ฤทธิ์ต้านเชื้อราก่อโรคพืชจากสารสกัดสมุนไพรไทยบางชนิด

นางสาวนงนุช เครือสนิท

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2547

ISBN 974-53-2112-5

ANTI-PHYTOPATHOGENIC FUNGAL ACTIVITY FROM SOME THAI MEDICINAL HERB EXTRACTS

Miss Nongnoot Khruasanit

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Biotechnology
Faculty of Science
Chulalongkorn University
Academic Year 2004

ISBN 974-53-2112-5

Thesis Title	Anti-Phytopathogenic Fungal Activity from Some Thai Medicinal Herb Extracts
By	Miss Nongnoot Khruasanit
Field of study	Biotechnology
Thesis Advisor	Professor Udom Kokpol, Ph.D.
Thesis Co-advisor	Assistant Professor Warinthorn Chavasiri, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

……Dean of the Faculty of Science
(Professor Piamsak Menasveta, Ph.D.)

THESIS COMMITTEE

…….Chairman
(Associate Professor Sirirat Rengpipat, Ph.D.)

……..……Thesis Advisor
(Professor Udom Kokpol, Ph.D.)

…….Thesis Co-advisor
(Assistant Professor Warinthorn Chavasiri, Ph.D.)

…… Member
(Associate Professor Nattaya Ngamrojnavanich, Ph.D.)

…… Member
(Panpim Vonkhorporn, Ph.D.)

นงนุช เครือสนิท : ฤทธิ์ต้านเชื้อราก่อโรคพืชจากสารสกัดสมุนไพรไทยบางชนิด. (ANTI-PHYTOPATHOGENIC FUNGAL ACTIVITY FROM SOME THAI MEDICINAL HERB EXTRACTS) อ. ที่ปรึกษา: ศ.ดร. อุดม ก๊กผล อ. ที่ปรึกษาร่วม: ผศ.ดร. วรินทร ชวศิริ, 78 หน้า. ISBN 974-53-2112-5.

การศึกษาฤทธิ์ต้านเชื้อราก่อโรคพืชเบื้องต้นของสารสกัดสมุนไพร 18 ชนิดต่อเชื้อรา 6 ชนิด ได้แก่ *Alternaria brassicicola*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum*, *Phytophthora palmivora*, *Pythium* sp. และ *Sclerotium* sp. โดยผสมผงพืชลงในอาหาร PDA ที่ความเข้มข้น 50,000 ppm พบว่า ว่านน้ำ (*Acorus calamus*) แสดงฤทธิ์ยับยั้งการเจริญของเส้นใยเชื้อราโรคพืชทั้ง 6 ชนิด มากกว่า 61% โดยแสดงฤทธิ์ยับยั้งต่อ *P. palmivora* ได้ดีที่สุด ส่วนพืชชนิดอื่นๆ แสดงฤทธิ์ยับยั้งต่อเชื้อราโรคพืชแต่ละชนิดมากน้อยแตกต่างกันไป และส่วนของพืชที่ต่างกันยังแสดงฤทธิ์การยับยั้งที่ต่างกัน ยิ่งไปกว่านั้นพืชบางชนิดยังแสดงฤทธิ์ยับยั้งจำเพาะต่อเชื้อราโรคพืชอีกด้วย การศึกษาสิ่งสกัดไดคลอโรมีเทนและเมทานอลของว่านน้ำโดยผสมลงในอาหาร PDA ที่ความเข้มข้น 10,000 ppm พบว่าสิ่งสกัดไดคลอโรมีเทนแสดงฤทธิ์ยับยั้งการเจริญของเส้นใยเชื้อราทั้ง 6 ชนิด 100% ขณะที่สิ่งสกัดเมทานอลไม่แสดงฤทธิ์ยับยั้งต่อเชื้อรา การศึกษาประสิทธิภาพการยับยั้งการเจริญเติบโตของเส้นใยของสิ่งสกัดไดคลอโรมีเทนที่ความเข้มข้นระดับต่างๆ พบว่าสามารถยับยั้งการเจริญของ *Pythium* sp. ได้ดีที่สุดที่ IC_{50} 22 ppm จากการศึกษาองค์ประกอบทางเคมีของสิ่งสกัดไดคลอโรมีเทนของว่านน้ำ พบสารออกฤทธิ์ต้านเชื้อราก่อโรคพืช 3 ชนิด ได้แก่ β-asarone, α-asarone และ 2,4,5-trimethoxybenzaldehyde สารบริสุทธิ์ทั้งสามชนิดยืนยันฤทธิ์ต้านเชื้อราโดยใช้เทคนิคไบโอออโตกราฟิกซึ่งทดสอบกับสปอร์จากเชื้อรา 3 ชนิด ได้แก่ *A. brassicicola*, *C. gloeosporioides* และ *F. oxysporum*

สาขาวิชา……..เทคโนโลยีชีวภาพ……ลายมือชื่อนิสิต……..……..

ปีการศึกษา……2547…….ลายมือชื่ออาจารย์ที่ปรึกษา……...…...

ลายมือชื่ออาจารย์ทีปรึกษาร่วม.....

4472291623 : MAJOR BIOTECHNOLOGY
KEY WORD: ANTIFUNGAL / HERBS / BIOAUTOGRAPHIC

NONGNOOT KHRUASANIT: ANTI-PHYTOPATHOGENIC FUNGAL ACTIVITY FROM SOME THAI MEDICINAL HERB EXTRACTS. THESIS ADVISOR: PROFESSOR UDOM KOKPOL, Ph.D. THESIS CO-ADVISOR : ASST. PROF. WARINTHORN CHAVASIRI, Ph.D. 78 pp. ISBN 974-53-2112-5.

Screening of antifungal activity from eighteen plant extracts were evaluated on six phytopathogenic fungi; *Alternaria brassicicola*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum*, *Phytophthora palmivora*, *Pythium* sp. and *Sclerotium* sp. Cultures of phytopathogenic fungi on PDA were incorporated with plant powder at concentration 50,000 ppm. *Acorus calamus* exhibited antifungal activity to all six phytopathogenic fungi higher up to 61%, especially for *P. palmivora*. The percentage of mycelial growth inhibition varied with species of plant materials. Moreover, different parts of plant materials revealed different levels on mycelial growth inhibiton. Furthermore, some plant species showed the selectivity to inhibit certain fungi. Investigation of antifungal activity of dichloromethane and methanol crude extracts at concentration 10,000 ppm found that only dichloromethane extract displayed 100% mycelial growth inhibition to all six phytopathogenic fungi while the methanol crude extract had no activity. For evaluation of dichloromethane crude extract on mycelial growth inhibition at various concentrations, *Pythium* sp was inhibited at the lowest concentration of IC_{50} 22 ppm. Chemical constituents of the dichloromethane extract of *A. calamus* gave 3 antifungal compounds characterized as β-asarone, α-asarone and 2,4,5-trimethoxybenzaldehyde which confirmed their antifungal activity by bioautographic technique with *A. brassicicola*, *C. gloeosporioides* and *F. oxysporum*.

Field of study.....Biotechnology..... Student's signature.....
Academic year.....2004..... .....Advisor's signature.....
Co-advisor's signature.....

ACKNOWLEDGEMENTS

I would like to express my appreciation to my advisor, Professor Dr. Udom Kokpol and my co-advisor, Assistant professor Dr. Warinthorn Chavasiri for his assistance and valuable advice in conducting this research.

I would also like to extend my gratitude to Associate Professor Dr.Sirirat Rengpipat, Associate Professor Dr. Nattaya Ngamrojnavanich and Dr. Panpim Vonkhorporn for their kindness and helpful suggesting for the complements of this thesis and serving as thesis committee.

Special appreciation is extended to Division of Plant Disease and Microbiology, Department of Agriculture, Thailand for isolated, identified, and maintained fungal culture use in the research and I would also like to express special thanks to Dr. Chainarong Rattanakreetakul, a staff of Environmental Science Laboratory, Kasetsart University for giving recommendation about antifungal activity experiment.

I also wish to express my sincere appreciation to the Program of Biotechnology, Faculty of Science and Natural Product Research Unit, Department of Chemistry, Chulalongkorn university for providing facilities and throughout the course of my research. In addition, I also thanks Miss Wanida Munbunjong and Miss Sujittra Deesamer for their friendship, suggestion, comment and help.

Finally, my deepest gratitude is to all my family members for their support and encouragement throughout my study.

CONTENTS

Page

LIST OF FIGURES

Figure **Page**

LIST OF TABLES

LIST OF SCHEMES

LIST OF ABBREVIATIONS

^{o}C	degree celsius
CH_2Cl_2	dichloromethane
cm	centrimeter
^{13}C-NMR	carbon-13 nuclear magnetic resonance
COSY	correlated spectroscopy
DMSO	dimethylsulfoxide
EtOAc	ethyl acetate
g	gram
GC	gas chromatography
^{1}H-NMR	proton nuclear magnetic resonance
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High Performance Liquid Chromatography
IC	inhibition concentration
J	coupling constant
MeOH	methanol
mg	milligram
ml	milliliter
No.	number
NOESY	Nuclear Overhauser Enhancement Spectroscopy
PDA	potato dextrose agar
PDB	potato dextrose broth
ppm	part per million
sp.	species
TLC	thin-layer chromatography
w/w	weight by weight
µl	microliter
UV	ultraviolet
δ	chemical shift

CHAPTER I

INTRODUCTION

Thailand exported fruits and vegetables to abroad with the value higher than 882 billion baht in 2003-2004. However, pests are still a main problem for the extensive loss of agricultural products in many areas around the world although there is increasing of new pesticides against them. Environmental factors are cause of plant disease, such factor include temperature, soil moisture, soil nutrients, light, air humidity, soil structure and pH. That factors lack or excess of something that support their life cause of inducing infection by plant diseases. Plant diseases caused by fungi are the largest problem to all plant stages of their life (*e.g.*, seed, seedling, mature plant, or fruit) and either preharvest or postharvest. In Thailand, there were good environmental factors for growing plants and their products. Especially fruits containing a lot of water with sweet taste such as banana, mango, mangosteen, papaya, rambutan, *etc*. are favored to be attacked by some kinds of fungi. Each of the parasitic fungi can attack one or many kinds of plants.

Moreover, Thailand had imported pesticide over approximate 7,000 tons in 1999 and have a tendency to increase annually. In 2004, Thailand imported pesticides in the value of 8,535 million baht. [1] Disease control fungicides are the most popular use to control severity of plant disease; however, a frequent use of fungicides can also lead to the formation of resistant pathogen strains. Besides, the pesticide residue on agricultural products is also created 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 are generating a strong demand for biologically grown fruit 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 alternative method to use for plant disease control instead of synthetic fungicides.

1.1 Plant diseases caused by fungi [2,3]

More than 10,000 species of fungi can cause diseases in plants. All plants are attacked by some kinds of fungi and each of the parasitic fungi can attack one or many kinds of plants. Most of phytopathogenic fungi attack the foliage of plants by means of conidia. On the infected area, numerous conidia are produced that spread to other plants by winds, wind-blown rain, water, and insects and cause more infections. In most cases, the fungi overwinter primarily as conidia or mycelium in fallen leaves or other plant debris. When perennial plants are infected, the pathogens may overwinter as mycelium in infected tissue of the plant. Furthermore, almost all of plant pathogenic fungi spend a part of their lives on their host and the rest in soil. Plant diseases caused by fungi in Thailand are a main loss problem of many kinds of economic crops. Thus, in this research, six phytopathogenic fungi causing diseases in many Thai crops were selected as target fungi for evaluation of antifungal activity of some Thai medicinal herbs.

1.1.1 Leaf spot cause by *Alternaria brassicicola*

This pathogen affect most cruciferous crops, including broccoli and cauliflower (*Brassica oleracea* L. var. *botrytis* L.), Chinese or celery cabbage (*B. pekinensis*), cabbage (*B. oleracea* var. *capitata*), and radish (*Raphanus sativus*). Distribution of these pathogens are cosmopolitan in their distribution but occur sporadically. The pathogens are greatly influenced by weather with the highest disease incidence reported in mild, wet seasons and in areas with relatively high rainfall. For symptoms, *A. brassicicola* can affect host species at all stages of growth, including seeds. On seedlings symptoms include dark stem lesions immediately after germination, that can result in damping-off, or stunted seedlings. When older plants become infected, *Alternaria* symptoms often occur on the older leaves, since they are closer to the soil and are more readily infected as a consequence of rain splash or wind blown rain. Late infection, or infection of older leaves, does not characteristically reduce yields, and can be controlled through intensive removal of infected leaves. Fruit-bearing branches and seed pods showed dark or blackened spots that result in yield loss due to premature pod ripening and shedding of the seeds. Infection can also occur on the fruit, before or after harvest. A common symptom of

broccoli and cauliflower infection is a browning that occurs on the head. This pathogen is destructive diseases for seed growers. The pathogens can shrivel seeds within the pods or kill the pod stalks before seed formation. In addition to destruction of a seed crop, the pathogen can live within the seed, spread the disease to other fields, and cause a loss of seedling. Biology of *A. brassicicola*, it sporulates in temperature range of 8 to 30 °C, where mature spores occur after 43 and 14 hours, respectively. Moisture in the presence of rain, dew, or high humidity is essential for infection. *A. brassicicola* germinates (*in vitro*) at temperature range of 7 to 31 °C) and begins to germinate 98% of its spores at 15°C after 10 hours of incubation. Plants wound inoculated with *A. brassicicola* develop symptoms most quickly at 25°C, while seedlings from infected seeds develop symptoms most quickly at 30 °C. No germination occurs at 35°C for all three pathogens. Free water or high humidity is required for germination and infection. Germination also requires the presence of moisture in the form of free water or high relative humidity (at least 95%). The development of disease was shown in Fig 1.1.

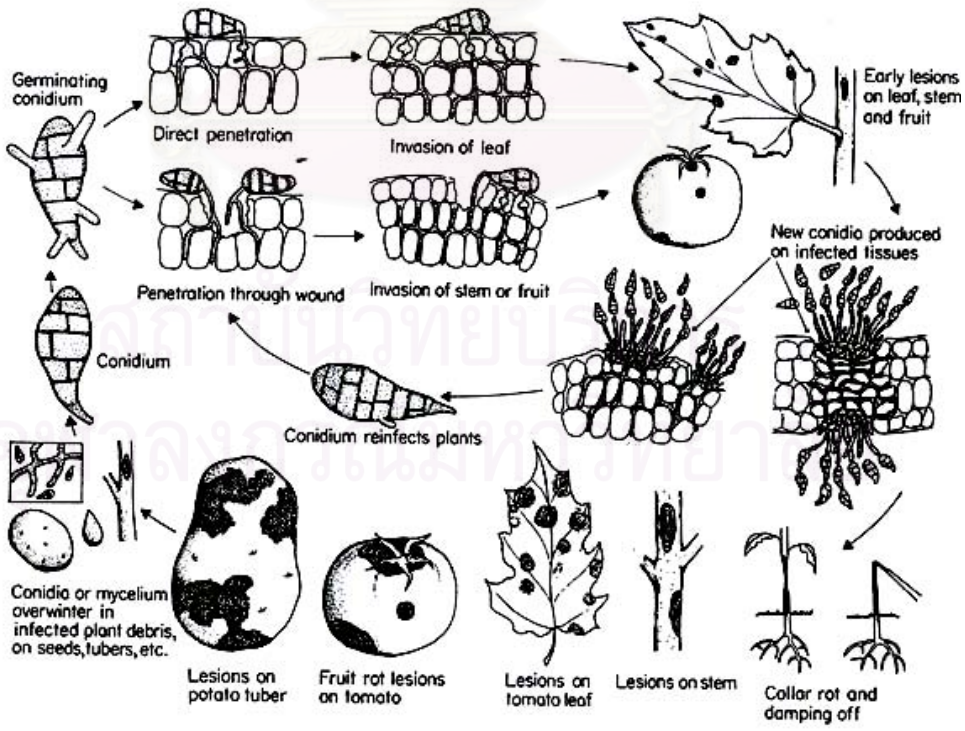


Fig 1.1 Disease cycle of *Alternaria* sp.[2]

A. brassicicola remain viable for a long period of time as spores on seed coat or as mycelium in seed as well as in infected plant debris. Seeds infected with *A. brassicicola* are known to have active surface spores for up to 2 years when the seeds are stored at 10°C with 50% relative humidity. Internal mycelium can remain viable for up to 12 years. Infected plant debris is also a major source of spores, which can survive up to 12 weeks. This is a potential problem for crops that are planted in a recently harvested field. Moreover, they also survive in the form of microsclerotia and chlamydospores which appear after infected leaves have partially decayed. Microsclerotia and chlamydospores of both pathogens can be formed within conidial cells. Both microsclerotia and chlamydospores develop best at low temperatures (3 °C) and are resistant to freezing and dessication (*in vitro* studies). Chlamydospores also can develop in conidial cells on natural soil at room temperature. *Alternaria* diseases are controlled primarily through the use of resistant varieties, disease-free or treated seed, and chemical sprays with fungicides such as chlorothalonil, maneb, captafol and mancozeb.

1.1.2 Anthracnose disease caused by *Colletotrichum gloeosporioides*

C. gloeosporioides is known to infect a wide variety of hosts such as mango, strawberry, guava, banana, olive, pepper, tomato, papaya, and chili. Symptoms of this disease is regularly seen in the field on ripe or overripe fruits they are not a serious problem with unrefrigerated fruit sold in the local markets. It is most important on fruits that are refrigerated and exported to overseas markets. For example the first symptoms of papaya anthracnose are round, watersoaked, and sunken spots on the body of the ripening fruit. Lesions may become as large as 5 cm in diameter. Pinkish-orange areas are formed by the conidial masses that cover the lesion center and are frequently produced in a concentric ring pattern.

Symptoms also may appear as irregular to circular spots 1 to 10 mm in diameter, sharply defined, occasionally slightly depressed and reddish-brown in color. These lesions are referred to as "chocolate spots". As the fruit ripens, these spots rapidly enlarge (up to 20 mm in diameter), to form the characteristic circular sunken lesions. *C. gloeosporioides* also causes anthracnose on papaya leaves.

Biology of *C. gloeosporioides*, a facultative parasite belongs to the order Melanconiales. The fungus produces hyaline, one- celled, ovoid to oblong, slightly curved or dumbbell shaped conidia, 10-15 µm in length and 5-7 µm in width. Masses of conidia appear in pink or salmon colored. The waxy acervuli produced in infected tissue are subepidermal, typically with setae, and simple, short, erect conidiophores. Isolations from the two different lesion types result in the isolation of *C. gloeosporioides* indistinguishable culturally from one another. Each isolate is capable of producing the two different lesion types. The factors involved in influencing a single isolate to produce these different lesion types are not known. Disease cycle of *C. gloeoporioides* was depicted in Fig 1.2.

