzoic acid (**5-19**), 5,7-dihydroxyflavone (**5-18**), melodorinol (**5-10**), melodorinones A (**5-21**) and B (**5-22**). All isolated compounds from selected Thai plants were summarized in **Table 7.1**.

As part of antifungal research against four selected fungi, mansonone C displayed the highest antifungal activity against *P. parasitica*, followed by mansonone E (**4-5**), mansorin B (**4-21**), benzoic acid (**5-19**), mansonone G (**4-7**), melodorinol (**5-10**) and ACA (**3-1**) with IC_{50} as 3.2, 62.5, 87.7, 107.5, 110.0, 130.0 and 200 μg·mL^{-1}, respectively. Whereas in case of *F. oxysporum* and *C. gloeosporioides*, mansonone G (**4-7**) showed the antifungal activity higher than the other compounds with the IC_{50} of 11.5 and 87.5 μg·mL^{-1}, respectively. However, mansonone C (**4-3**) and mansorin B (**4-**

21) were obtained in such a small amount so that they could not be enough for further study. For the inhibition of spore germination of *F. oxysporum*, ACA (**3-1**) also gave the highest result against spore germination, followed by mansonones E (**4-5**) and G (**4-7**).

Table 7.1 List of isolated compounds from selected Thai plants

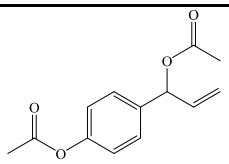
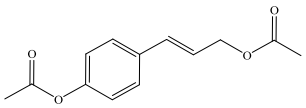
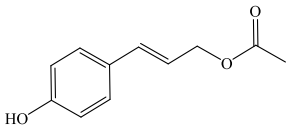
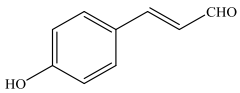
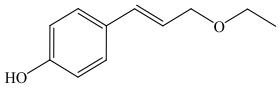
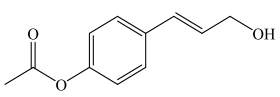
Plants*	no.	Compounds	Remarks	Molecular formula	Structure
AGR	1	1'-acetoxychavicol acetate (3-1)	colorless oil	$C_{13}H_{14}O_4$	
	2	*trans-p*-coumaryl diacetate (3-8)	light yellow oil	$C_{13}H_{14}O_4$	
	3	*trans-p*-hydroxycinnamyl acetate (3-6)	colorless oil	$C_{11}H_{12}O_3$	
	4	*trans-p*-hydroxycinnamaldehyde (3-5)	yellow semi-solid	$C_9H_8O_2$	
	5**	*trans-p*-coumaryl alcohol ethyl ether (3-24)	yellow oil	$C_{11}H_{14}O_2$	
	6**	*trans-p*-acetoxycinnamyl alcohol (3-17)	white crystal	$C_{11}H_{11}O_3$	

Table 7.1 (cont.)

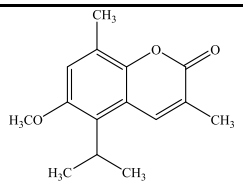
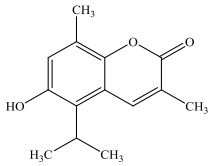
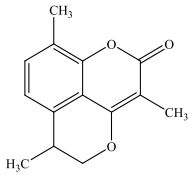
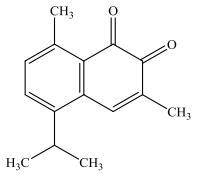
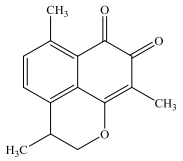
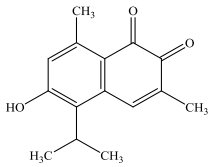
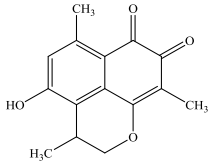
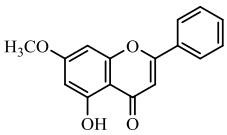
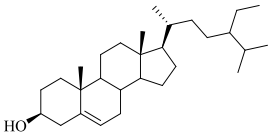
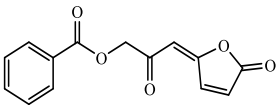
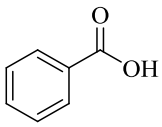
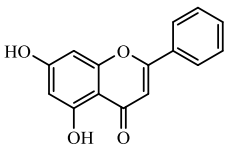
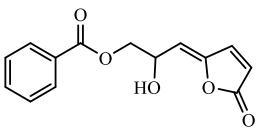
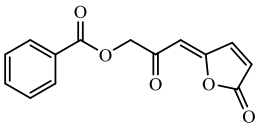
Plants*	no.	Compounds	Remarks	Molecular formula	Structure
MGH	7	mansorin A (4-20)	light yellow crystal	$C_{15}H_{18}O_3$	
	8	mansorin B (4-21)	pale yellow powder	$C_{14}H_{16}O_3$	
	9	mansorin C (4-22)	white crystal	$C_{14}H_{14}O_3$	
	10	mansonone C (4-3)	orange needle	$C_{15}H_{16}O_2$	
	11	mansonone E (4-5)	orange solid	$C_{15}H_{14}O_3$	
	12	mansonone G (4-7)	orange needle	$C_{15}H_{16}O_3$	
	13	mansonone H (4-8)	red platelet	$C_{15}H_{14}O_5$	
MFF	14	1-hexacosanol (5-25)	white powder	$C_{26}H_{54}O$	

Table 7.1 (cont.)

Plants*	no.	Compounds	Remarks	Molecular formula	Structure
MFF	15	5-hydroxy-7-methoxyflavone (5-26)	yellow crystal	$C_{16}H_{12}O_4$	
	16	**β**-sitosterol (5-9)	colorless needle	$C_{29}H_{50}O$	
	17	melodorinone B (5-22)	colorless bulky crystal	$C_{14}H_{10}O_5$	
	18	benzoic acid (5-19)	colorless crystal	$C_7H_6O_2$	
	19	5,7-dihydroxyflavone (5-18)	yellow powder	$C_{15}H_{10}O_4$	
	20	Melodorinol (5-10)	yellow liquid	$C_{14}H_{12}O_5$	
	21	melodorinone A (5-21)	white needle	$C_{14}H_{10}O_5$	

*AGR: *A. galanga*l rhizomes, MGH: *M. gagei* heartwood and MFF: *M. fruticosum* flower.

**the isolated compounds from ACA solvolysis

Since ACA, mansonones E and G, benzoic acid and melodorinol displayed strong antifungal activity *in vitro*, they were chosen to test *in vivo* against pineapple heart rot disease caused by *P. parasitica*. The antifungal activity *in vivo* were tested by detached leave method and ACA (**3-1**) displayed completely against heart rot lesion on pineapple leave at 3000 $\mu g \cdot mL^{-1}$. Whereas the other compounds could not inhibit the disease lesion even at the highest concentration. For the upscale *in vivo* test, using pineapple suckers, ACA (**3-1**) showed slightly against heart rot disease. The combination of ACA (**3-1**), mansonone E (**4-5**) and melodorinol was tested against *P. parasitica*. These compounds did not show the synergist effect. However, this is the excellent outcome of this research work and the best knowledge about the selected Thai plants to utilize these natural resources not only for medicinal uses, but also for antiphytopathogenic fungal. Furthermore this is the opportunity to discover the new antifungal agent for sustainable agro-chemical in Thailand.