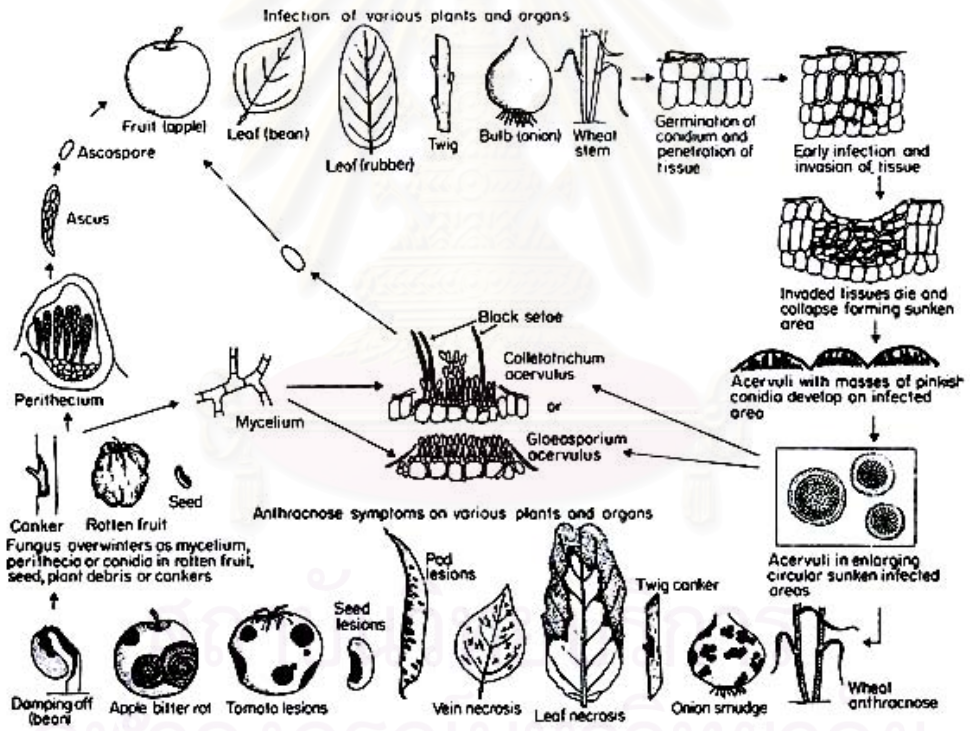


Fig 1.2 Disease cycle of *Colletotrichum* sp.[2]

The petioles of papayas support the abundant development of *C. gloeosporioides* and its perfect stage, *Glomerella cingulata*. The pathogen initially infects intact, non-wounded immature green fruit in the field. Spores germinate and form appressoria on the fruit surface. The fungus, using its appressorium, enzymatically penetrates the cuticle and then remains as sub-cuticular hyphae until

the post climacteric stage of fruit growth is attained. At this point, for reasons that are not understood, the fungus resumes growth and causes the characteristic symptoms. Thus, papaya anthracnose has a latent stage in its development that is similar to many other anthracnose diseases of tropical fruits. Environmental conditions favoring the pathogen are high temperatures, 28 °C being optimal, and high humidity. Spores must have free water to germinate; germination is negligible below 97% relative humidity. Spores are only released from acervuli when there is an abundance of moisture. Splashing from rain is a common means of spread. Severity of disease is related to weather and the fungus is relatively inactive in dry weather. Sunlight, low humidity and temperature extremes (below 18 °C or greater than 25 °C) rapidly inactivate spores. Primary inoculum can be disseminated by wind or rain.

1.1.3 Vascular wilt caused by *Fusarium oxysporum*

Hosts of *F. oxysporum* include: potato, sugarcane, garden bean, cowpea, and Musa sp. Like various other plant pathogens, *F. oxysporum* has several specialized forms - known as formae specialis (f.sp.) - that infect a variety of hosts causing various diseases. In Hawaii, these include: *F. oxysporum* f.sp. *asparagi* (fusarium yellows on asparagus); f.sp. *callistephi* (wilt on China aster); f.sp. *cubense* (Panama disease/wilt on banana); f.sp. *dianthi* (wilt on carnation); f.sp. *koae* (on koa); f.sp. *lycopersici* (wilt on tomato); f.sp. *melonis* (fusarium wilt on muskmelon); f.sp. *niveum* (fusarium wilt on watermelon); f.sp. *pisi* (on edible-podded pea); f.sp. *tracheiphilum* (wilt on Glycine max); and f.sp. *zingiberi* (fusarium yellows on ginger).

Distribution of these disease, overall, the distribution of *F. oxysporum* is known to be cosmopolitan. However, the different special forms (f.sp.) of *F. oxysporum* often have varying degrees of distribution.

Symptom of disease caused by *F. oxysporum* and its various formae speciales have been characterized as causing the following symptoms: vascular wilt, yellows, corm rot, root rot and damping-off. The most important of these is vascular wilt. Of the vascular wilt-causing Fusaria, *F. oxysporum* is the most important species. Strains that are rather poorly specialized may induce yellows, rot, and damping-off, rather than the more severe vascular wilt. Since fusarium wilt is the most important disease caused by *F. oxysporum*, the focus of this section will be on this symptom. In general,

fusarium wilts first appear as slight vein clearing on the outer portion of the younger leaves, followed by epinasty (downward drooping) of the older leaves. At the seedling stage, plants infected by *F. oxysporum* may wilt and die soon after symptoms appear. In older plants, vein clearing and leaf epinasty are often followed by stunting, yellowing of the lower leaves, formation of adventitious roots, wilting of leaves and young stems, defoliation, marginal necrosis of remaining leaves, and finally death of the entire plant. Browning of the vascular tissue is strong evidence of fusarium wilt. Further, on older plants, symptoms generally become more apparent during the period between blossoming and fruit maturation.

For biology, in solid media culture, such as potato dextrose agar (PDA), the different special forms of *F. oxysporum* can have varying appearances. In general, the aerial mycelium first appears white, and then may change to a variety of colors - ranging from violet to dark purple - according to the strain (or special form) of *F. oxysporum. F. oxysporum* produces three types of asexual spores: microconidia, macroconidia, and chlamydospores. Microconidia are one or two cells, and are the type of spore most abundantly and freqeuntly produced by the fungus under all conditions. It is also the type of spore most frequently produced within the vessels of infected plants. Macroconidia are three to five cells, gradually pointed and curved toward the ends. These spores are commonly found on the surface of plants killed by this pathogen as well as in sporodochialike groups. Chlamydospores are round, thick-walled spores, produced either terminally or intercalary on older mycelium or in macroconidia. These spores are either one or two cells.

Disease cycle of *F. oxysporum* is an abundant and active saprophyte in soil and organic matter, with some specific forms that are plant pathogenic as shown in Fig 1.3. Its saprophytic ability enables it to survive in the soil between crop cycles in infected plant debris. The fungus can survive either as mycelium, or as any of its three different spore types.

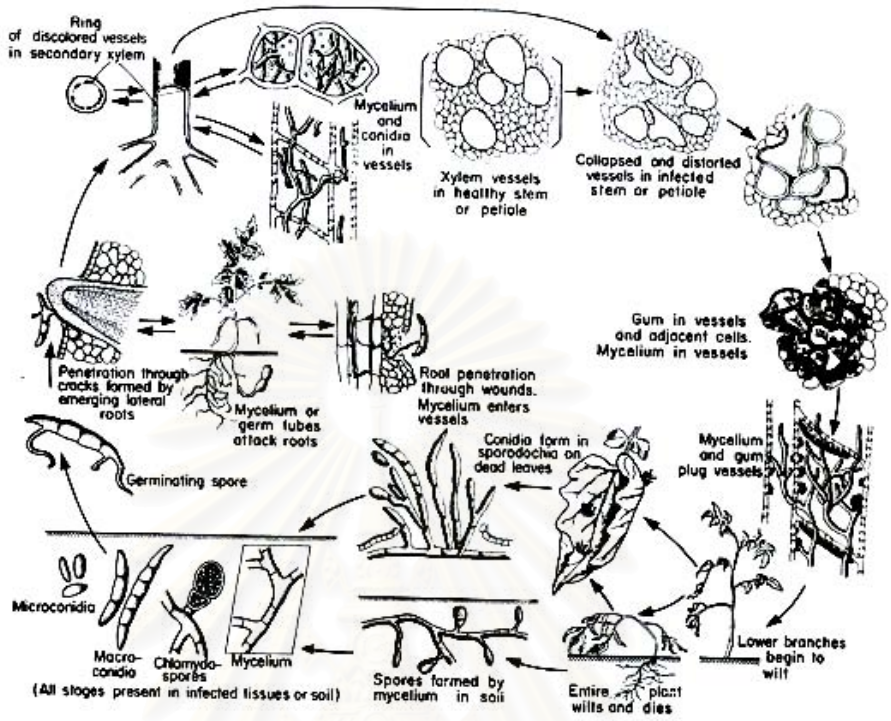


Fig 1.3 Disease cycle of *F. oxysporum* [2]

Healthy plants can become infected by *F. oxysporum* if the soil in which they are growing is contaminated with the fungus. The fungus can invade a plant either with its sporangial germ tube or mycelium by invading the plant's roots. The roots can be infected directly through the root tips, through wounds in the roots, or at the formation point of lateral roots. Once inside the plant, the mycelium grows through the root cortex intercellulary. When the mycelium reaches the xylem, it invades the vessels through the xylem's pits. At this point, the mycelium remains in the vessels, where it usually advances upwards toward the stem and crown of the plant. As it grows, the mycelium branches and produces microconidia, which are carried upward within the vessel by way of the plant's sap stream. When the microconidia germinate, the mycelium can penetrate the upper wall of the xylem vessel, enabling more microconidia to be produced in the next vessel. The fungus can also advance laterally as the mycelium penetrates the adjacent xylem vessels through the xylem pits.[1]

Due to the growth of the fungus within the plant's vascular tissue, the plant's water supply is greatly affected. This lack of water induces the leaves' stomata to close, the leaves wilt, and the plant eventually dies. It is at this point that the fungus invades the plant's parenchymatous tissue, until it finally reaches the surface of the dead tissue, where it sporulates abundantly. The resulting spores can then be used as new inoculum for further spread of the fungus.

Epidemiology of *F. oxysporum* is primarily spread over short distances by irrigation water and contaminated farm equipment. The fungus can also be spread over long distances either in infected transplants or in soil. Although the fungus can sometimes infect the fruit and contaminate its seed, the spread of the fungus by way of the seed is very rare. It is also possible that the spores are spread by wind.

1.1.4 Root and stem rot caused by *Phytophthora palmivora*

Durian fruit rot is caused by *P. palmivora* that also causes durian root and stem rot. Signs and symptoms of this disease under humid conditions with frequent rain, the fungus will spread to big branches where it will cause juicy spots, change of tissue color and yellowing of leaves. If the disease infests the leaves, brown spot appear and the disease will spread into the fruit. Usually brown spots are visible on the rotting fruit. This brown spot will expand and eventually the rotted fruit will fall down. This symptom is often found at about 1 month before harvesting. The mature fruit having brown spot will split and other fungi can infect and destroy the fruit. On the skin of rotted fruits which fall down on the ground, usually appears a white fluff, which is mycelium and zoosporangium of *P. palmivora*. Disease cycle of *P. palmivora* is shown in Fig 1.4.

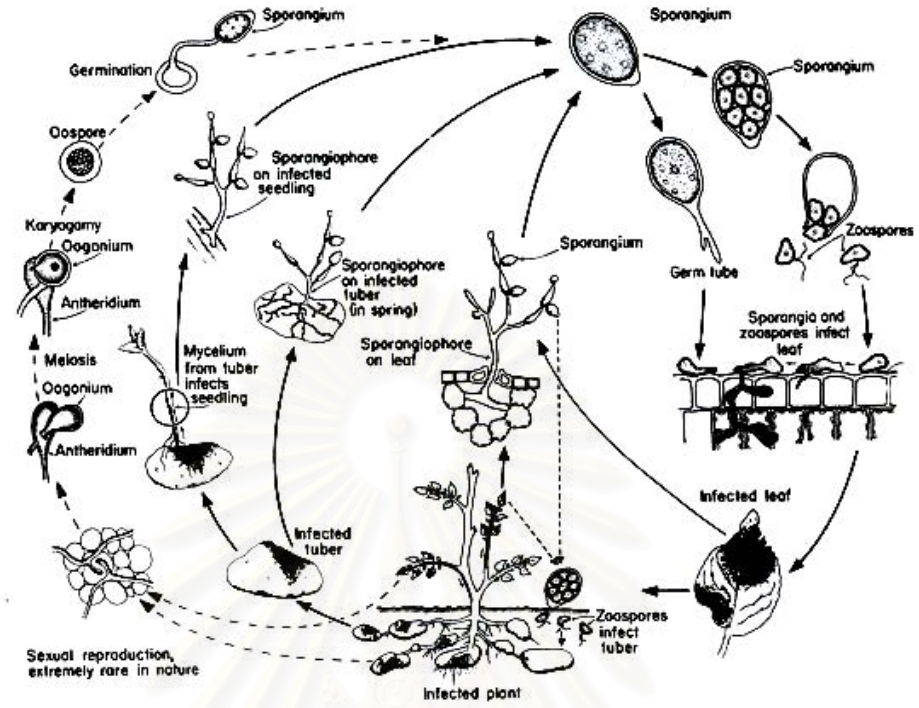


Fig 1.4 Disease cycle of *P. palmivora* [2]

1.1.5 Seed rot, root rot, soft rot, damping-off cause by *Pythium* sp.

Damping-off is the first seedling disease to occur in a growing season because this fungus prefers cold soil temperatures and excess soil moisture. Germinated seed that fails to emerge can be swollen, twisted and partially or wholly rotted. These rots are often referred to as "water molds". The occasional plant that does emerge may wilt, turn brown and die. Dead seedlings may be visible on the ground with infected plants killed before the first true leaf stage. Plants often have a rotted appearance. Leaves of infected seedlings are initially gray-green and then turn brown. A few days later, the plants die. Diseased plants are easily pulled from the soil because of rotted roots.

This disease is extremely severe if excessive rainfall occurs immediately after planting. It has been observed in well-drained and poorly drained soils; in sandy as well as heavier soils; and in low and high organic soils. The disease appears sporadically. It may occur in one part of a field in one year, but the following year may appear elsewhere. Moreover, a second planting in a disease area may (or may not) give a healthy stand. Symptoms may appear at any time of the year, but they will

always be associated with wet soil conditions either from excessive rainfall or irrigation. Poor drainage conditions will compound this problem. This is a root rot disease. The symptoms observed on the leaves are the result of fungal activity on the root system. Aboveground symptoms are typically a nonspecific decline in turf quality. Small or large turf areas will become a general yellow, light green or brown color and gradually decrease in density (thinning). However, the turf seldom dies from *Pythium* root rot, and no distinct patches are observed. Roots appear thin with few root hairs and have a general discoloration but are not black and rotted as they are with Take-all Root Rot. Microscopic examination of affected roots will determine if *Pythium* spp. are associated with the symptoms.

Cultural Controls: *Pythium* spp. are naturally present on warm-season turfgrass roots. The triggers for disease are wet soil conditions and stressed turfgrass. To prevent the disease, especially during low rainfall periods, improve drainage and reduce irrigation. Avoid irrigation management that maintains constantly wet soil. During periods of high rainfall, incorporate the following techniques into your management program. Mow the turfgrass at the correct height and as often as necessary so that only one third (1/3) of the leaf tissue is removed during any one mowing event. It may be necessary to raise the mowing height during periods of conducive weather. Improper mowing is a major stress on turfgrass. Balance nitrogen applications with equal amounts of potassium. Extra potassium may be useful in late summer and early fall for those areas that are routinely affected by *Pythium* root rot. Either use a slow-release potassium source, or apply a quick-release source more frequently. If the disease does occur, it may be beneficial to apply nutrients foliarly since the roots are not functioning efficiently.

Chemical Controls: azoxystrobin, chloroneb, ethazol, fosetyl-Al, mefenoxam, propamocarb. To increase effectiveness, these fungicides, except for fosetyl-Al should be either lightly watered into the root zone or applied in 5 gallons water per 1000 square feet. At least two applications will probably be required. Alternate between compounds to avoid development of fungicide-resistant strains of *Pythium.* Except for azoxystrobin, these fungicides are specific for *Pythium* spp. only. They are not useful against any other pathogens that attack turfgrass. Additionally, due to the

variation in *Pythium* spp. involved with this root rot, the mefenoxam manufacturer does not recommend its use for root rot control. Disease cycle of *Pythium* sp. is shown in Fig 1.5.

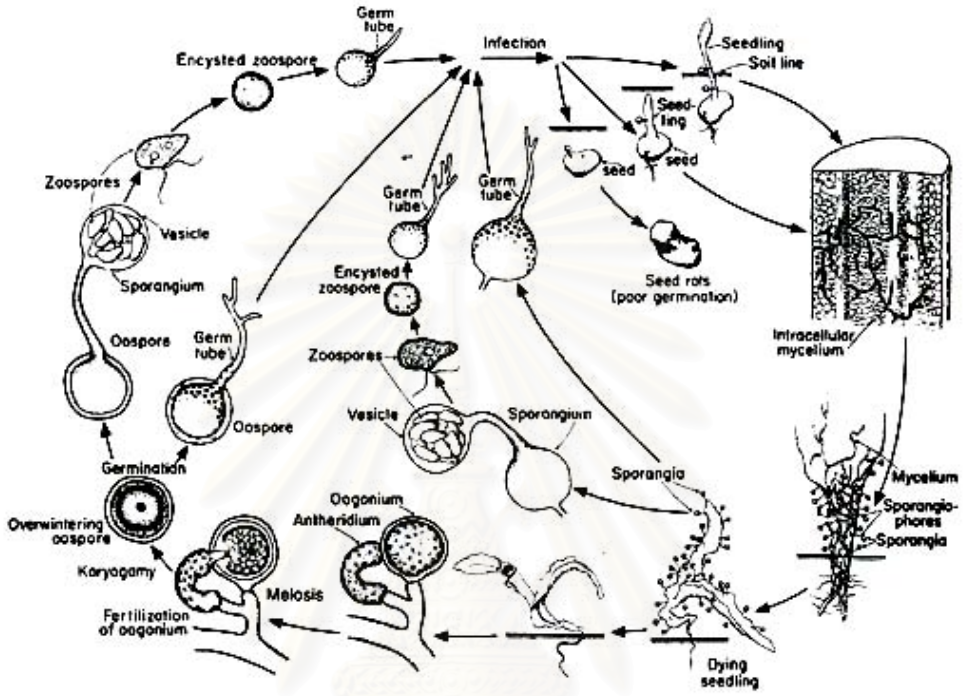


Fig 1.5 Disease cycle of *Pythium* sp.[2]

1.1.6 Sclerotium disease caused by *Sclerotium* sp.[2]

Sclerotium diseases occur in warm climates. They cause damping-off of seedlings, stem canker, crown blight, root, crown, bulb, and tuber rot, and fruit rots. *Sclerotium* frequently causes severe losses of fleshy fruits and vegetables during shipment and storage. They are called southern wilts or southern blights, and they affect a wide variety of plants, including most vegetables, flowers, legumes, cereals, forage plants, and weeds.

Seedlings are invaded by the fungus quickly and then die. Plants that have already developed some woody tissue are not invaded throughout, but the fungus grows into the cortex and slowly or quickly girdles the plants, which eventually die. Usually the infection begins on the succulent stem as a dark-brown lesion just below the soil line. Soon, at first the lower leaves and then the upper leaves turn yellow or

wilt or die back from the tips downward. In plants with very succulent stems, such as celery, the stem may fall over, whereas in plants with harder stems, such as tomato, the invaded stem stands upright and begins to lose its leaves or to wilt. In the meantime, the fungus grows upward in the plant, covering the stem lesion with a cottony, white mass of mycelium, with the upward advance of the fungus depending on the amount of moisture present. The fungus moves even more rapidly downward into the roots and finally destroys the root system. The white mycelium is always present in and on infected tissues, and from these it grows over the soil to adjacent plants, starting new infections. Invaded stem, tuber, and fruit tissues are usually pale brown and soft but not watery. When fleshy roots or bulbs are infected, a watery rot of the outer scales or root tissues may develop, or the entire root or bulb may rot and disintegrate and be replaced by debris interwoven with mycelium. If bulbs, roots, and fruits are infected late in their development, symptoms may go unnoticed at harvest, but the disease continues as a storage rot.

On all infected tissues, and even on the nearby soil, the fungus produces numerous small roundish sclerotia of uniform size that are white when immature, becoming dark brown to black on maturity. Each sclerotium is differentiated into an outer melanized rind, a middle cortex, and an innermost area of loosely arranged hyphae. The fungus, *Sclerotium* sp., produces abundant white,fluffy, branched mycelium that forms numerous sclerotia but is usually sterile, that is, does not produce spores.

The fungus overwinters mainly as sclerotia. It is spread by moving water, infested soil, contaminated tools, infected transplant seedlings, infected vegetables and fruits, and, in some hosts, as sclerotia mixed with the seed.

The fungus attacks tissues directly. However, the mass of mycelium it produces secretes oxalic acid and also pectinolytic, cellulolytic, and other enzymes, and it kills and disintegrates tissues before it actually penetrates the host. Once established in the plants, the fungus advances and produces mycelium and sclerotia quite rapidly, especially at high moisture and high temperature (between 30 and 35˚C)

Control of Sclerotium diseases is difficult. Crop rotation, cultural practices such as deep plowing to bury fungal sclerotia in surface debris, fertilizing with

ammonium-type fertilizers, applying calcium compounds, and, in some cases, application of fungicides such as pentachloronitrobenzene (PCNB) to the soil before planting, or in the furrow during planting, provide only partial control. Disease symtoms of *Sclerotium* sp. is shown in Fig 1.6.

Fig 1.6 Disease symtoms of *Sclerotium* sp.

1.2 Control of plant diseases

There was several methods to protect crop plants from plant diseases, these losses may be attenuated by control methods the eradicate or reduce pathogen such as crop rotation, sanitation or creating conditions unfavorable to the pathogen. The particular characteristics in the life cycle of each fungus, its habitat preferences, and its performance under certain environmental conditions are some of the most important points to be considered in attempting to control a plant disease caused by a fungus. However, although some diseases can be controlled completely by just on type of control measure, a combination of measures is usually necessary for satisfactory control of most diseases. Sometimes the only one available for their control is application of chemical sprays or dusts on the plants, on seeds, or into the

soil where the plants are to be grown. Soil-inhabiting fungi may be controlled by steam or electric heat and, in fields, by volatile liquids. In some diseases the fungus is carried in the seed, and control can be obtained only through treatment of the seed with systemic fungicides or hot water. Fungicides may be protectant or systemic in action. Protectant fungicides are effective against pathogens only at the site of application. This is usually the seed, tuber or foliage present at the time of application. Some fungicides may be taken up locally and can eradicate an established infection close to the site of application. Most systemic fungicides are, however, taken up and translocated throughout the plant in the xylem. The whole plant may therefore become resistant to infection. Many systemic fungicides also have a curative action and will eradicate already existing infections. Furthermore, these compounds may move into newly developing shoots and prevent invasion there by pathogens. As mentioned above, mode of action of fungicides may be divided to be 3 group by mode of action are protective action (protectants), curative or eradictive action (eradicants) and systemic fungicides.[1]

1.3 Natural products from plants [4,5]

Plants produce a vast and diverse assortment of organic compounds, the great majority of which do not appear to participate directly in growth and development. These substances, traditionally referred to as secondary metabolites. Studies of natural products stimulated development of the separation techniques, spectroscopic approaches to structure elucidation, and synthetic methodologies that now constitute the foundation of contemporary organic chemistry.

Based on their biosynthesic origins, plant natural products can be divided into three major groups: the terpenoids, the alkaloids and the phenylpropanoids and allied phenolic compounds. Terpenoids represent the largest class of natural products with over 40,000 defined compounds, including both primary and secondary metabolites, and they are composed of various numbers of isoprene (C5) units derived from the fundamental terpenoid precursor isopentenyl diphosphate (IPP). Both alkaloids and phenylpropanoids are generally produced from amino acids or their derivatives. In particular, phenylpropanoids are formed aromatic amino acid metabolism (*i.e.*, the

shikimate pathway), while alkaloids are largely derived from a wider range of amino acids. Bioactive natural products include compounds extracted from organisms, usually plants and microorganisms, which exhibit beneficial response. Recognition of biological properties of myriad natural products has fueled the current focus of this field, namely, the search for new drugs, antibiotics, insecticides, and herbicides. Thailand is located in a tropical region of the world where a vast biodiversity exists and which possesses a large number of medicinal plants that have been in used traditional treatment in the primary health care system. Therefore natural products research in Thailand is targeted toward the goal of studying the biological activity and chemical constituents of medicinal plants for both to obtain new drug leads and to contribute to the economic development of Thailand by developing them into new pharmaceuticals that provide new industry.[6]

In generally, plants defend themselves against pathogens by a combination of weapons from two arsenals: (1) Structural characteristics that act as physical barriers and inhibit the pathogen (2) biochemical reactions that take place in the cells and tissues of the plant and produce substances which either are toxic to the pathogen or create conditions that inhibit growth of the pathogen in the plant.

Phytoalexins are toxic antimicrobial substances produced in appreciable amounts in plants only after stimulation by various types of phytopathogenic microorganisms or by chemical and mechanical injury. Most know phytoalexins are toxic to and inhibit the growth of fungi pathogenic to plants. The chemical structures of phytoalexins produced by plants of a family are usually quite similar; for example, in most legumes phytoalexins are isoflavonoids, and in the Solanaceae are terpenoids. Some of the better studied phytoalexins include phaseollin in bean, pisatin in pea, glyceollin in soybean, alfalfa, and clover, rishitin in potato, gossypol in cotton, and capsidiol in pepper. Moreover, it has often been observed that certain common phenolic compounds that are toxic to pathogens are produced and accumulated at a faster rate after infection for example chlorogenic acid, caffeic acid, and ferulic acid.[2]

Using bioresources against phytopathogenic fungal diseases is not new. Several studies of them are available but not few phytochemical fungicides have been developed into product from and fewer still have made any real impact in crop

protection. However, the interest in developing products from natural sources is increasing rapidly, driven mainly by their commercial and, to a lesser degree, in response to consumer attitudes to pesticide use.

From literature review, antifungal substances are in several classification based on the chemical structure such as terpenoid group (iridoids, sesquiterpenoids, saponins), nitrogen and/or sulphur containing constituents (alkaloids, amines, amides), aliphatics especially long-chain and fatty acids, and aromatics (phenolics, flavonoids, stilbenes, bibenzyls, xanthones and benzoquinones).[7]

Antifungal constituents in several plant species have been presented as following examples. Garcinia acid derivatives, 2-(butoxycarbonylmethyl)-3-butoxycarbonyl-3-hydroxy-3-propanolide and 1′,1′-dibutyl methyl hydroxycitrate, were isolated from fruits of *Garcinia atroviridis* guided by TLC bioautography against *Cladosporium herbarum*.[8]

Essential oil from *Tagetes patual* Linn. was evaluated for fungicidal properties by poison food technique against five st*rains Aspergillus niger, Penicillium feniculosa, Fusarium solani, Rhizomucor sp.,* and *Trichoderma viride* fully inhibition at concentration 1600 and 3200 ppm.[9]

Sesquiterpene lactones constitute a large family of more than 5000 compounds mainly isolated from members of the Compositae and it possess a wide spectrum of biological activity in which they appear to play a role in plant defense mechanisms. Due to their toxicity, some sesquiterpene lactones with eudesmanolide, pseudoquaianolide, and *trans*,*trans*-germacranolide skeletons have been evaluated for fungicidal activity.[10]

Cinnamaldehyde at 30 ppm showed completely inhibit mycelial growth and germination of conidia of *Botryodipodia theobromae*, *Colletotrichum gloeosporioides* and *Gliocephalotrichum microchlamydosporum* which cause of postharvest diseases of rambutan (*Nephelium lappaceum* L.).[11]

A kaempferide triglycoside which showed a strong activity in restricting fungal parasite development has been found as a constitutive component in an uninfected carnation (*Dianthus caryophyllus*) of the cultivar Novada.[12]

Chemical studies carrie out on Brazilian Piperaceae species have isolated

several amides, mainly those bearing isobutyl, pyrrolidine, dihydropyridone and piperidine moieties. Two antifungal cinnamoyl derivatives from seeds and leaves of *Piper tuberculatum* were also have isolated. [13]

Four benzoic acid derivatives: genuine acid, taboganic acid, methyl ester of 2,2-dimethyl-6-carboxychroman-4-one and methyl ester of 2,2-dimethyl-3-hydroxy-6-carboxychroman have been isolated from the dichloromethane extract of the leaves of *Piper dilatatum* (Piperaceae) displaying antifungal activity against *Cladosporium cucumerinum* in direct bioautography on TLC plates.[14]

Hexane and ethyl acetate fractions of the methanol extract of *Macaranga monandra* (Euphorbiaceae) showed fungal growth inhibition of *Colletotrichum acutatum, C. fragariae* and *C. gloeosporioides, Fusarium oxysporum, Botrytis cinerea* and *Phomopsis viticola*. Bioassay-guided fractionation led to the isolation of two active clerodane-type diterpenes: kolavenic acid and 2-oxo-kolavenic acid. Both of them displayed moderate growth inhibition against *Botrytis cinerea* and *Phomopsis viticola*.[15]

Shikonin, a quinone from the root bark of *Lithospermum erythrorhizon*, demonstrated good to moderate antifungal activity to *Colletotrichum* sp. Moreover, *C. fragariae* was most sensitive species to quinone-based chemistry, *C. gloeosporioides* had intermediate sensitivity while *C. acutatum* was the species least sensitive to these compounds.[16]

Affinin from primary component of the lipidic fraction is the main alkamide from *Heliopsis longipes*(Gray) Blake (Asteraceae) roots demonstrates the fungistatic activities against four of the assayed fungi showed an important sensitivity to the presence of affinin: *Sclerotium rolfsii, S. cepivorum, Phytophthora infestans* and *Rhizoctonia solani* displaying a growth inhibition of 100%.[17]

Methanol fraction of acetone extract of stem bark of *Lannea coromandelica* remarkably inhibited motility followed by lysis of zoospores of *Aphanomyces cochlioides*. Bioassay-guided fractionation and chemical characterization revealed that the active constituents were angular type polyflavonoid tannins.[18]

Bioassay-directed isolation of antifungal compounds from an ethyl acetate extract of *Ruta graveolens* leaves yielded two furanocoumarins, one quinoline

alkaloid, and four quinolone alkaloids, including a novel compound, 1-methyl-2-[6′-(3″,4″-methylenedioxyphenyl)hexyl]-4-quinolone. Antifungal activities of the isolated compounds, together with 7-hydroxycoumarin, 4-hydroxycoumarin, and 7-methoxycoumarin, which are known to occur in Rutaceae species, were evaluated by bioautography and microbioassay. Four of the alkaloids had moderate activity against *Colletotrichum* species. These compounds and the furanocoumarins 5- and 8-methoxypsoralen had moderate activity against *Fusarium oxysporum*. The novel quinolone alkaloid was highly active against *Botrytis cinerea*. Phomopsis species were much more sensitive to most of the compounds, with *P. viticolabeing* highly sensitive to all of the compounds.[19]

The hexane extract from leaves of *Vitex trifolia* L. (Verbenaceae) completely inhibited the growth of the fungal plant pathogen *Fusarium* sp. within the first 2 day of experiment, but dropped significantly at day 6 (15% inhibition).[20]

Oils of bay, cinnamon leaf, clove, lemongrass, mustard, orange, sage, thyme and two rosemary oils were tested by two methods: (1) a rye bread-based agar medium (2) real rye bread was exposed. Antifungal effects of the essential oils depended on the application method. Larger phenolic compounds such as thymol and eugenol (thyme, cinnamon and clove) had best effect applied directly to medium, whereas smaller compounds such as allyl isothiocyanate and citral (mustard and lemongrass) were most efficient when added as volatiles. [21]

From these aforementioned studies show that antifungal substances could be found in several plant and possessed several different chemical class of active compounds. Each compound may show antifungal activity to different phytopathogenic fungi and have either fungistatic or fungicidal mode of action to the phytopathogenic fungi. Moreover, Thailand locate in a tropical rainforest region taking advantage of the diversity of secondary metabolites responsible for antifungal activity and many varieties of plants have been used as medicinal herbs which containing various bioactive compounds.

Therefore, the main objectives of this research are as follows:

1. To study for anti-phytopathogenic fungal activity from some Thai medicinal herbs.
2. To search for bioactive compound possessing antifungal activity.

CHAPTER II

MATERIALS AND METHODS

2.1 Plant materials

The dried plant materials were purchased from medicinal herb shops, Bangkok Thailand as listed in Table 2.1.

Table 2.1 Selected plant materials used in this research

No	Plants	Common name	Part used
	Acanthaceae		
1	*Andrographis paniculata* (Burn) Wall. ex Ness.	king of bitters, kirayat	aerial part
	Amaranthaceae		
2	*Achyrathes aspera* Linn.	achyranthes	aerial part
	Araceae		
3	*Acorus calamus* Linn.	myrtlegrass, sweetflag	rhizome
	Cleomaceae		
4	*Cleome viscose* Linn.	wild spider	whole plant
	Cyperaceae		
5	*Cyperus rotundun* Linn.	nutgrass, cocograss	tuber
	Meliaceae		
6	*Azadirachta indica* A. Juss.	neem	fruit
	Piperaceae		
7	*Piper ribesoides* Wall	sakhan	stem
8	*Piper chaba* Hunter.	long pepper	fruit
9	*Piper chaba* Hunter.	long pepper	stem
10	*Piper cubeba*	cubeb	fruit
11	*Piper nigrum*	black pepper	fruit

Table 2.1 (continued)

No	Plants	Common name	Part used
	Piperaceae		
12	*Piper sarmentosum* Roxb.	variegatum	leaf
13	*Piper sarmentosum* Roxb.	variegatum	fruit
	Plumbaginaceae		
14	*Plumbago indica* Linn.	leadwort	whole plant
	Rutaceae		
15	*Zanthoxylum limonella*	sichuan pepper	fruit
	Sterculiaceae		
16	*Mansonia gagei* Drumm.	chan-cha-mod	heartwood
	Zingiberaceae		
17	*Curcuma longa* Linn	turmeric	rhizome
	Zygophyllaceae		
18	*Tribulus terrestris* Linn.	ground bur-nut	aerial part

Dried plant materials were milled to powder and subjected to preliminary screening for antifungal tests. The attractive samples exhibiting interesting preliminary screening tests were chosen for further investigation to search for biological active compounds.

2.2 Chemicals

Merck's TLC was performed on aluminium sheets precoated with silica gel 60 F254 (20 x 20 cm, layer thickness 0.2 mm) for qualitative analysis purpose and the bioautographic assay. Adsorbents such as silica gel (Kieselgel 60, Merck) No.7731, No.7734 and No.9385 were used for quick column chromatography, open column chromatography and flash column chromatography, respectively. All organic solvents: hexane, dichloromethane, ethyl acetate and methanol were commercial grade and were redistilled prior to use except those being HPLC grade for HPLC analysis and deuterated chloroform (chloroform-*d*) solvent for NMR spectra. For dipping reagent, the 10% sulfuric acid in ethanol was used for detecting spots of some compounds with no uv absorption.

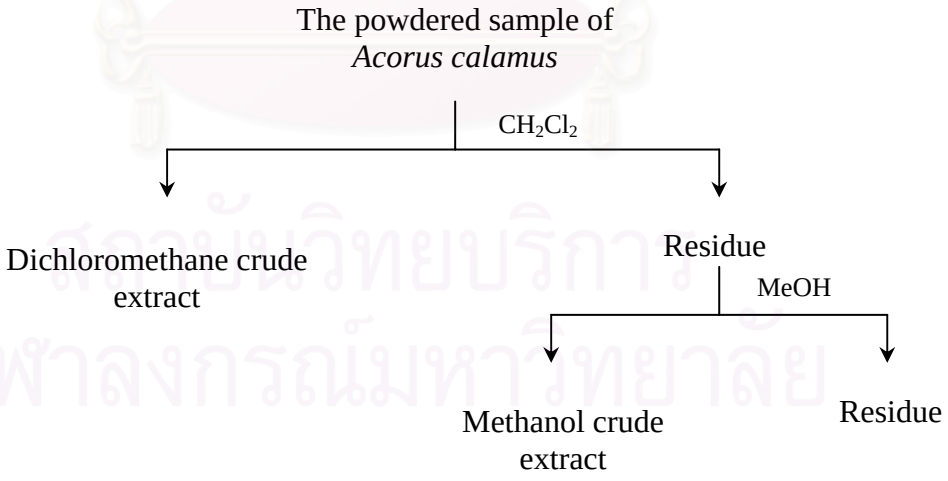
2.3 Equipments

Gas chromatograph was performed on Shimadzu 9A, HP-5 column (0.32 mm i.d., 30 m length and 0.25 mm. thickness). The temperature program was set at 60 °C for 4 min, 60-220 °C at 15°C /min and 5 min hold at 220°C. HPLC (Waters) was conducted on semipreparative using Cosmosil 5C18-AR-II column (250 mm length, 10 mm i.d.; All Tech Associates,Waters). The proton and carbon-13 nuclear magnetic resonance (^{1}H and ^{13}C-NMR) including 2D-NMR experiments were carried out with a Varian Mercury + 400 NMR spectrometer.