REFERENCES

[1] Wannamolee, W. Development of Good Agricultural Practices (GAP) for Fruit and Vegetables in Thailand. Training of Trainers in Good Agricultural Practices (GAP) and Benchmarking:. GLOBALGAP for Fruit and Vegetables (2008).

[2] Oreke, E.C., Dehne, H.W., Schonbeck, F., Weber, A. Crop Production and Crop Protection: Estimated Loses in Major Food and Cash Crops. Amesterdam: Elsevier, 1994.

[3] Agrios, G. Plant Pathology. 5th edition ed.: Elsevier, 2004.

[4] Khruasanit, N. Anti-phytopathogenic fungal activity from some Thai medicinal herb extracts. Master's thesis, Program in Biotechnology, Faculty of Science Chulalongkorn University, 2004.

[5] Anonemous. *Alternaria* sp. Available from: http://www.redpalmweevil.com/Course/PPath/Alternaria.jpg [15th October]

[6] Khorpaditsakul, W.a.R., C. . Effective of the Active compounds from Medicinal Plants to Control Anthracnose Disease in Mango. Kasetsart University, 2005.

[7] Khang, C.H., Park, S.Y., Lee, Y.H. and Kang, S. A dual selection based, targeted gene replacement tool for *Magnaporthe grisea* and *Fusarium oxysporum.* Fungal Genetics and Bilogy 42 (2005): 483-492.

[8] Kuwaki, S., Ohhira, I., Takahata, M., Hirota, A., Murata, Y. and Tada, M. Effects of the fermentation product of herbs by lactic acid bacteria against phytopathogenic filamentous fungi and on the growth of host plants. Journal of Biosci and Bioengineering 98 (2004): 187-192.

[9] Parinya Panuweta, W.S., Tippawan Prapamontolc, P. Barry Ryana, Nancy and Fiedlerd, M.G.R., and Dana Boyd Barra. Agricultural Pesticide Management in Thailand: Situation and Population Health Risk. Environment Science Policy 17 (2012): 72-81.

[10] Keith, J.B.a.D., W Hollomon. Fungicide resistance in crop pathogens: How can it be managed?, ed. 2007, F.R.A.C. Belgium: Newline Graphics, 2007.

[11] Fisher, D.J. and Hayes, A.L. Studies of mechanisms of metalaxyl fungitoxicity and resistance to metalaxyl. Crop Protection 3(2) (1984): 177-185.

[12] Davidse, L.C., Gerritsma, O.C.M., Ideler, J., Pie, K., and Velthuis, G.C.M. Antifungal modes of action of metalaxyl, cyprofuram, benalaxyl and oxadixyl in phenylamide-sensitive and phenylamide-resistant strains of *Phytophthora megasperma* f. sp. *medicaginis* and *Phytophthora infestans.* Crop Protection 7(6) (1988): 347-355.

[13] Mukalazi, J., Adipala, E., Sengooba, T., Hakiza, J.J., Olanya, M., and Kidanemariam, H.M. Metalaxyl resistance, mating type and pathogenicity of *Phytophthora infestans* in Uganda. Crop Protection 20(5) (2001): 379-388.

[14] Zhu, G.-n., et al. Sensitivities of *Phytophthora infestans* to Metalaxyl, Cymoxanil, and Dimethomorph. Agricultural Sciences in China 7(7) (2008): 831-840.

[15] Benner, J.P. Pesticidal compounds from higher plants. Pesticide Science 39(2) (1993): 95-102.

[16] Chandel B.S., A.K., Singh A.P. and Chauhan A.K.S. Antifeedant activity of Aframomum melegueta Rosc., *Alpinia galanga* (L.), *Zingiber officinale* L. and *Curcuma longa* L. against *Athlia proxima* Klugens. Indian J Entomol. 71 (2009): 203-205.

[17] Bajpai, V.K., Shukla, S., and Kang, S.C. Chemical composition and antifungal activity of essential oil and various extract of Silene armeria L. Bioresource Technology 99(18) (2008): 8903-8.

[18] Tiew, P., Ioset, J.R., Kokpol, U., Chavasiri, W., and Hostettmann, K. Antifungal, antioxidant and larvicidal activities of compounds isolated from the heartwood of *Mansonia gagei.* Phytotheraphy Research 17(2) (2003): 190-3.

[19] Amornrat Poopaibool, P.T., Thawee Kaosiri. Variation in *Phytophthora palmivara* (Butl.) isolated from Durian: morphology and mating type. Journal of Vichakarnkaset 21 (2003): 72-89.

[20] Tangjitcharoenkul, J. Bioactive compounds from Ma-Khan *Zanthoxylum limonella* Alston. Doctor of Philosophy, Program in Biotechnology, Faculty of Science Chulalongkorn University, 2010.

[21] Tegegne, G., Pretorius, J.C., and Swart, W.J. Antifungal properties of *Agapanthus africanus* L. extracts against plant pathogens. Crop Protection 27(7) (2008): 1052-1060.

[22] Karkacier, M., Erbas, M., Uslu, M.K., and Aksu, M. Comparison of different extraction and detection methods for sugars using amino-bonded phase HPLC. Journal of Chromatographic Science 41(6) (2003): 331-3.

[23] Guan, J., Yang, F.Q., and Li, S.P. Evaluation of carbohydrates in natural and cultured Cordyceps by pressurized liquid extraction and gas chromatography coupled with mass spectrometry. Molecules 15(6) (2010): 4227-41.

[24] Cavka, A. and Jo¨nsson, L.J. Comparison of the growth of filamentous fungi and yeasts in lignocellulose-derived media. Biocatalysis and Agricultural Biotechnology (2014).

[25] Sati, S.a.J., S. Aspects of antifungal potential of ethnobotanically known medicinal plants. Research Journal of Medicinal Plant 5(4) (2011): 377-391.

[26] Mukherjee P.K., K.V., Mal M., Houghton P.J. *Acorus calamus*: Scientific validation of ayurvedic tradition from natural resources. Pharmaceutical Biology 45(8) (2007): 651-666.

[27] Manniche, L. An Ancient Egyptian Herbal. Cairo: American University in Cairo Press, 2006.

[28] Devi, S.A.D.G. Antioxidant Activities of Methanolic Extracts of Sweet-Flag (*Acorus calamus*) Leaves and Rhizomes. Journal of Herbs, Spices & Medicinal Plants 17(1) (2011): 1 – 11.

[29] Ganjewala, A.D.S.a.D. Antimicrobial activity of *Acorus calamus* (L.) rhizome and leaf extract. Acta Biologica Szegediensis 53(1) (2009): 45-49.

[30] Paneru, R.B., le Patourel, G.N.J., and Kennedy, S.H. Toxicity of *Acorus calamus* rhizome powder from Eastern Nepal to *Sitophilus granarius* (L.) and *Sitophilus oryzae* (L.) (Coleoptera, Curculionidae). Crop Protection 16(8) (1997): 759-763.