2.4 Extraction of *A. calamus* rhizomes

2.4.1 Extraction for preliminary screening test

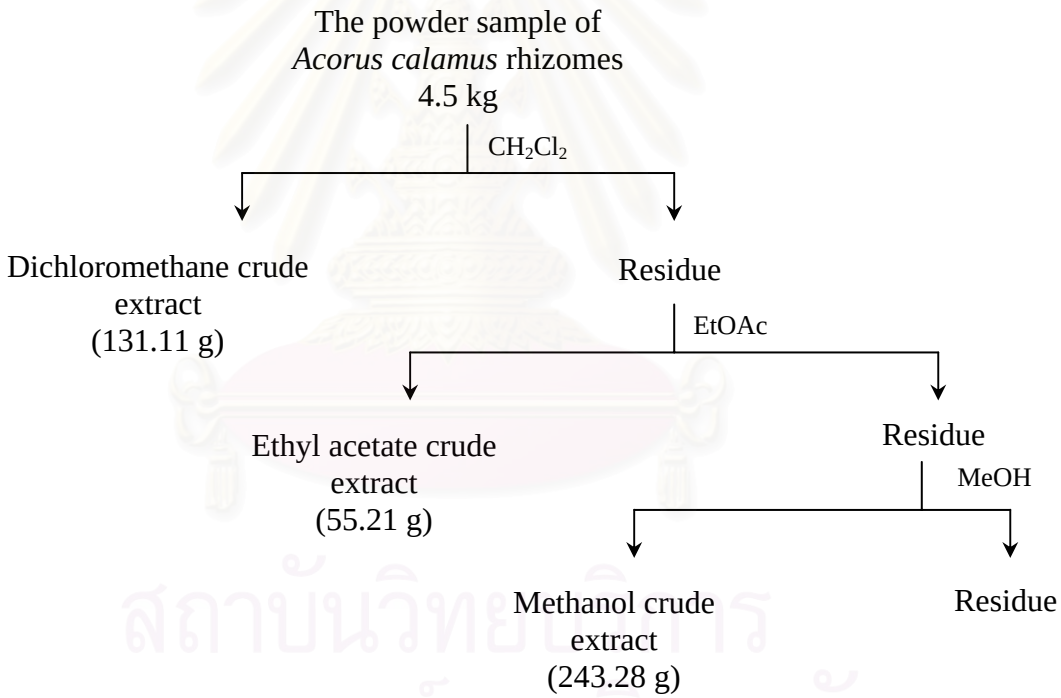
The dried rhizomes (250 g) were milled and extracted with dichloromethane and then methanol by soxhlet apparatus as presented in Scheme 2.1. Yield of the dichloromethane extract was 3.61% while that of methanol extract was 7.56%. The dichloromethane and methanol crude extracts at 10,000 ppm were used for preliminary test for antifungal activity. The positive fraction was further isolated for the active compounds.



Scheme 2.1 The extraction procedure for preliminary study of *Acorus calamus*

2.4.2 Extraction for separation

Dried rhizomes (4.5 kg) were milled to powder, then macerated with dichloromethane for 7 days at room temperature and filtered. The residue was repeated to macerate three times with the same solvent following the procedure as presented in Scheme 2.2 to give dichloromethane crude extract. The residue was further extracted with ethyl acetate and then methanol using the same procedure to give ethyl acetate and methanol crude extracts, respectively. The combined filtrate of each solvent was concentrated to dryness under vacuum by rotary evaporator. Yields of the dichloromethane, ethyl acetate and methanol extracts were 131.11 g (2.91%), 55.21 g (1.23%) and 243.28 g (5.41%), respectively.

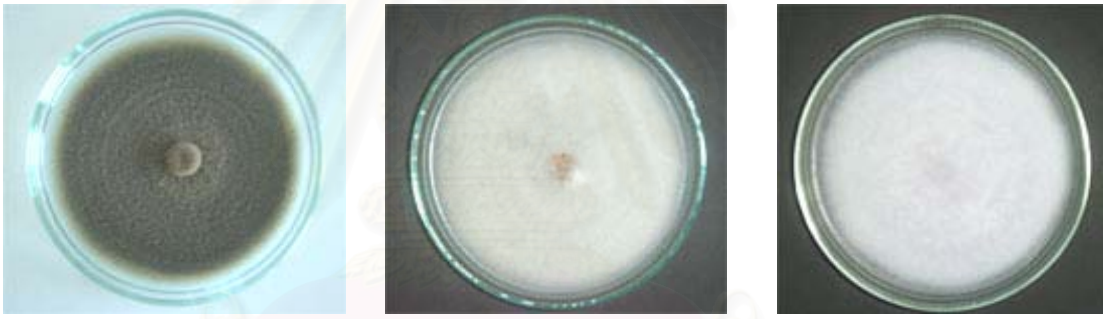


Scheme 2.2 The extraction procedure for *Acorus calamus* rhizomes

2.5 General methods for bioassays

2.5.1 Fungal cultures

Six phytopathogenic fungi used as test microorganisms in the experiments were *Alternaria brassicicola, Colletotrichum gloeosporioide*s, *Fusarium oxysporum, Phytophthora palmivora, Pythium* sp. and *Sclerotium* sp., supplied by the Division of Plant Disease and Microbiology, Department of Agriculture, Bangkok, Thailand. The colony characteristics of six phytopathogenic fungi were shown in Fig. 2.1. Fungal cultures were grown on potato dextrose agar (PDA) which the formula as presented in appendix A at room temperature (25-30°C). All cultures were maintained and subcultured every month.



Alternaria brassicicola *Colletotrichum gloeosporioides* *Fusarium oxysporum*

Phytophthora palmivora *Pythium* sp. *Sclerotium* sp.

Fig 2.1 The colony characteristics of the six phytopathogenic fungi on PDA used in this research

2.5.2 Conidia suspension preparation

Conidial suspension preparation of three phytopathogenic fungi: *A. brassicicola, C. gloeosporioides* and *F. oxysporum* was prepared from 7-to-14-day old cultures grown on PDA by flooding plates with 10 ml of sterile distilled water and scrapping the colonies on the agar surface with a glass rod. Aqueous conidial suspensions were filtered through steriled cotton wool to remove mycelia. Conidia concentrations in the suspensions were determined with a haemocytometer and then adjusted with sterile distilled water to the required concentration. The conidial suspensions were used for conidial germination inhibition and TLC bioautographic assay.

2.6 Bioassays for antifungal activity

2.6.1 Mycelial growth inhibition

2.6.1.1 Preliminary screening plants

The bioassay was determined by the modified method from agar medium assay [22] with six phytopathogenic fungi. Seventeen plant samples were milled to powder for preliminary antifungal tests. The powder was directly added to PDA at the final concentration of 50,000 ppm. Control plates contained only PDA. After sterilization at 15 psi and 121 °C, the medium was poured into petri plates. A disc (7 mm diameter) from pure culture of phytopathogenic fungi was aseptically transferred to the center of the petri plate. The plates were incubated at room temperature. Redial measurements of growth were taken when fungi reached the edge of the control plate, colony diameter was measured in centimeters and calculated for percent inhibition. All treatments were replicated four times.

$$\text{Percentage inhibition} = \frac{(C - T) \times 100}{C}$$

C : colony diameter of control plate (cm)

T : colony diameter of treatment (cm)

2.6.1.2 *In vitro* evaluation of crude extracts

The bioassay was conducted using the agar medium assay. The crude extract was dissolved in dimethylsulfoxide (DMSO) (final concentration not exceed 0.1%) and then was incorporated into PDA after autoclaving at a final concentration of 10,000 ppm. A disc (7 mm diameter) with the phytopathogenic fungal culture was transferred aseptically to culture on amended plates. Control plates containing only DMSO were included. Four replicate plates of each treatment were incubated at room temperature. Colony diameter was measured after the fungi reached to the edge of the control plates and calculated for percent inhibition.

2.6.1.3 Effect of dichloromethane crude extract for antifungal activity

The dichloromethane crude extract were dissolved in DMSO (final concentration not exceed 0.1%) and added to PDA at the following concentration: 2,000, 1,000, 100, 10 and 1 ppm then mixed after autoclaving while the media still warm and poured onto petri plates. A disc (7 mm diameter) cut from the edge of cultures of the phytopathogenic fungi was placed on the center of each plate, control plates containing only DMSO were prepared. Four replicate plates of each treatment were incubated. Colony diameter was measured after the fungi reached to the edge of the control plates and calculated for percent inhibition.

2.6.2 Bioautographic assay

Bioactivity testing on TLC by modifications of bioautographic assays [23,24] direct bioautography was used for screening of the extracts and subsequent activity-guided fractionation to determine the relative actions of the antifungal compounds. *C. gloeosporioides* and *F. oxysporum* grown on PDA for 7 days were used to prepare the conidial suspension as previous described, estimated by haemocytometer and diluted to the concentration of approximately 10^5 conidia/ml. Each fraction was collected and determined the antifungal activity on TLC plate (silica gel $60GF_{254}$ Merck, 0.25 mm thickness). Three pieces of TLC were prepared as follows. On each TLC, each fraction to be tested was applied and then developed with the appropriate solvent system. After the solvent was completely evaporated, the plate was marked after visualizing the spot position by UV. Conidia of *C. gloeosporioides* and *F. oxysporum*

suspended in PDB was sprayed directly onto each piece of the previously developed plate and the other piece of the developed plate, no fungi suspension was applied. 10% Sulfuric acid in ethanol was sprayed, followed by heating to detect those compounds with no UV absorption. After incubation in moisture chamber for 2 days at the end of the incubation period, the plate was stained with 1%(v/v) lactophenol cotton blue in 5%(v/v) acetic acid for 5 minutes and then destained with 5%(v/v) acetic acid for 10 minutes. A clearly visible growth inhibiton zone was observed against a blue background to display antifungal activity. All treatment performed replicatedly three times.

CHAPTER III

RESULTS AND DISCUSSION

The preliminary screening of eighteen Thai plant materials for antifungal activity was examined against six phytopathogenic fungi. The plant material which revealed most broad spectrum of antifungal activity would be selected to evaluate its activity in details and further examined for active compounds. The effects of the plant extracts on mycelial growth of phytopathogenic fungi were also explored. The fractionation of crude extract was tested to follow the activity by bioautographic assay. The active fractions which exhibited clear inhibition zone would lead to the discovery of bioactive compounds.

3.1 The results of preliminary screening test for antifungal activity

The powder of eighteen plant materials as aforementioned were incorporated into the PDA medium and then inoculated with six phytopathogenic fungi to evaluate their antifungal activity at the concentration of 50,000 ppm. The results are demonstrated in Table 3.1.

Table 3.1 The results of antifungal activity of some selected plants

No.	Plant	Plant part	Fungi[a]					
			A	B	C	D	E	F
1	*Achyrathes aspera* Linn.	aerial part	++	-	-	-	-	+
2	*Acorus calamus* Linn.	rhizome	+++	+++	+++	++++	++++	++++
3	*Andrographis aniculata* (Burn) Wall. ex Ness.	aerial part	+	+	+	+	+	-
4	*Azadirachta indica A.* Juss.	fruit	+	++	+	++	+	-
5	*Cleome viscosa* Linn.	whole plant	-	-	-	-	-	+
6	*Curcuma longa* Linn.	rhizome	++	++	++	+++	+++	+
7	*Cyperus rotundus* Linn.	tuber	+	+	+	+++	+	+
8	*Mansonia gagei* Drumm.	heartwood	+	+++	+++	++++	++	-
9	*Piper ribesoides* Wall	stem	++	+	+	-	+	+
10	*Piper chaba* Hunter.	fruit	++	++	+	+++	++	+++
11	*Piper chaba* Hunter.	stem	+	++	+	+++	+	++
12	*Piper cubeba*	fruit	-	+	-	+++	++	+
13	*Piper nigrum*	fruit	+++	++	+	+++	++	++
14	*Piper sarmentosum* Roxb.	leaf	++	+	+	++++	+++	++
15	*Piper sarmentosum* Roxb.	fruit	+++	+++	++	++++	+++	+
16	*Plumbago indica* Linn.	whole plant	+	+	++	+++	++	+
17	*Tribulus terrestris* Linn.	aerial part	+++	+++	-	-	+	++
18	*Zanthoxylum limonella*	fruit	++	+++	++	+++	++	+

Note: (+) =1-30 %inhibition, (++) = 31-60 %inhibition, (+++) = 61-85 %inhibition, (++++) = 86-100 %inhibition, (-) = not significantly different from control

[a]A, *Alternaria brassicicola*; B, *Colletotrichum gloeosporioides*; C, *Fusarium oxysporum*; D, *Phytophthora palmivora*; E, *Pythium sp.*; F, *Sclerotium sp.*

As the results of screening tests presented in Table 3.1, all eighteen plant meterials showed antifungal activity with different extents. The percentage of mycelial growth inhibition varied with species of plant materials and phytopathogenic fungi. Moreover, different parts of plant materials revealed different level on mycelial growth inhibiton against phytopathogenic fungi. To illustrate this, the stems of *P. chaba* exhibited less antifungal activity than fruits. The powder of *P. samentosum* fruits also displayed more effect on fungi growth inhibition than leaves. It may be possible that the fruit of both plant materials may have some active constituents different from the leave. According to the literature, it was reported that the essential oil from *P. chaba* (fruits) showed mycelial growth inhibition to *F. oxysporum* and *Phytophthora* sp. while *P. samentosum* (leaves) had no activity to both fungi.[7]

It was noteworthy to point that some plants showed the selectivity to inhibit certain fungi; for example *A. aspera* inhibited just *A. brassicicola* and *Sclerotium* sp and *C. rotundun* revealed the growth inhibition over 61% just only *Sclerotium* sp. while other fungi were inhibited less than 30%. In some particular cases, it was also observed that mycelial growth of *Pythium* sp. and *P. palmivora* on the media which incorporated with some plant materials, e.g. *T. terrestris* and *P. nigrum* revealed the mycelium occurance not as dense as that detected in the controlled plate (Figs 3.1 and 3.2). This may imply that those plant materials may contain less bioactive compounds. For *F. oxysporum*, the abnormal growth could be clearly visualized compared with the control treatment as shown in Fig 3.3. In the last case, the mycelial growth of some plant materials; *A. aspera* and *C. viscos*a was stimulated to grow better than the control.

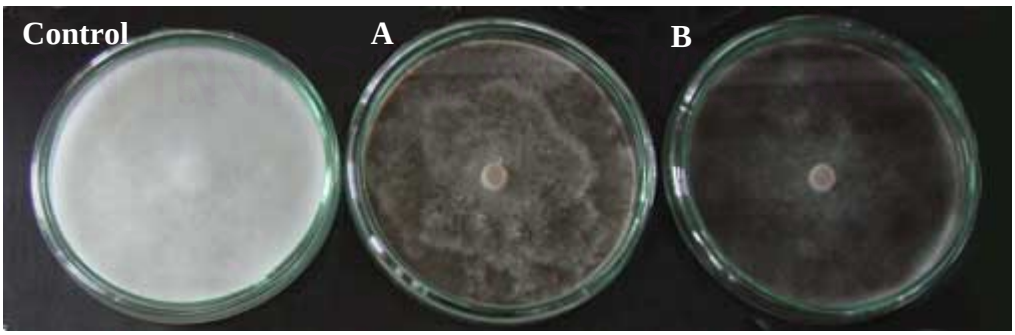


Fig 3.1 *Pythium* sp. cultured on PDA which incorporated plant powder of *T. terrestris* (**A**) and *P. nigrum* (**B**) compared with control.

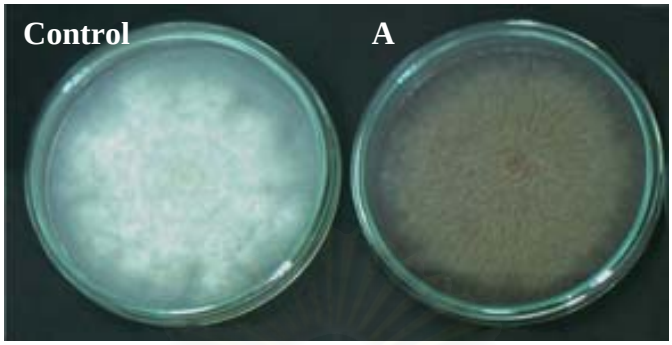


Fig 3.2 *P. palmivora* cultured on PDA which incorporated plant powder of *T. terrestris* compared with control.

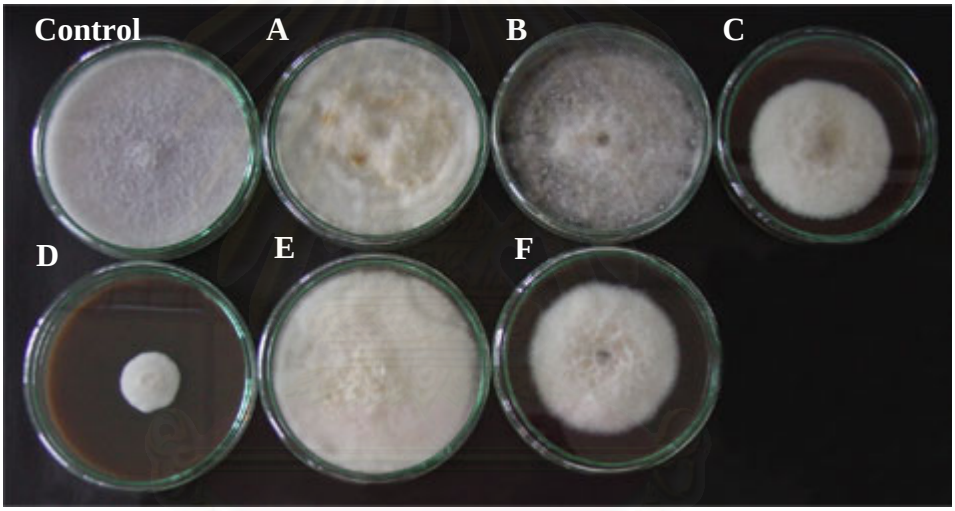


Fig 3.3 *F. oxysporum* cultured on PDA which incorporated plant powder of *T. terrestris* (A), *P. cubeba* (B), *P. chaba* (C), *A. calamus* (D), *P. samentosum* (E) and *P. nigrum* (F).

A. indica (fruits) had no effect on the mycelial growth of *Sclerotium* sp., therefore it showed mycelial growth and sclerotial production better than control. However, in this experiment it was found that some plant materials might not suppress mycelial growth of *Sclerotium* sp. but affected on sclerotial production such as *A. aspera*, and *C. viscosa* as presented in Fig 3.4.

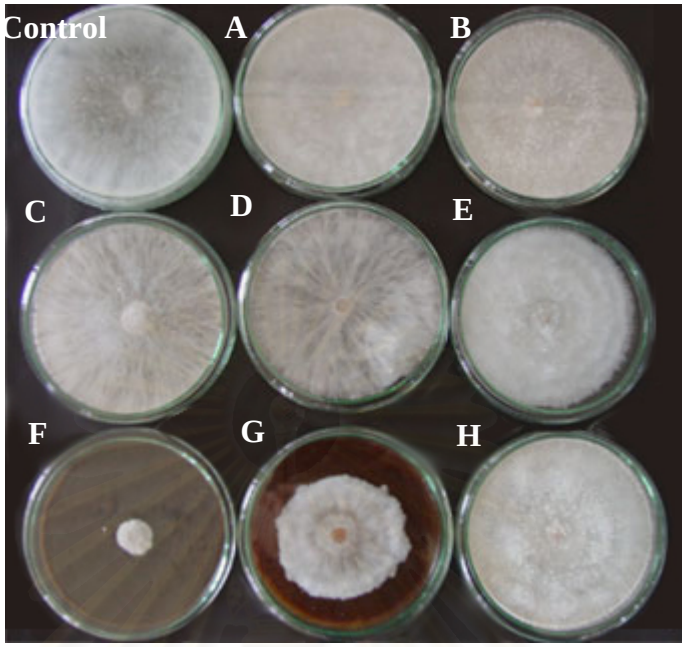
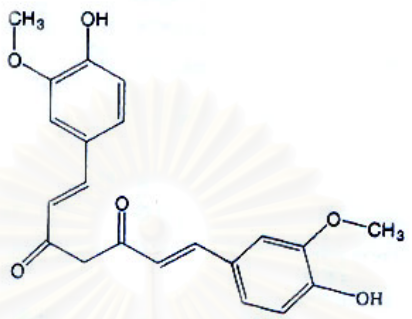


Fig 3.4 The mycelial growth inhibition and sclerotial production of *Sclerotium* sp. cultured on plant materials of *P. cubeba* (A), *C. viscosa* (B), *T. terrestris* (C), *P. chaba* (stem) (D), *P. samentosum* (E), *A. calamus* (F), *P. chaba* (fruits) (G), and *A. aspera* (H).