[31] Lee, M.-H., Chen, Y.-Y., Tsai, J.-W., Wang, S.-C., Watanabe, T., and Tsai, Y.-C. Inhibitory effect of β-asarone, a component of *Acorus calamus* essential oil, on inhibition of adipogenesis in 3T3-L1 cells. Food Chemistry 126(1) (2011): 1-7.

[32] Weisburger, E. Natural carcinogenic products: Environmental Science & Technology. ACS Publications, 1979.

[33] Reddy, G.B.S., et al. Chemical and Pharmacological Investigations of *Limnophila conferta* and *Limnophila heterophylla.* Pharmaceutical Biology 29(2) (1991): 145-153.

[34] Rao, K., Narasu L.M. and Giti A. Antibacterial activity of *Alpinia galanga* (L) Willd crude extracts. Applied Biochem Biotechnol 162 (2010): 871-884.

[35] Brahmachari, G. Limnophila (Scrophulariaceae): chemical and pharmaceutical aspects. The open natural products Journal 1 (2008): 34-43.

[36] Trakranrungsie, N., Chatchawanchonteera, A., and Khunkitti, W. Ethnoveterinary study for antidermatophytic activity of *Piper betle*, *Alpinia galanga* and *Allium ascalonicum* extracts in vitro. Research in Veterinary Science 84(1) (2008): 80-4.

[37] Mayachiew, P. and Devahastin, S. Antimicrobial and antioxidant activities of Indian gooseberry and galangal extracts. LWT - Food Science and Technology 41(7) (2008): 1153-1159.

[38] Indrayan A.K., A.P., Rathi A.K., Shatru A., Agrawal N.K. and TyAGRi D.K. Nutritive value of some indigenous plant rhizomes resembling Ginger. Natural Product Radiance 8 (2009): 507-513.

[39] Matsuda, H., Morikawa, T., Managi, H., and Yoshikawa, M. Antiallergic principles from *Alpinia galanga*: structural requirements of phenylpropanoids for inhibition of degranulation and release of TNF-α and IL-4 in RBL-2H3 cells. Bioorganic & Medicinal Chemistry Letters 13(19) (2003): 3197-3202.

[40] Jaju S., I.N., Sakarkar D., Fuloria N. and Ali M. Isolation of galangogalloside from rhizomes of *Alpinia galanga.* International Journal Green Pharmaceutical 3 (2009): 144-147.

[41] Jaju S., I.N., Sakarkar D., Fuloria N., Ali M., Das S. and Basu S. Galangoflavonoid isolation from rhizomes of *Alpinia galanga* (L) Sw (Zingiberaceae). Tropical Journal of Pharmacology Research 6 (2009): 545-550.

[42] Jaju S., I.N., Sakarkar D., Fuloria N., Ali M. and Basu S. Isolation of β-sitosterol diglucosyl caprate from *Alpinia galanga.* Pharmacognosis Research 2 (2010): 264-266.

[43] Kaur A., S.R., Dey C., Sharma S., Bhutani K. and Singh I. Antileishmanial phynylpropanoids from *Alpinia galanga* (Linn) Willd. Indian Journal of Experimental Biological 48 (2010): 314-317.

[44] Zhu X.L., D.Y.H., Huang X.F. and Kong L.Y. . Studies on the chemical constituents of *Alpinia galanga* Willd. Modern Chinese Medicine 11 (2008): 13-15.

[45] Zhao Ling, C.L.-Y., Liang Jing-Yu. Two new phenylpropanoids isolated from the rhizomes of Alpinia galanga. Chinese Journal of Natural Medicines 10(5) (2012): 0370-0373.

[46] De Pooter, H.L., Omar, M.N., Coolsaet, B.A., and Schamp, N.M. The essential oil of greater galanga (*Alpinia galanga*) from Malaysia. Phytochemistry 24(1) (1985): 93-96.

[47] Mori H., K.K.a.K.A. A potent aroma components of rhizomes from *Alpinia galanga* Willd. L. Nippon Shokuhin Kogyo Gakkaishi. 42 (1995): 989-995.

[48] Jirovertz L., B.G., Shafi M. and Leela N. Analysis of the essential oils of the leaves, stems, rhizomes and roots of the medicinal plant *Alpinia galanga* from southern India. Acta Pharmaceutica Hungarica 53 (2003): 73-81.

[49] Chukanhom K., B.P.a.H.K. Antifungal activities of aroma components from *Alpinia galanga* against water molds. Biocontrol Science 10 (2005): 105-109.

[50] Pripdeevech, P. and Chukeatirote, E. Chemical compositions, antifungal and antioxidant activities of essential oil and various extracts of *Melodorum fruticosum* L. flowers. Food and Chemical Toxicology 48(10) (2010): 2754-2758.

[51] Kikue Kubota, K.N., Akio Kobayashi. Acetoxy-1,8-cineoles as Aroma Constituents of *Alpinia galanga* Willd. Journal of Agricultural and Food Chemistry 46 (1998): 5244–5247.

[52] Janssen, A.M. and Scheffer, J.J. Acetoxychavicol Acetate, an Antifungal Component of *Alpinia galanga.* Planta Medica 51(6) (1985): 507-11.

[53] Khattak, S., Saeed ur, R., Ullah Shah, H., Ahmad, W., and Ahmad, M. Biological effects of indigenous medicinal plants *Curcuma longa* and *Alpinia galanga*. Fitoterapia 76(2) (2005): 254-257.

[54] Wong L.F., L.Y.Y.a.O.M. Antioxidant and antimicrobial activity of some Alpinia species. Journal of Food Biochemistry 33 (2009): 835-851.

[55] Oonmetta-aree, J., Suzuki, T., Gasaluck, P., and Eumkeb, G. Antimicrobial properties and action of galangal (*Alpinia galanga* Linn.) on *Staphylococcus aureus*. LWT - Food Science and Technology 39(10) (2006): 1214-1220.

[56] Latha, C., Shriram, V.D., Jahagirdar, S.S., Dhakephalkar, P.K., and Rojatkar, S.R. Antiplasmid activity of 1'-acetoxychavicol acetate from *Alpinia galanga* against multi-drug resistant bacteria. Journal of Ethnopharmacology 123(3) (2009): 522-5.

[57] Pompimon W., J.J., Prawat U., Mankhetkorn S. Anti-*Phytophthora capsici* activities and potential use as antifungal in agriculture of *Alpinia galanga* Swartz, *Curcumar longa* Linn, *Boesenbergia pandurata* Schut and *Chromolaena odorata*: Bioactivities guided isolation of active ingredients. American Journal of Agriculture and Biological Science 4 (2009): 83-91.

[58] Mahae, N.a.C., S. Antioxidant activities and antioxidative components in extracts of *Alpinia galanga* (L.) Sw. Kasetsart Journal, Natural Sciences 43 (2009): 358-369.

[59] Mayachiew, P., Devahastin, S., Mackey, B.M., and Niranjan, K. Effects of drying methods and conditions on antimicrobial activity of edible chitosan films enriched with galangal extract. Food Research International 43(1) (2010): 125-132.

[60] S., B.L.a.S. In vitro free radical scavenging and antimicrobial activity of some selected Thai medicinal plants. Research Journal of Medicinal Plant 5 (2011): 254-265.

[61] Chan, E.W.C., et al. Antioxidant and tyrosinase inhibition properties of leaves and rhizomes of ginger species. Food Chemistry 109(3) (2008): 477-483.

[62] Hanish Singh, J.C., Alagarsamy, V., Diwan, P.V., Sathesh Kumar, S., Nisha, J.C., and Narsimha Reddy, Y. Neuroprotective effect of *Alpinia galanga* (L.)

fractions on Aβ(25–35) induced amnesia in mice. Journal of Ethnopharmacology 138(1) (2011): 85-91.

[63] Juntachote, T. and Berghofer, E. Antioxidative properties and stability of ethanolic extracts of Holy basil and Galangal. Food Chemistry 92(2) (2005): 193-202.

[64] Phongpaichit, S., Subhadhirasakul, S., and Wattanapiromsakul, C. Antifungal activities of extracts from Thai medicinal plants against opportunistic fungal pathogens associated with AIDS patients. Mycoses 48(5) (2005): 333-8.

[65] Ye, Y. and Li, B. 1'S-1'-acetoxychavicol acetate isolated from *Alpinia galanga* inhibits human immunodeficiency virus type 1 replication by blocking Rev transport. Journal of General Virology 87(7) (2006): 2047-2053.

[66] Tamura, S., et al. New Rev-export inhibitor from *Alpinia galanga* and structure-activity relationship. Bioorganic and Medicinal Chemistry Letters 19(9) (2009): 2555-7.

[67] Mitsui, S., Kobayashi, S., Nagahori, H., and Ogiso, A. Constituents from seeds of *Alpinia galanga* Wild. and their anti-ulcer activities. Chemical and Pharmaceutical Bulletin 24(10) (1976): 2377-82.

[68] Lee, C.C. and Houghton, P. Cytotoxicity of plants from Malaysia and Thailand used traditionally to treat cancer. Journal of Ethnopharmacology 100(3) (2005): 237-243.

[69] Satish, R. and Dhananjayan, R. Evaluation of anti-inflammatory potential of rhizome of *Alpinia galanga* Linn. Biomedicine 23(1-2) (2003): 91-96.

[70] HN., N.M.S. Anti-inflammatory and analgesic activity of the topical preparation of *Alpinia galanga* willd. Biomedical Chromatography 1 (2006): 63-68.

[71] Noro, T., et al. Studies of enzyme inhibitors. V. Inhibitors of xanthine oxidase from *Alpinia galanga*. Chemical and Pharmaceutical Bulletin 36(1) (1988): 244-8.

[72] Tanakada Noro, T.S., Masako Katoh, Yasushi Oda, Toshio Miyase, Masanori Kuronayaki, Akira Ueno and Seiko Fukushima. Inhibitors of Xanthine oxidase from *Alpinia galanga*. Chemical and Pharmaceutical Bulletin 36(1) (1999): 244-248.

[73] Kiuchi, F., et al. Screening of natural medicines used in Vietnam for trypanocidal activity against epimastigotes of *Trypanosoma cruzi*. Natural Medicine (Tokyo, Japan) 56(2) (2002): 64-68.

[74] Leem, J.Y., Jeong, I.J., Park, K.T., and Park, H.Y. Isolation of p-hydroxycinnamaldehyde as an antibacterial substance from the saw fly, *Acantholyda parki* S. . FEBS Letters 442(1) (1999): 53-56.

[75] Sukhirun, N., Pluempanupat, W., Bullangpoti, V., and Koul, O. Bioefficacy of *Alpinia galanga* (Zingiberaceae) rhizome extracts, (*E*)-p-acetoxycinnamyl alcohol, and (*E*)-p-coumaryl alcohol ethyl ether against *Bactrocera dorsalis* (Diptera: Tephritidae) and the impact on detoxification enzyme activities. Journal of Economic Entomology 104(5) (2011): 1534-40.

[76] Khongake Siringam, T.T., Srunya Vajrodaya and Chalermpol Kirdmanee. Influence of plantation area and rhizome age on variation of 1'-Acetoxychavicol acetate (ACA) in galangal. Journal of Science and Technology Phranakorn Rajabhat University 1(1) (2011): 2-15.

[77] Azuma, H., et al. Lipase-catalyzed preparation of optically active 1'-acetoxychavicol acetates and their structure-activity relationships in apoptotic activity against human leukemia HL-60 cells. Bioorganic and Medicinal Chemistry 14(6) (2006): 1811-8.

[78] Ara Kirakosyan, T.M.S., Donna Marie Gibson and Peter B. Kaufson The production of hypericins and yperforin by in vitro cultures of St. John's wort (Hypericum perforatum). Biotechnology and Applied Biochemistry 39 (2004): 71-81.

[79] Jeffery, E.H., et al. Variation in content of bioactive components in broccoli. Journal of Food Composition and Analysis 16(3) (2003): 323-330.

[80] Pripdeevech, P. Analysis of odor constituents of *Melodorum fruticosum* flowers by solid-phase microextraction-gas chromatography-mass spectrometry. Chemistry of Natural Compounds 47(2) (2011): 292-294.

[81] Herman L. De Pooter, M.N.O., Brigite A. Coolsaet and Niceas M. Schamp. The essential oil of greater galanga (*Alpinia galanga*) from Malaysia. Phytochemistry 14(1) (1985): 93-96.

[82] Jantan, I.b., Ahmad, F.b., and Ahmad, A.S. Constituents of the rhizome and seed oils of greater galangal *Alpinia galanga* (L.) Willd. from Malaysia. Journal of Essential Oil Research 16(3) (2004): 174-176.

[83] Raina, A.P., Verma, S.K., and Abraham, Z. Volatile constituents of essential oils isolated from *Alpinia galanga* Willd. (L.) and *A. officinarum* Hance rhizomes from North East India. Journal of Essential Oil Research 26(1) (2014): 24-28.

[84] Mallavarapu, G.R., et al. Composition of the Volatile Oils of *Alpinia galanga* rhizomes and Leaves from India. Journal of Essential Oil Research 14(6) (2002): 397-399.

[85] Ibrahim, H., et al. Essential oils of Alpinia conchigera Griff. and their antimicrobial activities. Food Chemistry 113(2) (2009): 575-577.

[86] Arambewela, L.S.R., Arawwawala, M., Owen, N.L., and Jarvis, B. Volatile Oil of *Alpinia galanga* Willd. of Sri Lanka. Journal of Essential Oil Research 19(5) (2007): 455-456.

[87] Wannissorn, B., Jarikasem, S., Siriwangchai, T., and Thubthimthed, S. Antibacterial properties of essential oils from Thai medicinal plants. Fitoterapia 76(2) (2005): 233-6.

[88] Suputtra Jamkratoke, C.R., Chalida Leksomboon, Nuanwan Farungsang, Udom Farungsang Zingiberaceous Plant Crude Extracts Unfavourable towards Growth and Spore Germination of Posthavest Disease Fungi. (2002).

[89] Yamsakul P., K.S., Yano T., Sukprasitch V., Prakattagomol W. and Ogonoki S. The antibacterial and bactericidal activity of *Alpinia galanga* extracts to referent strain of pathogenic bacteria of pig *in vivo*. in Proceedings of the 47th Kasetsart University Annual Conference, Veterinary, Subject: Medicine, pp. 208-215. Kasetsart, 2009.