As the other reports presented that *A. indica* was ineffective against *R. solani in vivo*. On the other hand, the leaf extracts was effective against *R. solani* especially in hot water extract. It inhibited sclerotial production but did not inhibit mycelial growth.[25] In this case, the fungi *Rhizoctonia* and *Sclerotium* are soil inhabitants and known as sterile fungi because they produce only sclerotia and incapable of producing any kind of spores and they cause losses on many kinds of plants by attacking primarily the roots and lower stems of plants and showing sclerotia on their infected host.[2] For these reasons, the results of mycelial growth inhibition and sclerotial production of *Sclerotium* sp. in this experiment revealed in line with those reports, thus the characteristics of the fungi might relative with antifungal activity.

A literature search revealed that Curcumin which was isolated from the ethyl acetate fraction of *Curcuma longa* showed fungicidal activities at 500 mg/l against *Erysiphe graminis, Pyricularia grise, Phytophthora infestans* and *Botrytis cinerea*.[26]

This might explain antifungal activity of *Curcuma longa* in this experiment which against to all selected fungi.



Curcumin isolated from *C. longa* L. rhizome [26]

For another literature search, a piperidine alkaloid, pipernonaline derived from the hexane fraction of *Piper longum* L displayed a potent fungicidal activities against *Puccinia recondita* with 91% and 80% control values at the concentrations of 0.5 and 0.25 mg/ml, respectively.[27] Therefore, this substance might be an active compound which inhibited mycelial growth of selected phytopathogenic fungi in this experiment.

Pipernonaline isolated from *P. longum* L. [27]

P. palmivora was relatively sensitive to twelve plant materials and also suppressed in high percentage more than 61% of mycelial growth inhibition compared with other fungi. In addition, the maximum inhibition of fungal growth was observed among eighteen plant materials screened. *A. aspera*, *C. viscosa*, and *T. terrestris* nevertheless had no inhibitory effect on the mycelial growth of *P. palmivora*.

Some plant materials such as *A. aspera*, *A. paniculata* and *C. viscosa* did not show good activity and had no antifungal activity to some fungi. This might explain by following reasons: their plant materials in fact had no active constituent to suppress these phytopathogenic fungi, or their constituents had no good activity to

control phytopathogenic fungi, or the active constituents could not diffuse into PDA very well. Another reason may arise from the active constituents lost their activity after autoclaving. However, some the plant extracts were reported to retain the inhibitory effect even after autoclaving both mycelial growth and sclerotial inhibition to *R. solani*. In contrast, some plant extracts lost their inhibitiory effect after autoclaving. Moreover, the fungi toxicity of different plant extracts were thermostable up to 120 °C.[27] This may be good reason to explain the mycelial growth inhibition activity of *A. calamus* while still have good activity after autoclaving.

In this present study, the powder of *A. calamus* exhibited broad spectum activity over 60 % inhibition against tested fungi more than other plant materials at the same concentration and showed complete mycelial growth inhibition of *Pythium* sp. and *P. palmivora*. Good activities were also observed from *P. sarmentosum* Roxb. fruits which exhibited high activity against *A. brassicicola*, *C. gloeosporioides*, *P. palmivora* and *Pythium* sp. over 60%.

According to the result of preliminary screening test, the rhizomes of *A. calamus* were selected for further investigation.

3.2 The extraction of *A. calamus* rhizomes

3.2.1 The extraction results of *A. calamus* rhizomes (4.5 kg)

The dried *A. calamus* rhizomes were milled and extracted with various appropriate solvents: dichloromethane, ethyl acetate and methanol as described in chapter II. The results of the extraction are presented in Table 3.2.

Table 3.2 The results of extraction of *A. calamus* rhizomes (4.5 kg)

Crude extract	Weight (g)	%w/w
Dichloromethane	131.11	2.91
Ethyl acetate	55.21	1.23
Methanol	243.28	5.41

From the extraction results, the methanol extract gave the best yield, 243.28 g following by the dichloromethane extract, 131.11 g and the ethyl acetate extract, 55.21 g, respectively. The dichloromethane and ethyl acetate crude extracts were checked for their chemical constituents on TLC using ethyl acetate/hexane (1:1) as a developing solvent system. Their TLC exhibited the same pattern as shown in Fig 3.5. Thus both crude extracts were mixed and used for further separation and biological activity tests.

Fig. 3.5 TLC pattern of the crude extracts of *A. calamus* rhizomes
(a) dichloromethane extract (b) ethyl acetate extract (c) methanol extract

3.3 Evaluation of crude extract for antifungal activity

The dichloromethane and methanol crude extracts derived from extraction by soxhelt apparatus were tested as preliminary study for antifungal activity with six phytopathogenic fungi at 10,000 ppm. The results are displayed as shown in Table 3.3 and Fig 3.6.

Table 3.3 Preliminary antifungal activity study of the dichloromethane and methanol extracts of *A. calamus* at 10,000 ppm.

Crude extract	Mycerial growth inhibition (%)					
	A	B	C	D	E	F
Dichloromethane extract	100	100	100	100	100	100
Methanol extract	0	0	0	0	0	0

Note : A, *Alternaria brassicicola*; B, *Colletotrichum gloeosporioides*; C, *Fusarium oxysporum*; D, *Phytophthora palmivora*; E, *Pythium sp.*; F, *Sclerotium sp.*

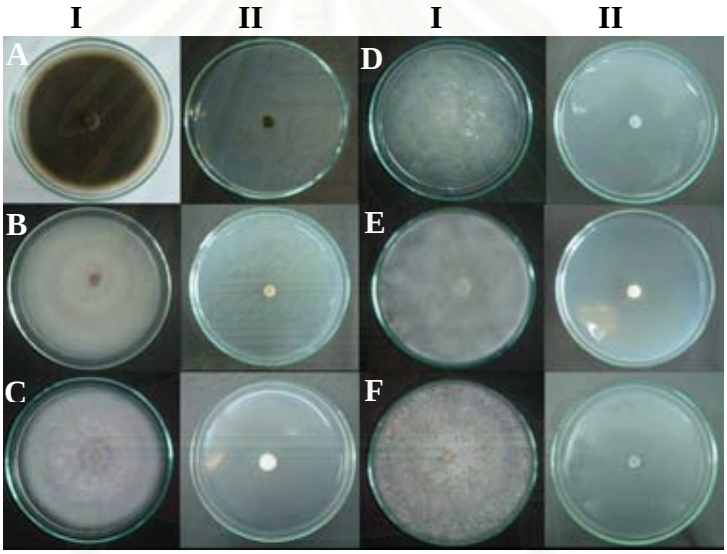


Fig 3.6 Evaluation of dichloromethane extract (**I**) and methanol extract (**II**) for antifungal activity with *A. brassicicola* (**A**), *C. gloeosporioides* **(B)**, *F. oxysporum* (**C**) *P. palmivora*(**D**), *Pythium* sp.(**E**) and *Sclerotium* sp.(**F**).

The results revealed that the dichloromethane extract revealed 100% mycelial growth inhibition to all six phytopathogenic fungi at 10,000 ppm while the methanol crude extract did not show mycelial growth inhibition to any phytopathogenic fungi. Thus, dichloromethane crude extract was used for further study.

3.4 Effect of dichloromethane crude extract for antifungal activity

3.4.1 Mycelial growth inhibition

Dichloromethane crude extract were prepared at final concentration as following concentration; 2,000, 1,000, 100, 10, 1 ppm. After a mycelial disc inoculated each plated, and the fungi were allowed to grow. The antifungal activity was observed after the fungi reached to the edge of control plates. The result was shown in Table 3.4.

Table 3.4 The mycelial growth inhibition and IC_{50} of dichloromethane crude extract

Fungi	% inhibition					IC_{50} (ppm)
	1 ppm	10 ppm	100 ppm	1000 ppm	2000 ppm	
Alternaria brassicicola	0	0	0	46.89	90.83	1047
Colletotrichum gloeosporioides	0	1.11	8.33	79.72	87.00	708
Fusarium oxysporum	0	0	20.56	73.89	83.56	501
Phytophthora palmivora	0	3.45	52.18	86.09	100	89
Pythium sp.	0	37.67	73.78	100	100	22
Sclerotium sp.	0	0	0	100	100	500

As Table 3.4, the results revealed that dichloromethane crude extract showed the most inhibition on *Pythium* sp. at the lowest concentration of IC_{50} at 22 ppm, followed by *P. palmivora* at 89 ppm, respectively.

Dichloromethane crude extract showed complete mycelial growth inhibition of *Pythium sp.* and *Sclerotium sp.* at 1,000 ppm and *P. palmivora* at 2,000 ppm. For *A. brassicicola*, *C. gloeosporioides* and *F. oxysporum* did not show complete mycelial growth inhibition. The mycelial growth on the media with several concentration of dichloromethane crude extract were presented as shown in Fig 3.7. Moreover, increase of crude extract concentration showed more inhibition than the others while the mycelial growth inhibition of all fungi on the media with 1 ppm of dichloromethane crude extract did not show antifungal activity (Fig 3.8).

As literature data of efficacy of crude extract from *A calamus* revealed that ethanol crude extract of 500 and 1000 ppm inhibited both vegetative growth of *C. gloeosporioides* (63% and 82%, respectively) and spore germination (75.5% and 100%, respectively). Moreover, after partition of ethanol crude extract that report presented that ethyl acetate soluble gave better inhibitory effect than water soluble.[22] The inhibitory effect of the extract exhibited similar result to the result achieved from this experiment.

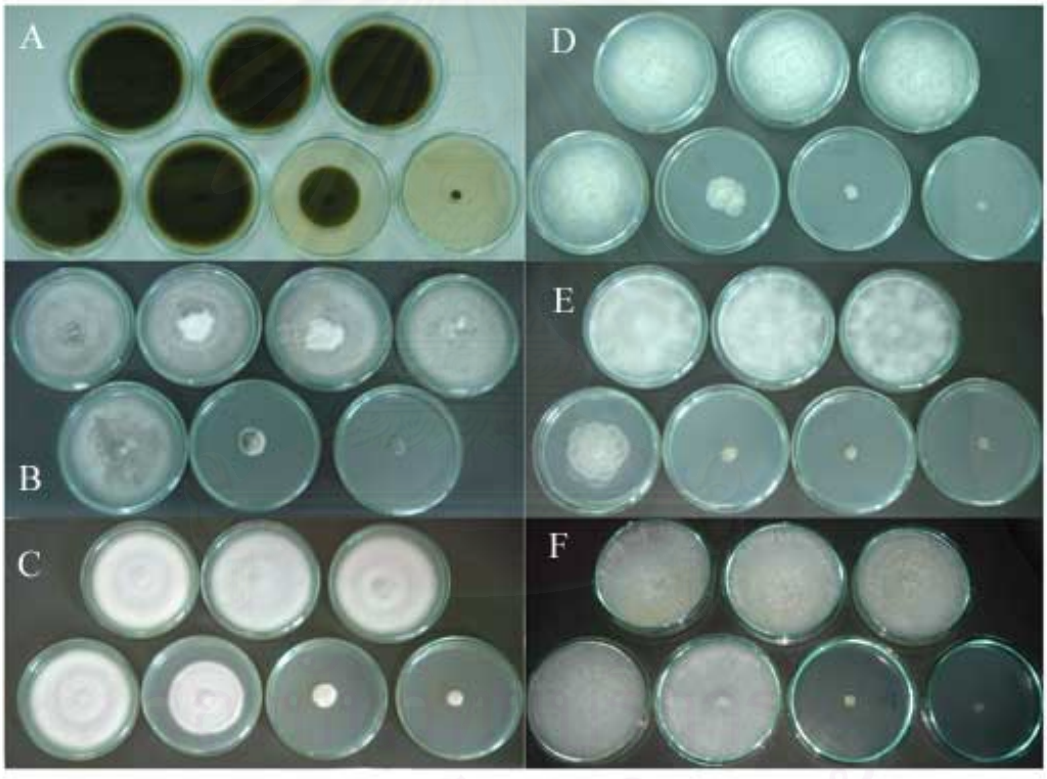


Fig. 3.7 The mycelial growth inhibition of crude dichloromethane extract to phytopathogenic fungi : A, . *brassicicola*; B, *C. gloeosporioides*; C, *F. oxysporum*; D, *P.palmivora*; E, *Pythium sp.*; F, *Sclerotium sp.*

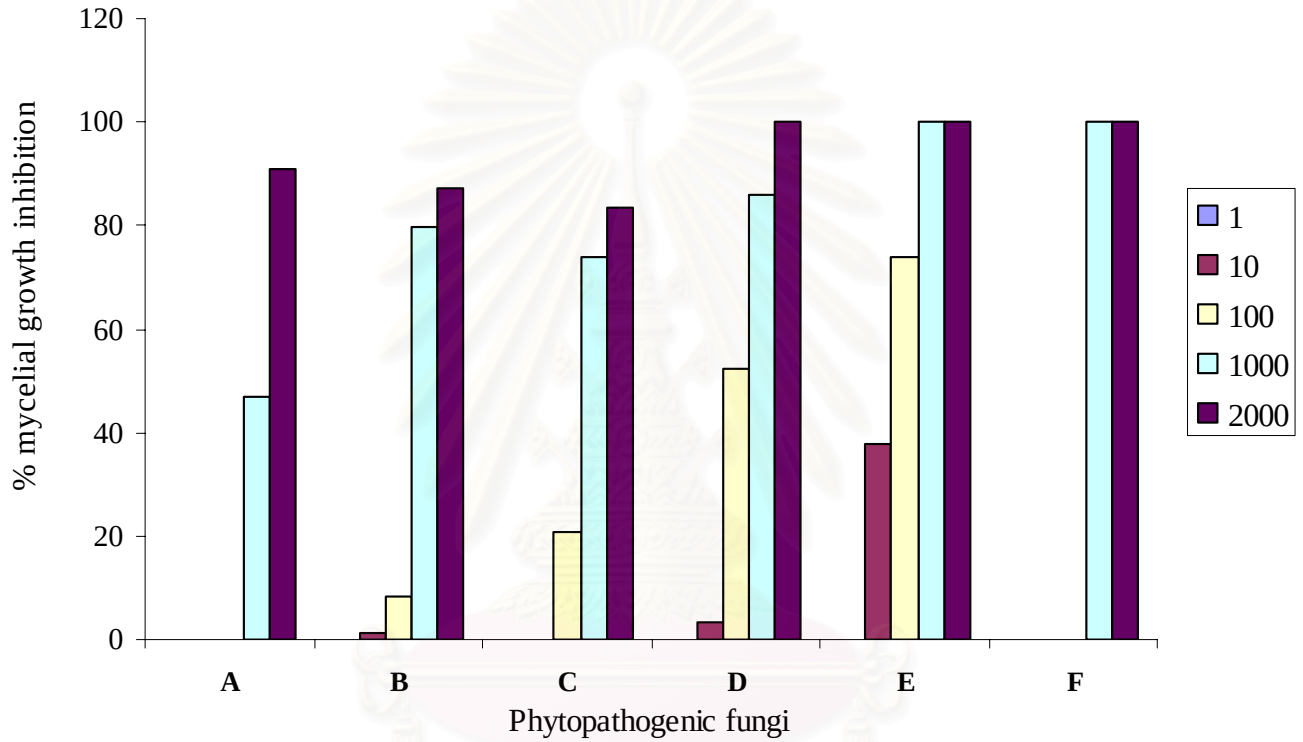


Fig 3.8 The mycelial growth inhibition of dichloromethane crude extract

Note : A, *Alternaria brassicicola*; B, *Colletotrichum gloeosporioides*; C, *Fusarium oxysporum*; D, *Phytophthora palmivora*; E, *Pythium sp.*; F, *Sclerotium sp.*

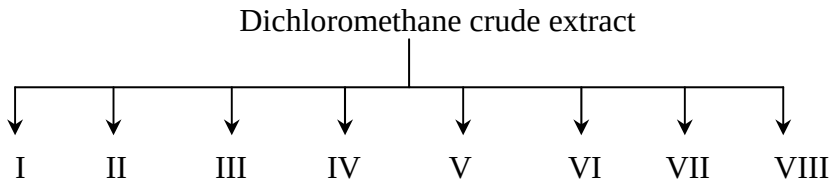
3.5 The fractionation of Dichloromethane crude extract

Based upon the result of preliminary test, the dichloromethane extract was selected for further separation for active compound. A part of dichloromethane extract (87 g) was mixed with silica gel and then dried under reduced pressure. It was then fractionated by quick column chromatograph, eluted with step gradient of hexane-EtOAc and EtOAc-MeOH as solvent systems (approximately 2000 ml per system). Each fraction was monitored by TLC using 50% ethyl acetate in hexane as a developing solvent. Fractions with similar chromatographic patterns were combined to yield eight fractions as presented in Table 3.5 and Scheme 3.1.

TLC bioautographic assay was used to ascertain the active constituent in each fraction and guide to determine the relative locations of the bioactive compounds.

Table 3.5 The fractionation of dichloromethane extract by quick column chromatograph.

Fraction code	Eluent	Remarks	Weight (g)
I	Hexane – 1:9 EtOAc/Hexane	orange brown viscous liquid	65.41
II	1.5:8.5 EtOAc/Hexane	dark brown viscous liquid +precipitate	4.86
III	2:8 EtOAc/Hexane	dark brown viscous liquid	4.99
IV	3:7 EtOAc/Hexane	dark brown viscous liquid	2.20
V	3:7 EtOAc/Hexane	dark brown solid	0.87
VI	4:6 EtOAc/Hexane	dark brown solid	2.46
VII	6:4 EtOAc/Hexane	dark brown solid	1.86
VIII	8:2 EtOAc/Hexane - 2:8 MeOH/EtOAc	dark brown solid	1.91



Scheme 3.1 The separation of dichloromethane crude extract

As the result, the dichloromethane extract could be subfractionated to 8 fractions. Fraction I of dichloromethane extract gave the highest yield. This fraction also was indicated the possibility to contain bioactive substances owing to the TLC bioautographic assay with three phytopathogenic fungi (Fig 3.9).

3.5.1 TLC bioautographic assay of fractionation of the dichloromethane crude extract

The eight fractions of the dichloromethane crude extract were subjected to bioactivity testing on TLC. The eight fractions were loaded on TLC and developed with ethyl acetate/hexane (1:1). One plate was dipped into 10% H_2SO_4 in ethanol followed by heating to visualize the spots separated of the compounds as presented in Fig 3.9(A). For the other plates were used for the bioautographic assay system, the plates were dried to completely remove solvent and then sprayed with conidial suspension of *A. brassicicola*, *C. gloeosporioides* and *F. oxysporum* in liquid media and incubated in moisture chamber for 2 days. The results were presented as shown in Fig 3.9.