[90] Aziz, A.N., Ibrahim, H., Rosmy Syamsir, D., Mohtar, M., Vejayan, J., and Awang, K. Antimicrobial compounds from *Alpinia conchigera.* Journal Ethnopharmacol 145(3) (2013): 798-802.

[91] Akavipak, K. Pun Mai Ni Suan Pa Sirikit Pak Klang (Ratchaburi). Bangkok: Chutima Press, 1992.

[92] Pongboonrod, S. Mai Tet Muang Thai. in, pp. 162-163. Bangkok: Amarin Printing, 1976.

[93] Santisuk, T. Suan Tid Lad Da Puck Sa Pua. Bangkok: Amarin Printing, 1995.

[94] Bettòlo, G.B.M., Casinovi, C.G., and Galeffi, C. A new class of quinones: sesquiterpenoid quinones of *Mansonia Altissima* chev. Tetrahedron Letters 6(52) (1965): 4857-4864.

[95] Tanaka, N., Yasue, M., and Imamura, H. The quinonoid pigments of *Mansonia altissima* wood. Tetrahedron Letters 7(24) (1966): 2767-2773.

[96] Shimada, K., Yasue, M., Imamura, H. A new pigment from *Mansonia altissima* wood. Mokuzai Gakkaishi 13 (1967): 126.

[97] Galeffi, C., delle Monache, E.M., Casinovi, C.G., and Bettòlo, G.B.M. A new quinone from the heartwood of *Mansonia Altissima* chev: Mansonone L. Tetrahedron Letters 10(40) (1969): 3583-3584.

[98] Tiew P., I.J., Kokpol U., Schenk K., Jaiboon N., Chaichit N., Chavasiri W., Hostettmann K. . Four new sesquiterpenoid derivatives from the heartwood of *Mansonia gagei.* Journal of Natural Products 65 (2002): 1332-1335.

[99] Tiew, P. Bioactive compounds from *Mansonia gagei* Drumm. Degree of Doctoral for Philosophy in Chemistry, Chemistry Chulalongkorn University, 2002.

[100] Tiew, P., Takayama, H., Kitajima, M., Aimi, N., Kokpol, U., and Chavasiri, W. A novel neolignan, mansoxetane, and two new sesquiterpenes, mansonones R and S, from *Mansonia gagei.* Tetrahedron Letters 44(35) (2003): 6759-6761.

[101] Tiengtham P., S.P., Chavasiri W., Otsuka H. Chemical constituents of the roots and the leaves of *Mansonia gagei* Drumm. and their bioactivities. in the 30th Congress on Science and Technology of Thailand (STT 30), 2004.

[102] Tiew P., P.A., Kokpol U., Chavasiri W. Coumarins from the heartwood of *Mansonia gagei* Drumm. Phytochemistry 60 (2002): 773–776.

[103] Kongcharoensuntorn W., S.C., Bootsayatrus., Katawee P. The potential co-treatment effects of three plant extracts and three antibiotics on multidrug-resistant bacteria. Handbook of The 32th Congress on Science and Technology of Thailand (STT 32) (2006).

[104] Changwong, N., Sabphon, C., Ingkaninan, K., and Sawasdee, P. Acetyl- and butyryl-cholinesterase inhibitory activities of mansorins and mansonones. Phytotheraphy Research 26(3) (2012): 392-6.

[105] Chang, W.-T., Chen, Y.-C., and Jao, C.-L. Antifungal activity and enhancement of plant growth by Bacillus cereus grown on shellfish chitin wastes. Bioresource Technology 98(6) (2007): 1224-1230.

[106] Banerjee, A.K., Laya, M.S., and Poon, P.S. Sesquiterpenes classified as phytoalexins. Study Natural Product Chemistry 33(Bioactive Natural Products (Part M)) (2006): 193-237.

[107] Yang, D., Jeng, R.S., and Hubbes, M. Mansonone accumulation in elm callus induced by elicitors of Ophiostoma ulmi, and general properties of elicitors. Can. J. Bot. 67(12) (1989): 3490-7.

[108] Overeem, J.C. and Elgersma, D.M. Accumulation of mansonones E and F in *Ulmus Hollandica* infected with *Ceratocystis ulmi.* Phytochemistry 9(9) (1970): 1949-1952.

[109] Dumas, M.T., Strunz, G.M., Hubbes, M., and Jeng, R.S. Inhibition of Ceratocystis ulmi by mansonones A, C, D, E, F, and G isolated from *Ulmus americana.* European Journal of Forest Pathology 16(4) (1986): 217-22.

[110] Dumas, M.T., Strunz, G.M., Hubbes, M., and Jeng, R.S. Isolation and identification of six mansonones from *Ulmus americana* infected with *Ceratocystis ulmi.* Experientia 39(10) (1983): 1089-90.

[111] Duchesne, L.C., Jeng, R.S., and Hubbes, M. Accumulation of phytoalexins in *Ulmus americana* in response to infection by a nonaggressive and an aggressive strain of *Ophiostoma ulmi.* Canadian Journal Botany 63(4) (1985): 678-80.

[112] Merrill, E.D. On the application of the generic name Melodorum of Loureiro. The Phillippine Journal of Science 15 (1919): 125-137.

[113] Smitinand, T. Thai plant names (Botanical manes-Vermacular names). Second printing ed. Vol. limited partnership: Funny publishing, 1980.

[114] Rujjanawate, C., Hargreave, O.D., Sansomchai, P., Wongnut, P., Hongsing, P. . The Essence of Thai Herbs. Bangkok, Thailand: PTT Publish Company Limited, , 2008.

[115] Jung, J.H., Pummangura, S., Chaichantipyuth, C., Patarapanich, C., and McLaughlin, J.L. Bioactive constituents of *Melodorum fruticosum.* Phytochemistry 29(5) (1990): 1667-1670.

[116] Jung, J.H., et al. New bioactive heptenes from *Melodorum fruticosum* (annonaceae). Tetrahedron 46(15) (1990): 5043-5054.

[117] Tuchinda, P., et al. Bioactive butenolides from *Melodorum fruticosum.* Phytochemistry 30(8) (1991): 2685-2689.

[118] Tiyaworanan, S. Chemical constituents of *Melodorum fruticosum* flowers. Master, Department of Pharmacognosy, Graduate School Chulalongkorn University, 1998.

[119] Chaichantipyuth, C., et al. Oxidized heptenes from flowers of *Melodorum fruticosum.* Phytochemistry 58(8) (2001): 1311-5.

[120] Juengwatanakul, T. Chemical constituents of *Melodorum fruticosum* root and seeds. Master, Department of Pharmacognosy , Faculty of Pharmaceutical Science Chulalongkorn University, 2000.

[121] Jung, J.H., Chang, C.J., Smith D.L. and McLaughlin, J.L. . Additional bioactive heptenes from *Melodorum fruticosum.* Journal of Natural Products 54 (1991): 500-505.

[122] Joshi, S. and Poudel, T.N. Isolation and characterization of the chemical constituents of *Sonchus wightianus* of Nepalese origin. J. Nepal Chem. Soc. 28 (2011): 115-120.

[123] Astudillo S, L., et al. Biologically active compounds from Chilean propolis. Boletin de la Sociedad Chilena de Quimica 45(4) (2000): 577-581.

[124] Yahya, M.A.A., Yaacob, W.A., & Nazlina, I. Isolation of chemical constituents from rhizomes of *Etlingera sphaerocephala* var *grandiflora.* Malaysian Journal of Analytical Sciences 15(1) (2011): 22-26.

[125] Rahman, S., Mukta, Z. and Hossain, M. . Isolation and characterization of β-sitosterol-D-glycoside from petroleum extract of the leaves of *Ocimum sanctum* Asian Journal of Food and Agro-Industry 1 (2009): 39-43.

[126] Pateh, U., Haruna, A., Garba, M., Iliya, I., Sule, I., Abubakar, M. and Ambi, A. . Isolation of stigmasterol, β-sitosterol and 2-hydroxyhexadecanoic acid methyl ester from the rhizomes of the *Stylochiton lancifolius* Pyer and Kotchy (Araceae). Nigerian Journal of Pharmaceutical Sciences 8 (2009): 19-25.

[127] Akhtar, P., Ali, M., Sharma, M., Farooqi, H. and Khan, H. . Phytochemical investigation of fruits of *Corylus colurna* Linn. Journal of Phytology 2 (2010): 89-100.

[128] Kamboj, A.a.S., A. . Isolation of stigmasterol and β-sitosterol from petroleum ether extract of aerial parts of *Ageratum conyzoides* (Asteraceae). International Journal of Pharmacy and Pharmaceutical Sciences 3 (2011): 94-96.

[129] Chaturvedula, V.a.P., I. . Isolation of stigmasterol and β-sitosterol from the dichloromethane extract of *Rubus suavissimus.* International Current Pharmaceutical Journal 1 (2012): 239-242.

[130] Wibbertmann, A., Kielhorn, J., Koennecker, G., Mangelsdorf, I. and Melber, C. Benzoic acid and sodium benzoate. Concise international chemical assessment. 2005, WHO library cataloguing in publication data: Geneva. 52

[131] Pornchalermpong, P.a.R., N. Benzoic acid 2014. Available from: http://www.foodnetworksolution.com/wiki/word/1132/benzoic-acid

[132] Youn, U.J., et al. A pyridyl alkaloid and benzoic acid derivatives from the rhizomes of *Anemarrhena asphodeloides.* Natural Product Sciences 16(4) (2010): 203-206.

[133] Latijnhouwers, M. and Govers, F. A *Phytophthora infestans* G-Protein Subunit Is Involved in Sporangium Formation. Eukaryotic Cell 2(5) (2003): 971-977.

[134] Marshall, J.A., Dennis, A.L., Kumazawa, T., Haynes, A.M., and Nes, W.D. Soybean sterol composition and utilization by Phytophthora sojae. Phytochemistry 58(3) (2001): 423-428.

[135] Amit Sen, P.D., Kshitiz Kumar Shukla, Sanjay Sing, G. Tejovathi. Analysis of IR, NMR and Antimicrobial Activity of β-Sitosterol Isolated from *Momordica charantia.* Science Secure Journal of Biotechnology 1 (2012): 9-13.

[136] Cécile Robin, D.G. Characterisation of pathogenicity of *Phytophthora parasitica* isolates by stem and detached-leaf inoculations in four tobacco cuitivars. New Zealand Journal of Crop and Horticultural Science 22 (1994): 159-166.

[137] Mongkol, R. Antifungal activity of benzoic acid and cinnamic acid derivatives against pineapple heart rot disease. Master's Degree, Program in Biotechnology, Faculty of Science Chulalongkorn University, 2007.

[138] Takaya, S. Studies on Epidemiology of Pineapple Heart Rot Diseases and Ecology of It's Pathogen, Phytophtora Nicotianae Var. Parasitica (Dastur) Waterhouse: Under the Cooperative Research Work Between Thailand and Japan. 1980.

[139] Zhu Jie-hua, Z.Z.-m., Yang Zhi-hui. General research methods on pathogen of potato late blight(*Phytophthora infestans*). Journal of Agricultural University of Hebei (2001).

[140] Ellwood, S., et al. Inoculation and growth with foliar pathogenic fungi. Medicago truncatula handbook (2007): 1-14.

[141] Sukhada, M., Manjula, R., and Rawal, R.D. Evaluation of arbuscular mycorrhiza and other biocontrol agents against *Phytophthora parasitica* var. *nicotianae* infecting papaya (*Carica papaya* cv. *Surya*) and enumeration of pathogen population using immunotechniques. Biological Control 58(1) (2011): 22-29.

[142] Yan, H.-Z. and Liou, R.-F. Selection of internal control genes for real-time quantitative RT-PCR assays in the oomycete plant pathogen *Phytophthora parasitica.* Fungal Genetics and Biology 43(6) (2006): 430-438.

[143] Takahashi W., Y.M., T. Sasaki. A novel inoculation method for evaluation of grey leaf spot resistance in Italian ryegrass. Journal of Plant Pathology 91(1) (2009): 171-176.

[144] Mitchell, J.H.B.a.D.J. Relationship between inoculum level of *Phytophthora capsici* and mortality of peper. Phytopathology 81 (1991): 178-184.

[145] Cartwright, D.K. and Spurr jr, H.W. Biological control of *Phytophthora parasitica* var. *nicotianae* on tobacco seedlings with non-pathogenic binucleate Rhizoctonia fungi. Soil Biology and Biochemistry 30(14) (1998): 1879-1884.

[146] Bartholomew, D.P., Paull, R.E., and Rohrbach, K.G. The Pineapple: Botany, Production, and Uses. CABI, 2002.

[147] Weerakkody, N.S., Caffin, N., Lambert, L.K., Turner, M.S., and Dykes, G.A. Synergistic antimicrobial activity of galangal (*Alpinia galanga*), rosemary (*Rosmarinus officinalis*) and lemon iron bark (*Eucalyptus staigerana*) extracts. Journal of Science and Food Agricultural 91(3) (2011): 461-8.

APPENDIX

Table A1 Phytotoxicity of ACA against lettuce seedlings

Concentration*	Seedling growth	%Growth inhibition	Fresh weight (mg)	Seedlings length (mm.)		
				Shoot	Root	Cotyledon
control	10.0±0.0	0.0±0.0	174.4±12.8	5.1±0.7	24.6±3.3	12.4±1.3
10	10.0±0.0	0.0±0.0	170.2±24.6	4.2±0.8	32.5±15.6	11.6±1.5
50	10.0±0.0	0.0±0.0	169.9±0.6	4.6±0.8	17.0±6.6	11.4±1.9
100	10.0±0.0	0.0±0.0	161.6±12.7	4.2±1.3	14.0±4.4	10.6±1.3
250	9.0±1.4	10.0±0.0	96.9±12.4	2.7±0.8	1.7±1.3	11.7±3.6
500	6.0±0.0	40.0±0.0	54.5±9.6	1.0±0.0	0.0±0.0	8.2±3.3
1000	0.0±0.0	100.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

*concentration ($\mu g \cdot mL^{-1}$)