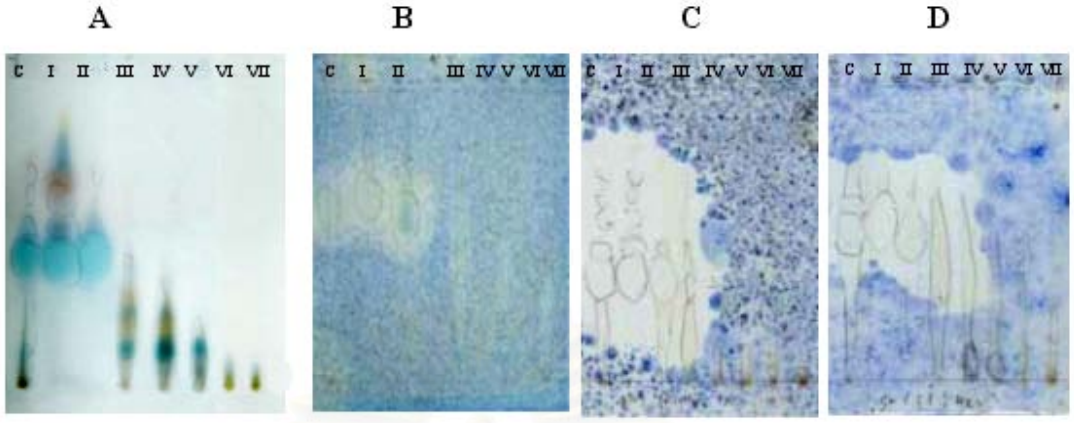


Fig 3.9 TLC bioautographic pattern of fractionation of dichloromethane crude extract, sprayed with *A. brassicicola* (B), *C. gloeosporioides* (C), and *F. oxysporum* (D) compared with control treatment (A).

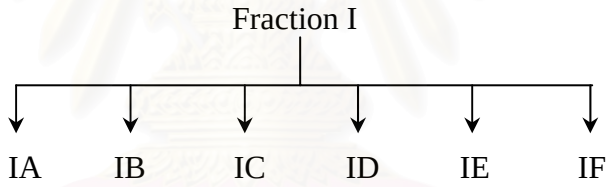
After dye with stained for 5 minute and then destained solution for 10 minute, bioautographic pattern were showed as Fig 3.9. Fraction I, II and III showed clear inhibition zone to all three fungi. For *A. brassicicola*, faction III showed slight activity. Fractionation of dichloromethane crude extract were not clearly separated so TLC pattern presented that in each fraction had mixture of some constituents similar as another fraction. Thus, as TLC profile of these 3 fractions, fraction I, II and III had similar pattern which were position of active constituents so just fraction I which gave highest yield was selected to further investigation of active constituents.

3.5.2 The separation of Fraction I

The portion of dichloromethane extract (Fraction I) 45 g as brown liquid was chromatographed on silica gel column chromatograph using Merck's silica gel 60 Art 7734 as an adsorbent. The column was initially eluted with hexane and increasing polarity by mixing with dichloromethane. Each portion was examined by TLC using 50% ethyl acetate in hexane as a developing solvent. Fraction with similar chromatographic patterns were combined to yield six fractions. The results of separation of Fraction I are presented in Table 3.6 and Scheme 3.2.

Table 3.6 The separation of fraction I by column chromatograph.

Fraction code	Eluent	Remarks	Weight (g)
IA	Hexane – 2:8 CH_2Cl_2/Hexane	brown liquid	2.65
IB	2:8 CH_2Cl_2/Hexane	brown liquid	0.33
IC	2:8 CH_2Cl_2/Hexane - 4:6 CH_2Cl_2/Hexane	brown liquid	13.63
ID	4:6 CH_2Cl_2/Hexane - 6:4 CH_2Cl_2/Hexane	brown viscous liquid	17.15
IE	6:4 CH_2Cl_2/Hexane – 8:2 CH_2Cl_2/Hexane	brown viscous liquid	5.60
IF	8:2 CH_2Cl_2/Hexane – 2:8 MeOH/CH_2Cl_2	brown liquid	0.23



Scheme 3.2 The separation of fraction I

As mentioned above, the separation of Fraction I by column chromatograph gave 6 fractions and fraction ID gave the highest yield (38.11%). The portions were then checked for active constituent by TLC bioautographic assay.

3.5.3 TLC bioautographic assay of fraction I

The six fractions of Fraction I were subjected to bioactivity testing on TLC and then developed with ethyl acetate/hexane (1:1). The plates were dried to completely remove solvent and then sprayed with conidial suspension of *A. brassicicola, C. gloeosporioides* and *F. oxysporum* in liquid media and incubated in moisture chamber for 2 days then dye with stained for 5 minute and then destained solution for 10 minute. The results were presented as shown in Fig 3.10.

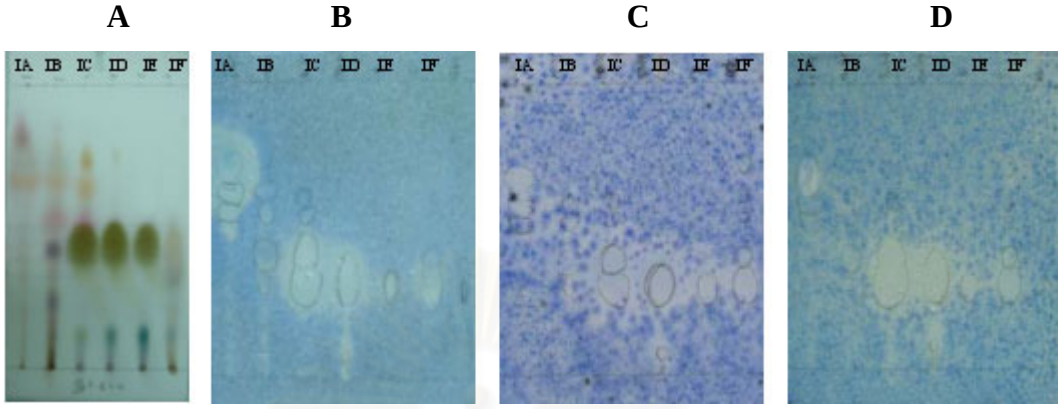


Fig 3.10 TLC bioautographic pattern of Fraction I, sprayed with *A. brassicicola* (B), *C. gloeosporioides* (C), and *F. oxysporum* (D) compared with control treatment (A).

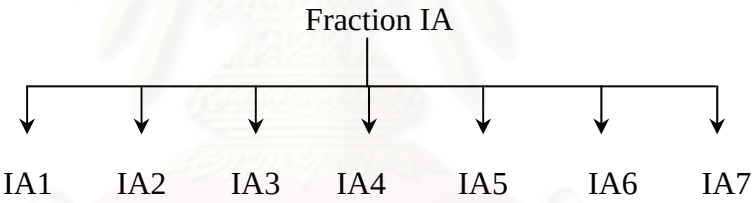
As TLC bioautographic result, all fraction illustrated position of inhibition zone of active compounds. Fraction IA, ID and IE showed broad spectrum of clear inhibition zone against *A. brassicicola* while *C. gloeosporioides*, and *F. oxysporum* exhibited narrow spectrum of inhibition zone. For Fraction IC exhibited inhibition zone same as position of major component in Fraction ID which could compare with control (A) as green spot with R_f value at 0.45 and a little spot at 0.12. TLC pattern of Fraction IE (A) seem no spot appear up on the major component so this fraction was further separated the major component. From the results, Fraction IA, ID and IE was selected for further study.

3.5.4 Separation of Fraction IA

Fraction IA (1.5 g) as brown viscous liquid was subjected to silica gel column chromatograph using silica gel 60 (No. 7734) as an adsorbent. The column was packed with hexane and then eluted with step gradient of dichloromethane-hexane, ethyl acetate and ethyl acetate-methanol. Each fraction was examined by TLC using 50% dichloromethane in hexane as a developing solvent system. Fractions with similar chromatographic pattern were combined to yield seven factions, as shown in Table 3.7 and Scheme 3.3.

Table 3.7 The separation of Fraction IA by column chromatograph.

Fraction code	Eluent	Remarks	Weight (g)
IA1	Hexane- 2:8 CH_2Cl_2/Hexane	pale yellow liquid+ precipitate	0.17
IA2	2:8 – 3:7 CH_2Cl_2/Hexane	white precipitate	0.11
IA3	3:7 CH_2Cl_2/Hexane	pale yellow viscous liquid	0.08
IA4	3:7-4:6 CH_2Cl_2/Hexane	pale yellow viscous liquid	0.02
IA5	6:4 CH_2Cl_2/Hexane-	brown viscous liquid	0.06
	2:8 EtOAc/CH_2Cl_2	brown viscous liquid +	0.14
IA6	2:8 EtOAc/CH_2Cl_2	colorless solid	
IA7	2:8 EtOAc/CH_2Cl_2 –	red brown	0.11
	1:9 MeOH/EtOAc		



Scheme 3.3 The separation of fraction IA

According to separation results of Fraction IA as shown in Table 3.7 and Scheme 3.3, Fraction IA was separated into seven fractions. Fraction IA1 gave highest yield (0.17 g). The precipitate in this fraction was soluble in hexane with heating. It was chosen for further study according to TLC bioautographic results which was displayed in Fig 3.11.

3.5.5 TLC bioautographic assay of fraction IA

The seven fractions of Fraction I were subjected to bioactivity testing on TLC and then developed with dichloromethane/hexane (1:1). The plates were dried to completely remove solvent and then sprayed with conidial suspension of *A. brassicicola, C. gloeosporioides* and *F. oxysporum* in liquid media and incubated

in moisture chamber for 2 days then dye with stained for 5 minute and then destained solution for 10 minute. The results were presented as shown in Fig 3.11.

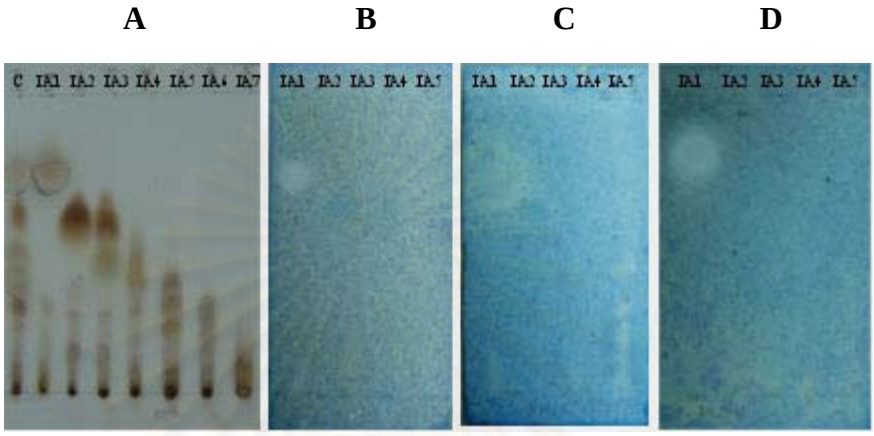


Fig 3.11 TLC bioautographic pattern of Fraction IA, sprayed with *A. brassicicola* (B), *C. gloeosporioides* (C), and *F. oxysporum* (D) compared with control treatment (A).

As TLC bioautographic results, Fraction IA1 appeared inhibition zone of major compound against all three phytopathogenic fungi with R_f value at 0.72 and showed little activity on spot at 0.12. The position of major component in Fraction IA1 could compare with control as brown spot (A) after dipped with 10% H_2SO_4 in ethanol. According to fraction IA1 gave trace yield and did not be pure compound so this fraction did not used to further separation.

3.5.6 Separation of Fraction IE

Fraction IE (3.5 g) as brown viscous liquid was subjected to silica gel column chromatography using Merck's silica gel 60 Art 7734 as an adsorbent. The column was packed with 20% ethyl acetate-hexane and then eluted with step gradient of ethyl acetate-hexane and ethyl acetate-methanol. Each fraction was examined by TLC using 80% ethyl acetate in hexane as a developing solvent. Fractions with similar chromatographic pattern were combined to yield eight factions, as shown in Table 3.8.

Table 3.8 The separation of Fraction IE by column chromatograph.

Fraction code	Eluent	Remarks	Weight (g)
IE1	2:8 EtOAc/Hexane	yellow liquid	0.03
IE2	2:8 EtOAc/Hexane	yellow liquid	0.17
IE3	2:8 EtOAc/Hexane	brown liquid	1.71
IE4	2:8 EtOAc/Hexane	brown viscous liquid	0.62
IE5	2:8 – 3:7 EtOAc/Hexane	brown viscous liquid	0.01
IE6	3:7- 4:6 EtOAc/Hexane	brown viscous liquid	0.03
IE7	4:6 EtOAc/Hexane	brown viscous liquid + colorless solid	0.08
IE8	EtOAc – 1:9 MeOH/EtOAc	brown viscous liquid	0.03

As the result, fraction IE4 which contained main compound and showed clear inhibition zone on TLC bioautographic assay as displayed in Fig 3.12.

3.5.7 TLC bioautographic assay of fraction IE

The eight fractions of Fraction IE were subjected to bioactivity testing on TLC and then developed with 50% ethyl acetate in hexane. The plates were dried to completely remove solvent and then sprayed with conidial suspension of *A. brassicicola, C. gloeosporioides* and *F. oxysporum* in liquid media and incubated in moisture chamber for 2 days then dye with stained for 5 minute and then destained solution for 10 minute. The results were presented as shown in Fig 3.12.

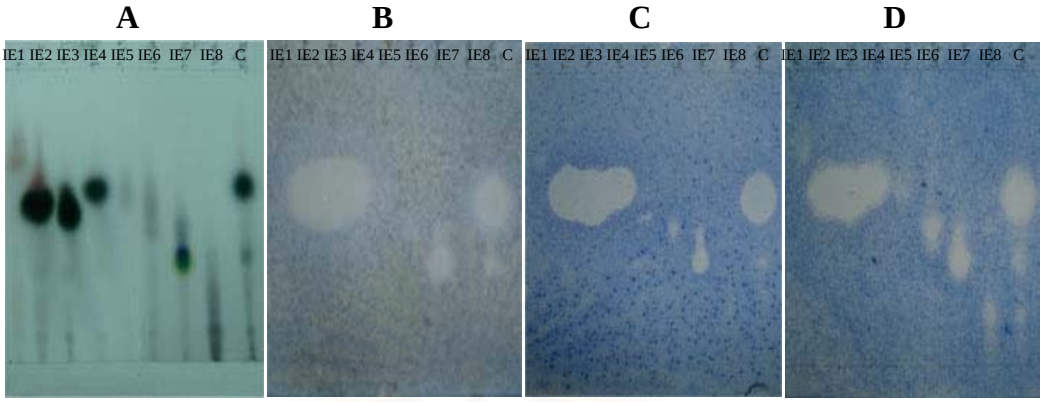
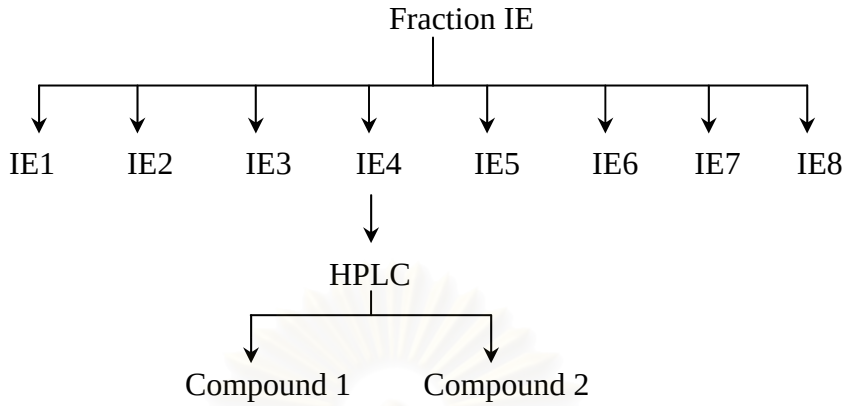


Fig 3.12 TLC bioautographic pattern of Fraction IE, sprayed with *A. brassicicola* (B), *C. gloeosporioides* (C), and *F. oxysporum* (D) compared with control treatment (A).

As presented above, TLC pattern of Fraction IE (A) appeared spots up on the major component. Fraction IE1 did not clear inhibit to the fungi that possibly contain small amount of the active constituents. Moreover, FractionIE1 might contain components same as Fraction IA when compared pattern between Fig 3.11 and 3.12. Fraction IE2, IE3 and IE4 clearly appeared inhibition zone of major compound against all three phytopathogenic fungi with R_f value at 0.57-0.60. Furthermore, there was observed little activity to the fungi at the same position of inhibition zone in Fraction IE6 and IE7 with R_f value at 0.45, 0.35, respectively. For Fraction IE8 showed inhibition zone with R_f value at 0.17 only on TLC which sprayed with *F. oxysporum*.

Fraction IE4 was then subjected to GC analysis for determine the mixture component. According to GC chromatogram, Fraction IE4 exhibited 2 peaks so Fraction IE4 was then performed by HPLC system on semipreparative Cosmosil 5C18-AR-II column using a solvent system of 65% MeOH in H_2O to separate the active compound as shown in Scheme 3.4.



Scheme 3.4 The separation of fraction IE4

After fraction IE4 was performed by HPLC, Compound **1** (0.172 g) was isolated as brown liquid and Compound **2** was isolated as a trace white solid and both compound showed similar position of a single spot of green color on TLC after dipped 10% H_2SO_4 in ethanol with an R_f value of 0.45 (5:5 EtOAc/Hexane).

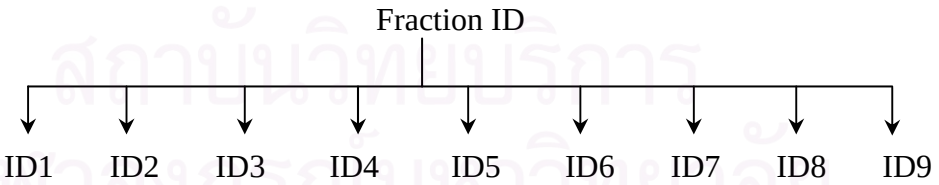
3.5.8 Fractionation of Fraction ID

The portion of Fraction ID 15 g as brown viscous liquid was subjected to silica gel column chromatography using Merck's silica gel 60 Art 7734 as an adsorbent. The column was packed with 60%ethylacetate-hexane and then eluted with step gradient of ethyl acetate-hexane and ethyl acetate-methanol. Each fraction was examined by TLC using 80% ethyl acetate in hexane as a developing solvent. Fractions with similar chromatographic pattern were combined to yield nine factions, as shown in Table 3.9.

Table 3.9 The separation of Fraction ID by column chromatograph.

Fraction code	Eluent	Remarks	Weight (g)
ID1	6:4 EtOAc/Hexane	pale yellow liquid	0.07
ID2	6:4 EtOAc/Hexane	yellow liquid	9.92
ID3	6:4 EtOAc/Hexane	pale yellow liquid	3.18
ID4	6:4 EtOAc/Hexane	brown viscous liquid	0.08
ID5	6:4 EtOAc/Hexane	brown viscous liquid and white precipitate	0.19
ID6	6:4 EtOAc/Hexane	brown viscous liquid	0.01
ID7	8:2-9:1 EtOAc/Hexane	brown viscous liquid	0.06
ID8	9:1 EtOAc/Hexane – 1:9 MeOH/EtOAc	brown viscous liquid	0.13
ID9	1:9-5:5 MeOH/EtOAc	red brown liquid	0.06

As presented in Table 3.9 and Scheme 3.5, fraction ID was separated into 9 fractions and Fraction ID2 gave the highest yield. Fraction ID1, ID2 and ID3 had similar TLC pattern of major active component with Fraction IE as presented in Fig 3.12. According to the antifungal activity by TLC bioautographic assay (Fig. 3.13), the Fraction ID5 as mixture of solid was then chosen for further investigation.



Scheme 3.5 The separation of fraction ID

3.5.9 TLC bioautographic assay of fraction ID

The nine fractions of Fraction I were subjected to bioactivity testing on TLC and then developed with ethyl acetate/hexane (1:1). The plates were dried to completely remove solvent and then sprayed with conidial suspension of *A. brassicicola*, *C. gloeosporioides* and *F. oxysporum* in liquid media and incubated in moisture chamber for 2 days then dye with stained for 5 minute and then destained solution for 10 minute. The results were presented as shown in Fig 3.13.

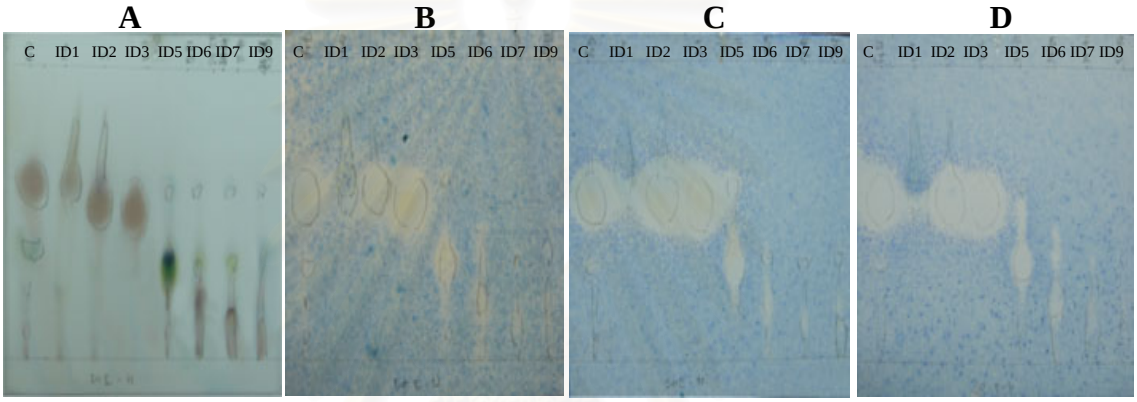


Fig 3.13 TLC bioautographic pattern of fractionation of Fraction ID, sprayed with *A. brassicicola* (B), *C. gloeosporioides* (C), and *F. oxysporum* (D) compared with control treatment (A).

According to the separation results, TLC bioautographic pattern of Fraction ID appeared inhibition zone of major spots in Fraction ID2 and ID3 with R_f value at 0.57 similar to TLC pattern of Fraction IE (Fig 3.12). Fraction ID6 and ID7 contained mixture of active constituents and showed clear zone similar position with Fraction ID5. Thus, Fraction ID5 which also showed clear inhibition zone with R_f value at 0.35 was selected to further separation.

3.5.10 Separation of Fraction ID5

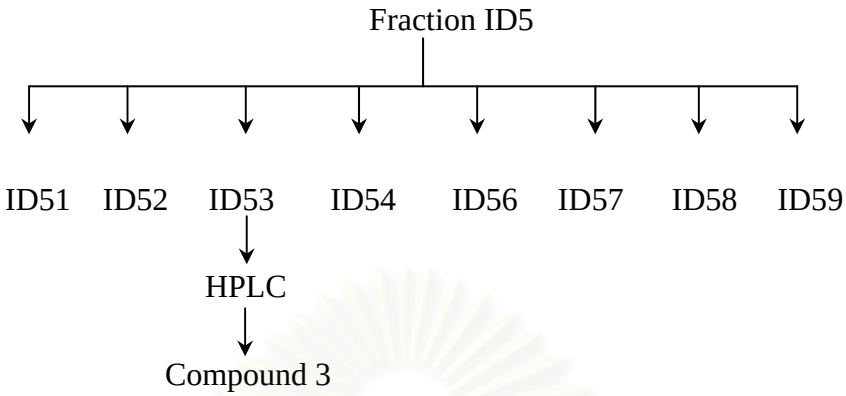
Fraction ID5 (0.19 g) as brown viscous liquid and white precipitate was subjected to silica gel flash column chromatograph using Merck's silica gel No.9385 as an adsorbent. The column was packed with 90% dichloromethane-hexane and then eluted with step gradient of ethyl acetate-dichloromethane and ethyl acetate-methanol.

The fractions was checked on TLC using 80% ethyl acetate in hexane as a solvent system. The outcomes are tabulated as presented in Table 3.10.

Table 3.10 Separation of Fraction ID5 by flash column chromatography

Fraction code	Eluent	Remarks	Weight (mg)
ID51	9:1 CH_2Cl_2/Hexane	brown viscous liquid	3.40
ID52	9:1 CH_2Cl_2/Hexane	white solid	22.70
ID53	9:1 CH_2Cl_2/Hexane	brown liquid + white solid	94.30
ID54	9:1 CH_2Cl_2/Hexane-CH_2Cl_2	brown viscous liquid	8.50
ID55	1:9 EtOAc/CH_2Cl_2	brown viscous liquid	4.70
ID56	1:9 EtOAc/CH_2Cl_2- 2:8 EtOAc/CH_2Cl_2	brown viscous liquid	28.30
ID57	2:8 EtOAc/CH_2Cl_2- 4:6 EtOAc/CH_2Cl_2	brown viscous liquid	4.70
ID58	8:2 EtOAc/CH_2Cl_2 – 1:9 MeOH/EtOAc	brown viscous liquid	7.10

As displayed in Table 3.10, eight fractions were obtained. TLC pattern are displayed in Fig 3.15. According to TLC bioautographic assay, fraction ID53 which gave highest yield and containing white solid was then purified by HPLC system on semipreparative Cosmosil 5C18-AR-II column using a solvent system of 65%MeOH in H_2O as Scheme 3.6.



Scheme 3.6 The separation of fraction ID5

After 0.09 g of fraction ID53 was performed by HPLC, Compound **3** (0.037 g) was isolated as a white crystal and showed a single spot of green color on TLC after dipped 10% H_2SO_4 in ethanol with an R_f value of 0.48 (8:2 EtOAc/Hexane).

3.5.11 TLC bioautographic assay of fraction ID5

The eight fractions of Fraction ID5 were subjected to bioactivity testing on TLC and then developed with ethyl acetate/hexane (8:2). The plates were dried to completely remove solvent and then sprayed with conidial suspension of *A. brassicicola*, *C. gloeosporioides* and *F. oxysporum* in liquid media and incubated in moisture chamber for 2 days then dye with stained for 5 minute and then destained solution for 10 minute. The results were presented as shown in Fig 3.14.

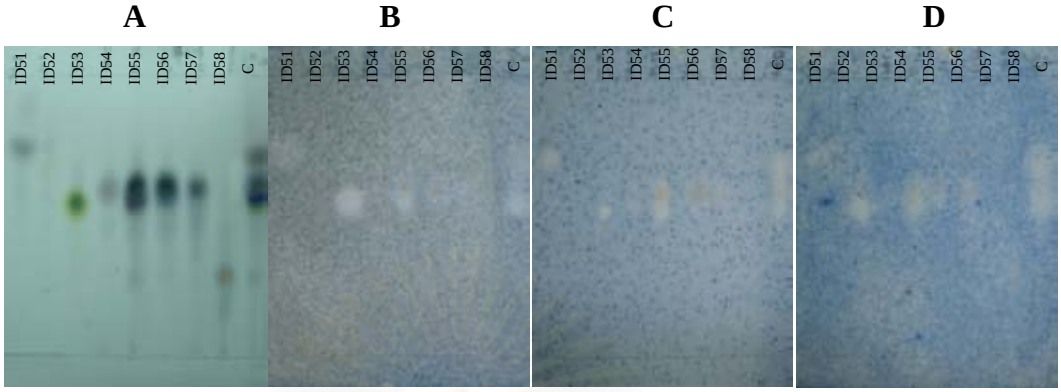
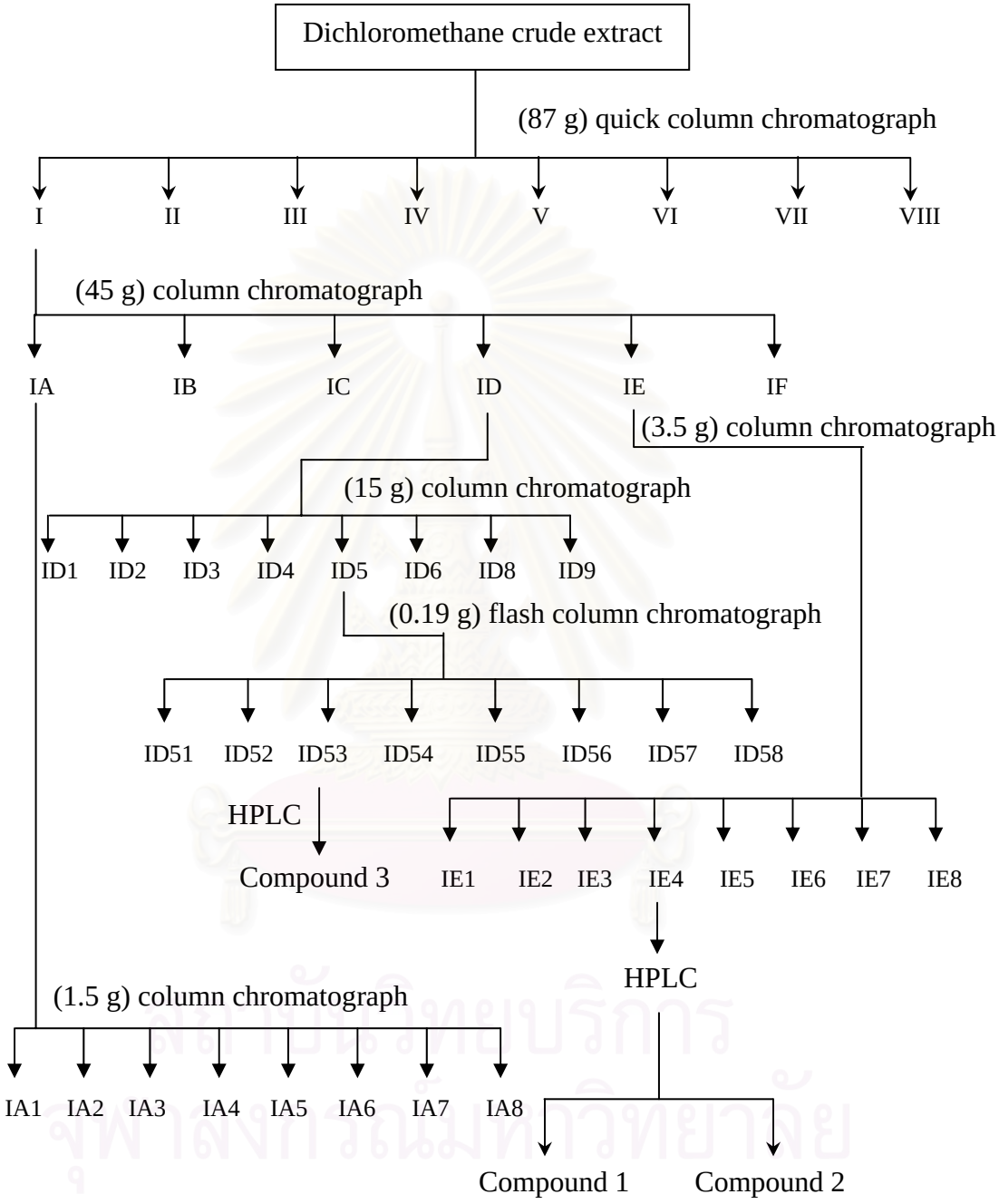


Fig 3.14 TLC bioautographic pattern of fractionation of Fraction ID5, sprayed with *A. brassicicola* (B), *C. gloeosporioides* (C), and *F. oxysporum* (D) compared with control treatment (A).

TLC bioautographic pattern of Fraction ID5 appeared spots of Fraction ID51, ID53, ID55, ID56 and ID57 against all three phytopathogenic fungi with R_f value at 0.70, 0.55, 0.57, 0.57 and 0.57, respectively. There was observed little activity to the fungi on TLC of Fraction ID56 and ID57 to all three fungi.

The summary of separation of dichloromethane extract is depicted as shown in Scheme 3.7.



Scheme 3.7 Isolation scheme of dichloromethane extract.

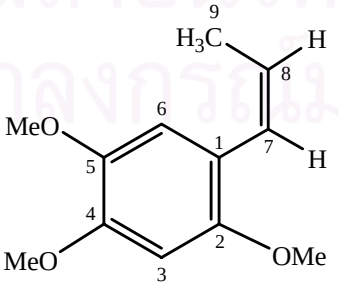
3.6 Structural Elucidation of Isolated Compounds.

3.6.1 Structural elucidation of Compound 1 and Compound 2

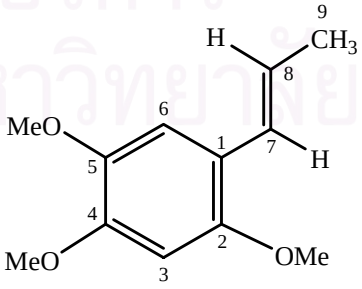
Compound **1** was identified by comparison of its ^{1}H and ^{13}C-NMR spectral data with those reported values.[29] The ^{1}H-NMR spectrum ($CDCl_3$) were observed the presence of two singlet aromatic protons at δ 6.57 (1H, s) and 6.88 (1H, s), three methoxyl protons at δ 3.85 (3H, s, OMe-2), δ 3.94 (3H, s,OMe-4) and δ 3.88 (3H, s, OMe-5), two olefinic protons at δ 6.52 (1H, dd, *J*=9.75, 1.61 Hz) and δ 5.82 (1H, dq, *J*=9.06, 1.61 Hz) and methyl protons at δ 1.88 (3H, dd, *J*=7.05, 1.77 Hz).

Compound **2** was identified by ^{1}H and ^{13}C-NMR spectral data and compared with those reported values.[29] The ^{1}H-NMR spectrum ($CDCl_3$) were observed the presence of two singlet aromatic protons at δ 6.53 (1H, s) and 6.98 (1H, s), three methoxyl protons at δ 3.85 (3H, s, OMe-2), δ 3.92 (3H, s,OMe-4) and δ 3.89 (3H, s, OMe-5), two olefinic protons at δ 6.69 (1H, dd, *J*=15.81, 1.69 Hz) and δ 6.13 (1H, dq, *J*=15.82, 1.63 Hz) and methyl protons at δ 1.92 (1H, dd, *J*=6.62, 1.76 Hz). Nine carbon signals observed in the ^{13}C-NMR spectrum ($CDCl_3$, Fig 3.10) which presented three aromatic methoxyl carbon at δ 56.1 (1C), δ 56.4 (1C) and δ 56.7(1C), four quaternary carbons at δ 119.0, 151.0, 149.0 and 143.5 and four methines at δ 125.0 (1C), 124.4 (1C), 109.7 (1C) and 97.9 (1C) and a methyl carbon at δ 18.8. The ^{1}H-^{13}C-NMR, HMBC, COSY and NOESY experiments (Appendix B) conducted for elucidating the structure of both compounds.

The structure of both compounds are shown below:



Compound **1**



Compound **2**

For ^{1}H and ^{13}C-NMR spectral data of *β*-asarone[29] were compared with Compound **1** and Compound **2**. The ^{1}H-NMR spectral data[29] were presented in Table 3.6. It was shown two singlet aromatic protons at δ 6.65 (1H, s) and 6.86 (1H, s), three methoxyl protons at δ 3.78 (3H, s, OMe-2), δ 3.85 (3H, s,OMe-4) and δ 3.77 (3H, s, OMe-5), two olefinic protons at δ 6.42 (1H, dq, *J*=12.0, 2.0 Hz) and δ 5.68 (1H, dq, *J*=12.0, 7.0 Hz) and methyl protons at δ 1.80 (3H, dd, *J*=7.0, 1.76 Hz). Twelve carbon signals in the ^{13}C-NMR spectral data[29] was assigned to four quaternary carbons at δ 119.6, 153.4, 150.4 and 143.7, three aromatic methoxyl carbon at δ 57.6 (CH_3), 56.7 (CH_3) and 56.9 (CH_3) and four methines at δ 126.1 (CH), 126.0 (CH), 99.4 (CH) and116.4 (CH) and a methyl carbon at δ 14.9 (CH_3).

Table 3.11 ^{1}H-NMR and ^{13}C-NMR spectral data of Compound **1**, Compound **2** and *β*-asarone[29]

Position	Chemical shift (ppm)				
	Compound 1	Compound 2		*β*-asarone	
	δH	δH	δC	δH	δC
1	-	-	119.0	-	119.6
2	-	-	151.0	-	153.4
3	6.57	6.53	97.9	6.65	99.4
4	-	-	149.0	-	150.4
5	-	-	143.5	-	143.7
6	6.88	6.98	109.7	6.86	116.4
7	6.52	6.69	125.0	6.42	126.1
8	5.82	6.13	124.4	5.68	126.0
9	1.88	1.92	18.8	1.80	14.9
OMe-2	3.85	3.85	56.1	3.78	57.6
OMe-4	3.94	3.92	56.4	3.85	56.7
OMe-5	3.88	3.89	56.7	3.77	56.9

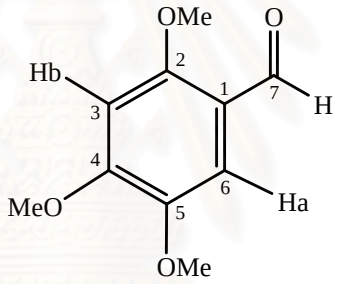
Table 3.12 ^{1}H-NMR and ^{13}C-NMR chemical shift assignments and 2D correlation of Compound **2**.

Position	Chemical shift (ppm)		HMBC	NOESY
	^{1}H	^{13}C		
1	-	119.0	C-1, C-6, C-7	-
2	-	151.0	-	-
3	6.53	97.9	C-1,C-7	OMe-2,OMe-4
4	-	149.0	-	-
5	-	143.5	-	-
6	6.98	109.7	-	OMe-5
7	6.69	125.0	C-3, C-9	H-8
8	6.13	124.4	C-1, C-9	H-6, H-7, H-9
9	1.92	18.8	C-1, C-6	H-8, H-7
OMe-2	3.85	56.1	-	-
OMe-4	3.92	56.4	-	-
OMe-5	3.89	56.7	-	-

As the ^{1}H-NMR of Compound 2 was consistent with Compound 1, their spectral data of β-asarone was similar to that obtained in both compound of this work (as shown in Table 3.11) except the positions of 7 and 8 of both compound exhibiting different coupling constant data. The structure of the antifungal compound, Compound **1** was identified to be β-asarone (*cis*-isomer of 2,4,5-trimethoxy-1-prophenylbenzene) and Compound **2** was identified to be α-asarone (*trans*-2,4,5-trimethoxy-1-prophenylbenzene). ^{1}H-NMR, ^{13}C-NMR and 2D correlation data of Compound 2 were confermed its structure.