Table A2 Phytotoxicity of ACA against Italian ryegrass

Concentration	Seedling growth	%Growth inhibition	Fresh weight (mg)	Length (mm.)	
				Shoot	Root
control	10.0±0.0	0.0±0.0	209.1±5.2	62.6±11.9	28.6±6.4
10	10.0±0.0	0.0±0.0	242.3±1.4	53.5±15.1	25.1±9.5
50	10.0±0.0	0.0±0.0	207.1±1.2	45.5±10.4	24.4±6.3
100	10.0±0.0	0.0±0.0	244.8±21.4	55.7±9.5	29.7±7.3
250	10.0±0.0	0.0±0.0	212.0±1.1	42.5±14.8	20.3±10.7
500	10.0±0.0	0.0±0.0	223.3±14.8	38.2±9.1	17.9±5.2
1000	5.0±0.0	50.0±0.0	85.7±5.7	29±10.7	6.1±3.5

Table A3 Phytotoxicity of *trans-p*-coumaryl diacetate against lettuce seedlings

Concentration	Seedling growth	%Growth inhibition	Fresh weight (mg)	Seedlings length (mm.)		
				Epicotyls	Hypocotyls	Cotyledon
control	10.0±0.0	0.0±0.0	154.6±7.0	2.7±0.5	19.5±1.9	12.0±1.6
10	10.0±0.0	0.0±0.0	163.3±5.8	4.0±0.5	26.0±9.1	12.3±1.1
50	10.0±0.0	0.0±0.0	140.8±11	3.7±0.9	24.0±6.2	12.2±1.3
100	10.0±0.0	0.0±0.0	140.4±8.9	2.9±0.3	34.2±9.4	11.7±1.5
250	9.0±0.0	10.0±0.0	99.3±8.7	3.1±0.7	0.0±0.0	12.1±0.6
500	9.0±0.0	10.0±0.0	83.3±1.0	1.8±0.6	0.0±0.0	10.4±1.6
1000	6.5±2.1	35.0±20.2	61.1±28.7	0.8±0.6	0.0±0.0	9.4±2.4

Table A4 Phytotoxicity of *trans-p*-coumaryl diacetate against Italian ryegrass

Concentration	Seedling growth	%Growth inhibition	Fresh weight (mg)	Length (mm.)	
				Shoot	Root
control	10.0±0.0	0.0±0.0	209.1±5.2	62.6±11.9	28.6±6.4
10	10.0±0.0	0.0±0.0	173.1±5.7	48.2±5.0	23.2±6.2
50	10.0±0.0	0.0±0.0	164.0±5.8	40.3±5.4	21.1±8.5
100	10.0±0.0	0.0±0.0	146.3±0.1	39.3±6.1	20.0±5.7
250	10.0±0.0	0.0±0.0	139.4±11	38.5±7.4	19.0±4.6
500	10.0±0.0	0.0±0.0	123.3±8.4	31.6±8.3	18.0±6.3
1000	5.0±0.0	50.0±0.0	93.9±1.7	21.2±2.6	6.7±0.9

Table A5 Phytotoxicity of *trans-p*-hydroxycinnamyl acetate against lettuce seedlings

Concentration	Seedling growth	%Growth inhibition	Fresh weight (mg)	Seedlings length (mm.)		
				Epicotyl	Hypocotyl	Cotyledon
control	10.0±0.0	0.0±0.0	154.6±7.0	2.7±0.5	19.5±1.9	12.0±1.6
10	10.0±0.0	0.0±0.0	152.3±13.9	3.5±0.8	19.6±3.6	11.4±2.2
50	10.0±0.0	0.0±0.0	164.2±0.5	3.3±0.8	30.7±8.2	10.9±2.1
100	10.0±0.0	0.0±0.0	145.9±12.6	3.5±1.3	27.3±8.2	10.9±0.7
250	10.0±0.0	0.0±0.0	107.1±0.8	2.9±0.8	3.6±3.6	10.7±1.2
500	10.0±0.0	0.0±0.0	89.1±1.8	0.9±0.7	1.5±2.2	8.7±2.2
1000	8.0±0.0	20.0±14.1	69.5±19.1	0.5±0.3	0.0±0.0	7.5±1.6

Table A6 Phytotoxicity of *trans-p*-hydroxycinnamyl acetate against Italian ryegrass

Concentration	Seedling growth	%Growth inhibition	Fresh weight (mg)	Length (mm.)	
				Shoot	Root
control	10.0±0.0	0.0±0.0	209.1±5.2	62.6±11.9	28.6±6.4
10	10.0±0.0	0.0±0.0	149.2±3.1	38.1±5.1	22.1±4.8
50	10.0±0.0	0.0±0.0	148.8±4.1	31.9±5.8	20.5±6.4
100	10.0±0.0	0.0±0.0	177.9±1.6	30.2±6.2	21.0±7.8
250	10.0±0.0	0.0±0.0	139.5±7.8	24.8±4.5	11.7±4.8
500	10.0±0.0	0.0±0.0	129.3±0.3	20.8±6.6	8.6±1.7
1000	10.0±0.0	0.0±0.0	99.2±3.5	15.8±3.4	6.1±2.2

Table A7 Phytotoxicity of *trans-p*-hydroxycinnamaldehyde against lettuce seedlings

Concentration	Seedling growth	%Growth inhibition	Fresh weight (mg)	Seedlings length (mm.)		
				Epicotyl	Hypocotyl	Cotyledon
control	10.0±0.0	0±0	174.4±12.8	5.1±0.7	26.5±15.6	12.4±1.3
10	10.0±0.0	0±0	127.9±4.7	3.4±0.5	24.3±3.1	8.8±1.3
50	10.0±0.0	0±0	144.1±0.8	3.3±0.5	25.2±5.9	9.6±1.1
100	10.0±0.0	0±0	140.7±0.7	3.5±0.7	22.4±5.6	9.9±1.5
250	10.0±0.0	0±0	135.7±0.8	3.1±0.3	22.4±7.0	7.9±0.8
500	10.0±0.0	0±0	131.2±2.7	2.8±0.4	9.6±3.5	7.7±1.2
1000	10.0±0.0	0±0	11.2±0.8	0.2±0.3	0.0±0.0	1.8±2.3

Table A8 Phytotoxicity of *trans-p*-hydroxycinnamaldehyde against Italian ryegrass

Concentration	Seedling growth	%Growth inhibition	Fresh weight (mg)	Length (mm.)	
				Shoot	Root
control	10.0±0.0	0.0±0.0	209.1±5.2	56.4±1.3	29.3±7.3
10	10.0±0.0	0.0±0.0	218.9±1.9	41.3±5.7	25.5±4.8
50	10.0±0.0	0.0±0.0	206.9±2.5	42.4±7.1	35.5±6.9
100	10.0±0.0	0.0±0.0	219.9±0.8	41.1±5.2	32.3±5.7
250	10.0±0.0	0.0±0.0	201.5±1.4	43.4±7.3	30.8±8.5
500	10.0±0.0	0.0±0.0	179.4±1.2	36.6±6.6	23.3±5.1
1000	10.0±0.0	0.0±0.0	172.4±4.1	24.0±1.6	9.5±0.9

Table A9 Phytotoxicity of *p*-acetoxycinnamyl alcohol against lettuce seedlings

Concentration	Seedling growth	%Growth inhibition	Fresh weight(mg)	Seedlings length (mm.)		
				Epicotyl	Hypocotyl	Cotyledon
control	10.0±0.0	0.0±0.0	154.6±7.0	2.7±0.5	19.5±1.9	12.0±1.6
10	10.0±0.0	0.0±0.0	162.8±1.2	4.0±0.7	21.6±10.0	12.2±1.1
50	10.0±0.0	0.0±0.0	136.1±13.6	3.8±1.7	21.0±3.9	10.1±2.1
100	10.0±0.0	0.0±0.0	149.9±1.1	3.9±0.9	24.3±6.0	11.5±0.8
250	9.0±1.4	10.0±0.0	141.9±2.3	3.7±1.3	19.9±4.6	11.0±1.2
500	6.0±0.5	40.0±0.0	110.6±22.6	1.1±0.3	1.1±3.5	10.1±1.7
1000	0.5±0.7	95.0±7.1	4.8±6.7	0.1±0.2	0.0±0.0	0.7±2.2

Table A10 Phytotoxicity of *p*-acetoxycinnamyl alcohol against Italian ryegrass

Concentration	Seedling growth	%Growth inhibition	Fresh weight (mg)	Length (mm.)	
				Shoot	Root
control	10.0±0.0	0.0±0.0	209.1±5.2	62.6±11.9	28.6±6.4
10	10.0±0.0	0.0±0.0	144.2±1.1	32.2±6.1	12.3±3.1
50	10.0±0.0	0.0±0.0	171.0±4.6	33.4±7.1	17.8±6.1
100	10.0±0.0	0.0±0.0	160.6±10.3	29.3±5.7	15.0±8.1
250	10.0±0.0	0.0±0.0	163.5±0.1	25.7±7.8	13.0±5.9
500	10.0±0.0	0.0±0.0	138.8±7.7	24.2±2.5	5.8±3.3
1000	10.0±0.0	0.0±0.0	112.7±0.7	16.6±4.1	4.1±1.9

Table A11 Phytotoxicity activity of coumaryl ethyl ether against Italian ryegrass

Concentration	Seedling growth	%Growth inhibition	Fresh weight (mg)	Length (mm.)	
				Shoot	Root
control	10.0±0.0	0.0±0.0	209.1±5.2	62.6±11.9	28.6±6.4
10	10.0±0.0	0.0±0.0	187.1±2.8	43.9±7.6	21.3±6.4
50	10.0±0.0	0.0±0.0	172.1±4.8	40.6±5.7	21.9±8.4
100	10.0±0.0	0.0±0.0	187.8±1.0	42.8±4.4	17.5±5.7
250	10.0±0.0	0.0±0.0	136.5±22.2	34.5±5.6	16.8±4.5
500	10.0±0.0	0.0±0.0	127.8±8.3	33.6±3.8	11.6±4.7
1000	10.0±0.0	0.0±0.0	125.7±6.7	25.2±4.9	5.2±2.7

Table A12 Phytotoxicity of coumaryl ethyl ether against lettuce seedling

Concentration	seedling growth	%Growth inhibition	Fresh weight(mg)	seedlings length (mm.)		
				Epicotyl	Hypocotyl	Cotyledon
control	10.0±0.0	0.0±0.0	174.4±12.8	5.1±0.7	32.5±15.6	12.4±1.3
10	10.0±0.0	0.0±0.0	132.9±11.1	2.3±0.4	30.1±3.5	10.2±1.2
50	10.0±0.0	0.0±0.0	147.2±8.9	2.6±0.8	29.9±7.8	9.8±0.9
100	10.0±0.0	0.0±0.0	138.4±8.3	2.2±0.4	26.3±6.2	9.0±1.1
250	10.0±0.0	0.0±0.0	131.6±3.2	2.3±0.4	23.7±7.3	9.9±0.7
500	10.0±0.0	0.0±0.0	124.1±4.2	2.1±0.3	13.1±2.3	8.3±1.2
1000	10.0±0.0	0.0±0.0	103.4±26.8	2.2±0.4	5.6±3.2	7.5±2.0

Table A13 Antifungal activity of isolated pure compounds from active fraction of *M. fruticosum* against *P. parasitica* at 1000 μg·mL^{-1}

Compounds no.	Mycelial growth (mm.)	%inhibition
acetone	90.0±0.0	0
5-25	90.0±0.0	0
5-26	90.0±0.0	0
5-9	90.0±0.0	0
5-19	0.0±0.0	100
5-18	76.7±1.6	14.8
5-10	0.0±0.0	100
5-21	72.0±2.1	20

VITA

Ms. Rachsawan Mongkol was born on January 7, 1982 in Petchaburi province, Thailand. She was the first daughter of Mr. Yoon Mongkol and Mrs. Samran Kitwang. She graduated primary school from Ban Pongyae school (Cha-Am, Petchaburi) in 1994, secondary school from Nongplub Vittaya school (Hua-Hin, Prachuabkhirikhan) in 1997, high school from Benjamatheputit school (Muang, Petchaburi) in 2000, Bachelor degree of Science in Animal Science and Agricultural Technology, Silapakorn University in 2004, Master degree of Science in Biotechnology, Chulalongkorn University in 2007. After that she started her study on Ph.D. degree of Program in Biotechnology, Faculty of Science, Chulalongkorn University. During her study, she received a grant under the program Strategic Scholarships for Frontier Research Network for the Ph.D. Program Thai Doctoral degree from Commission on Higher Education, Ministry of Education. Moreover, she got the scholarships from the 90th anniversary of Chulalongkorn University fund (Ratchadaphiseksomphot Endowment Fund) and the scholarships of Conference Grant for Ph.D. Student, Chulalongkorn University.

Academic presentation:

1. Mongkol, R. and Chavasiri, W. 2011. Efficiency of Thai medicinal plants against phytopathogenic fungi. Poster presentation of The 16th Biological Sciences Graduate Congress (BSGC), 12-14 December 2011, at National University of Singapore, Singapore.

2. Mongkol, R. and Chavasiri, W. 2012. Screening of Plant Extracts to Control Phytophthora parasitica. Oral presentation of the 10th National Plant Protection Conference, 22-24 February 2012 at Chiangmai Province, Thailand.

3. Mongkol, R., Ishida, M., Chavasiri, W., Morimoto, M. and Matsuda K. 2013. Phytotoxic and antiphytopathogenic activities of constituents from Alpinia galanga rhizomes. Journal of Weed Science and Technology. 58 (Sup.), 27-28. Oral presentation of the Weed Science and Technology 2013, 12-13 April 2013 at Kyoto University, Kyoto, Japan.

4. Mongkol, R., Ishida, M., Matsuda K., Morimoto, M., and Chavasiri, W. 2013. Efficiency of Constituents from Thai Alpinia Galanga (L.) Against Phytopathogenic Fungi and Structure Activity Relationship. Oral presentation of The International Chemical Ecology Conference 2013 on 19-23 August 2013 at the Melbourne Convention and Exhibition Centre, Victoria, Australia.

5. Pongkittiphan, V., Mongkol, R. and Chavasiri, W. 2012. Antifungal agents from Xylia xylocarpa Taub. Natural Products Research and Development Center (NPRDC), Science and Technology Research Institute (STRI) on 28-30 November 2012 at Chiang Mai University, Chiang Mai, Thailand.