3.5.2 Structural elucidation of Compound 3

The ^{1}H-NMR spectrum ($CDCl_3$, Fig 3.8) were observed the presence of three methoxy groups at δ 3.90 (3H, s, OMe-2), δ 3.95 (3H, s,OMe-4) and δ 4.00 (3H, s, OMe-5), two singlet aromatic protons at δ 6.52 (1H, s) and 7.35 (1H, s) and an aldehyde group at δ 10.34 (1H, s). Ten carbon signals observed in the ^{13}C-NMR spectrum ($CDCl_3$) which presented three aromatic methoxyl carbon at δ 56.3 (3C), two aromatic carbon at δ 158.7 and 109.0, four quaternary carbons at δ 95.9, 117.3, 143.5, and 155.8 and an aldehyde carbon at δ 188.1 (1C). The complete assignments of these protons and carbons were established from analysis of HMBC and COSY spectral data (Appendix B). The structure of this compound are shown below:



The ^{1}H-NMR and ^{13}C-NMR spectral assignments are tabulated in Table 3.13

Table 3.13 ^{1}H-NMR and ^{13}C-NMR chemical shift assignments and 2D correlation of Compound **3**.

Position	Chemical shift (ppm)		HMBC	COSY
	^{1}H	^{13}C		
1	-	117.3	-	-
2	-	143.5	-	-
3	6.52	95.9	C-1, C-2 ,C-6	-
4		158.7	-	-
5	-	155.8	-	H-4,H-6
6	7.35	109.0	C-1, C-2, C-3, C-5, CHO	-
7	10.34	188.1	C-1,C-2,C-6	-
OMe-2	3.90	56.3	C-2, C-3,C-4	-
OMe-4	3.95	56.3	C-6	-
OMe-5	4.00	56.3	C-5	-

From all the spectral data, the structure of Compound **3** was identified to be 2,4,5-trimethoxybenzaldehyde.

3.6 TLC bioautographic assay of active compounds from dichloromethane crude extract of *A. calamus*

After dichloromethane crude extract which were separated by several technique of chromatography such as quick column chromatography, open column chromatography, flash column chromatography and HPLC as summarize scheme 3.7. All three compound were evaluated for antifungal activity against *A. brassicicola*, *C. gloeosporioides* and *F. oxysporum* by TLC bioautographic assay. As the results of all separation of dichloromethane extract, three active compounds were obtained from dichloromethane crude extract and all spectroscopic data were indicated that Compound 1, 2 and 3 were β-asarone, α-asarone and 2,4,5-trimethoxybenzaldehyde, respectively. The antifungal activity of these three compounds against the phytopathogenic fungi were confirmed as presented in Fig 3.15 and 3.16.

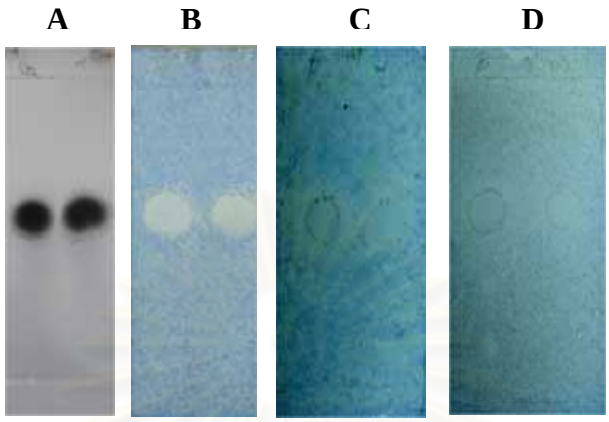


Fig 3.15 TLC profile (A) of α-and β-asarone (**A**) and bioautographic pattern against *C. gloeosporioides* (**B**), *F. oxysporum* (**C**) and *A. brassicicola* (**D**)

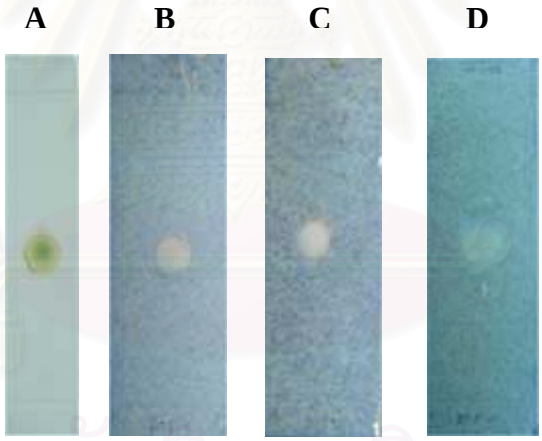


Fig 3.16 TLC profile of 2,4,5-trimethoxybenzaldehyde (**A**) and bioautographic pattern of against *C. gloeosporioides* (**B**), *F. oxysporum* (**C**) and *A. brassicicola* (**D**)

The literature search revealed that there are no reported antiphytopathogenic fungal activity to study on 2,4,5-trimethoxybenzaldehyde.

CHAPTER IV

CONCLUSION

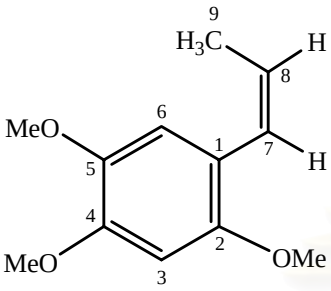
During the course of this research focusing on the search for antifungal active compound for agrochemicals, screening of antifungal activity from eighteen plant materials were evaluated on six phytopathogenic fungi; *Alternaria brassicicola*, *Colletotrichum gloeosporioides, Fusarium oxysporum*, *Phytophthora palmivora*, *Pythium* sp. and *Sclerotium* sp. Cultures of phytopathogenic fungi on PDA were incorporated with plant powder at concentration 50,000 ppm. *Acorus calamus* exhibited antifungal activity to all six phytopathogenic fungi higher up to 61%, especially for *P. palmivora*. The percentage of mycelial growth inhibition varied with species of plant materials and phytopathogenic fungi. Moreover, different parts of plant materials revealed different level on mycelial growth inhibiton. Furthermore, some plant species showed the selectivity to inhibit certain fungi.

Study of dichloromethane and methanol crude extract at concentration 10,000 ppm found that only dichloromethane extract part showed mycelial growth inhibition to all six phytopathogenic fungi 100% while methanol crude extract had no activity.

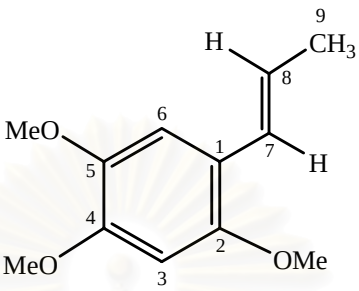
For evaluation of dichloromethane crude extract on mycelial growth inhibition, *Pythium* sp. was inhibited at the lowest concentration of IC_{50} 22 ppm.

After fractionation and purification of active compounds from the dichloromethane extract, three antifungal compounds were obtained. All isolated substances were further elucidated their structures by means of spectroscopic evidences to characterize as β-asarone, α-asarone and 2,4,5-Trimethoxybenzaldehyde and they were confirmed their antifungal activity by bioautographic technique with three phytopathogenic fungi *A. brassicicola*, *C. gloeosporioides* and *F. oxysporum*.

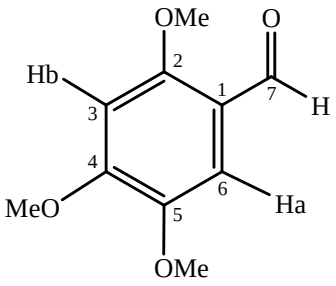
The structure of all the isolated substances from dichloromethane extract are summarized as followed :



β-asarone



α-asarone



2,4,5-Trimethoxybenzaldehyde

Proposal for future work

This research found antifungal activity of asarone derivatives. Further studies should involve the elucidation for other biological activity, study on structure activity relationship, mode of action of active compound to fungi and toxicity to human. Phytotoxicity of these active compounds and *in vivo* studies for further application uses are necessarily studied in futhur investigations. Moreover, β-asarone which is major compounds of dichloromethane extract of *A. calamus* and has toxicity might be lead compound to modify structure to reduce toxic and study their biological activity or antifungal activity relationship.

REFERENCES

1. Pesticides[online]. Available from : http://classroom.psu.ac.th/users/naran/536-412/content/Content.html [11/21/2004]
2. Agrios, N. G. *Plant Pathology*. 4th edition. San Diego: Academic Press, **1997**.
3. Ploetz, R.C. *Diseases of Tropical Fruit crops*, CABI Publishing, **2003**.
4. Christou, P. and Klee, H. *Handbook of Plant Biotechnology*, John Wiley&Sons, **2004**.
5. Buchanan, B., W. Gruissem, R. Jones. *Biochemistry & Molecular Biology of Plants*, American Society of Plant Physiologists, **2000**.
6. Tiew, P. Bioactive Compounds from Mansonia gagei Drumm. Doctoral dissertation, Department of Chemistry, Graduate School, Chulalongkorn University, **2002**.
7. Thammakasadsri, C. Anti-Phytopathogenic Fungal Agents from Essential Oil. Master's Thesis, Department of Biotechnology, Graduate School, Chulalongkorn University, **2002**.
8. Mackeen, M.M., Ali, A.M., Lajis, N.H., Kawazu, K., Kikuzaki, H. and Nakatani, N. Antifungal Garcinia Acid Esters from the Fruits of *Garcinia atroviridis*. Verlag der Zeitschrift fur Naturforschung, **2002**, 57c, 291-295.
9. Sridhar, S.R., Rajagopal, R.V., Rajavel,R., Masilamani, S., and Narasimhan, S. Antifungal Activity of Some Essential Oils. *J. Agric. Food Chem*., **2003**, 51, 7596-7599.
10. Wedge, D.E., Galindo, J.C.G., and Macias, F.A. Fungicidal Activity of Natural and Synthetic Sesquiterpene Lactones Analogs. *Phytochemistry*, **2000**, 53, 747-757.
11. Sivakumar, D., WilsonWijeranam, R.S., Wijesundera, R.L.C., Abeyesekere, M. Control of Postharvest Diseases of Rambutan Using Cinnamaldehyde. *Crop Protection*, **2002**, 21, 847-852.
12. Curir, P., Dolci, M., Lanzotti, V. and Taglialatela-Scafati, O. Kaempferide Triglycoside: a Possible Factor of Resistance of Carnation (*Dianthus*

caryophyllus) to *Fusarium oxysporum* f. sp. *dianthi*. *Phytochemistry*, **2001**, 56, 717-721.

13. Vasques da Silva, R., Debonsi Navickiene, H.M., Kato, M.J. Bolzani, V.S., Meda, C.I. and M. Young, M.C. Antifungal Amides from *Piper arboretum* and *Piper tuberculatum*. *Phytochemistry*, **2002**, 59, 521-527.
14. Terreaux, C., Gupta, M.P. and Hostettmann, K. Antifungal Benzoic Acid Derivatives from *Piper dilatatum*. *Phytochemistry*, **1998**, 49, 461-464.
15. Sarah, M.A., Bedir, E., Toyang, N.J., Khan, I.A., Dewayne Harries, M. and Wedge, D.E. Antifungal Clerodane Diterpenes from *Macaranga monandra* Muell. Et Arg. (Euphorbiaceae). *J. Agric. Food. Chem*., **2003**, 51, 7607-7610.
16. Meazza, G., Dayan, F.E., and Wedge, D.E. Activity of Quinones on *Colletotrichum* Species. *J. Agric. Food. Chem*., **2003**, 51, 3824-3828.
17. Molina-Torres,J., *et al*. Fungistatic and Bacteriostatic Activities of Alkamides from *Heliopsis longipes* Roots: Affinin and Reduced Amides. *J. Agric. Food. Chem*., **2004**, 52, 4700-4704.
18. Islam, M.T., Ito, T., Sakasai, M. and Tahara, S. Zoosporicidal Activity of Polyflavonoid Tannin Identified in *Lannea coromandelica* Stem Bark against Phytopathogenic Oomycete *Aphanomyces cochlioides*. *J. Agric. Food. Chem*., **2002**, 50, 6697-6703.
19. Oliva. A., Meepagala, K.M., Wedge, D.E., Harries, D., Hale, A.L., Aliotta, G. and Duke, S.O. Natural Fungicides from *Ruta graveolens* L. Leaves, Includeing a New Quinolone Alkaloid. *J. Agric. Food. Chem*., **2003**, 51, 890-896.
20. Hernandez, M.M, Heraso, C., Villarreal, M.L., Vargas-Arispuro, I. And Aranda, E. Biologocal Activities of Crude Plant Extracts from *Vitex trifolia* L. (Verbenaceae). *J. Ethanopharmacilogy*, **1999**, 67, 37-44.
21. Suhr, K.I. and Nielsen, P.V. Antifungal Activity of Essential Oils Evaluated by Two Different Application Techniques Against Rye Bread Spoilage Fungi. *Journal of Microbiology*, **2003**, 94, 665-674.
22. Bhasabutra, T. The Effects of Some Plant Extracts on Mango Anthracnose Fungus (*Colletotrichum gloeosporioides* (Penz.) Sacc.). Master's Thesis, Department of Plant Pathology, Graduate School, Kasetsart University, **1997**. p.14-21.

23. Beom, S.K., Surk, S.M. and Byung, K.H. Structure Elucidation and Antifungal Activity of an Anthracycline Antibiotic, Daunomycin, Isolated from *Actinomadura roseola*. *J. Agric. Food Chem*., **2000**, 48, 1875-1881

24. Wedge, D.E., Galindo, J.C.G. and Macias, F.A. Fungicidal Activity of Natural and Synthetic Sesquiterpene Lactone analogs. *Phytochemistry*, **2000**, 53, 747-757.

25. Kurucheve, V., Gerard, E., and Jayaraj, J. Screening of Higher Plants for Fungitoxicity against *Rhizoctonia solani in vitro*. *Indian Phytopath*., **1997**, 50, 235-241.

26. Kim, M., Choi, G. and Lee, H. Fungicidal Property of *Curcuma longa* L. Rhizome-Derived Curcumin Against Phytopathogenic Fungi in a Greenhouse. *J. Agric. Food Chem*., **2003**, 51, 1578-1581.

27. Lee, S., Park, B., Kim, M., Choi, W., Kim, H., Cho, K., Lee, S.G., and Lee, H.S. Fungicidal Activity of Pipernonaline, a Piperidine Alkaloid Derived from Long Pepper, *Piper longum* L., Against Phytopathogenic Fungi. *Crop protection*, **2001**, 20, 523-528.

28. Anandaraj, M. and Leela, N.K. Toxic effect of some plant extracts on *Phytophthora capsici*, the foot rot pathogen of black pepper. *Indian Phytopathology*, **1996**, 49, 181-184.

29. Yeon Lee, J., Yeop Lee, J., Yun, B.S. and Hwang, B.K. Antifungal Activity of β-Asarone from Rhizomes of *Acorus gramineus*. *J. Agric. Food Chem*., **2004**, 52, 776-780.

APPENDIX A

Fungal Culture Media

Potato Dextrose Agar	
Potato	200 g
Dextrose	20 g
Agar	15 g
Distilled water add to	1000 ml

Stained Solution

Acetic acid	5%
Lactophenol	1%

Destained Solution

Acetic acid	5%

APPENDIX B

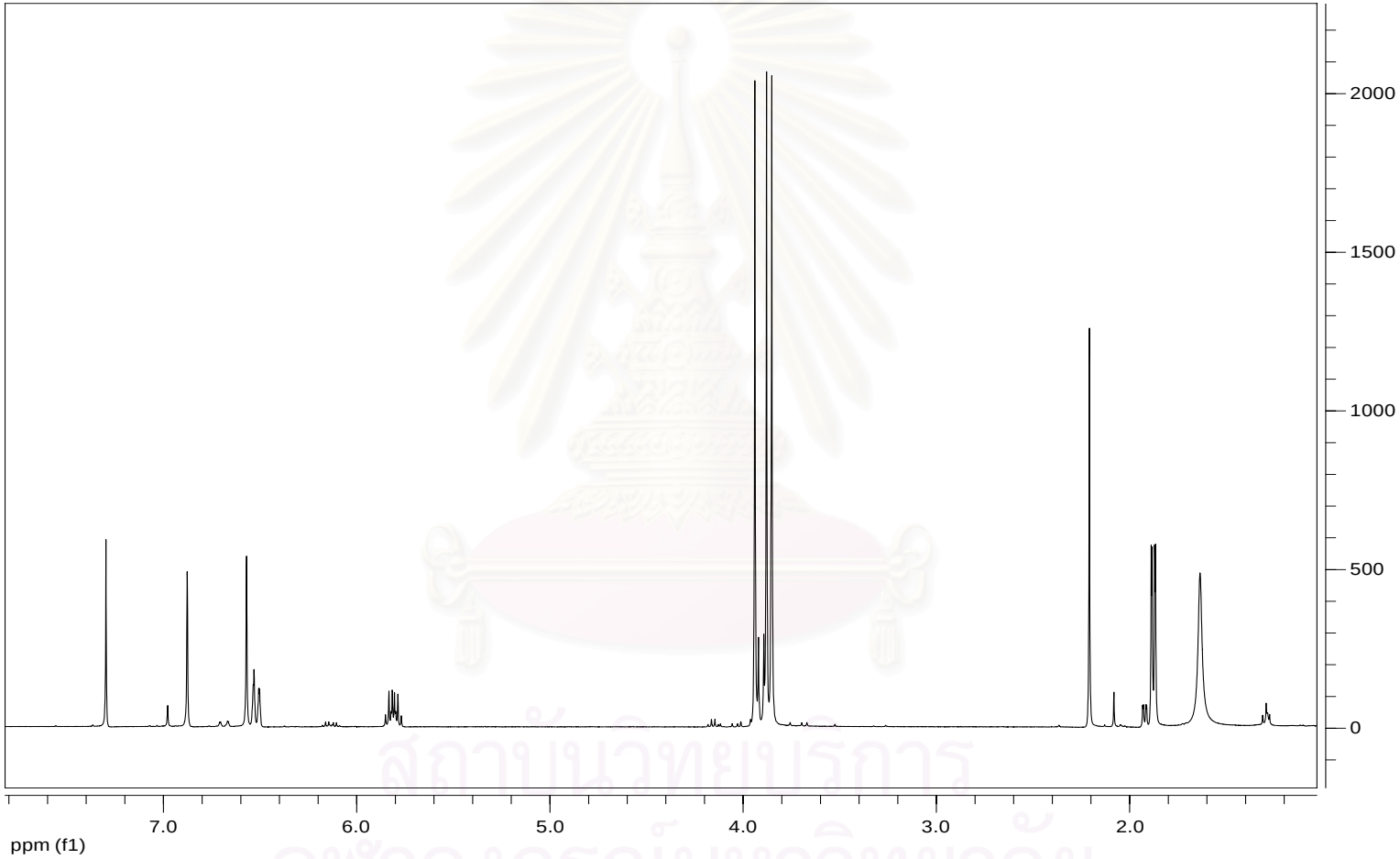


Fig. 3.17 ^{1}H-NMR spectrum of Compound **1**

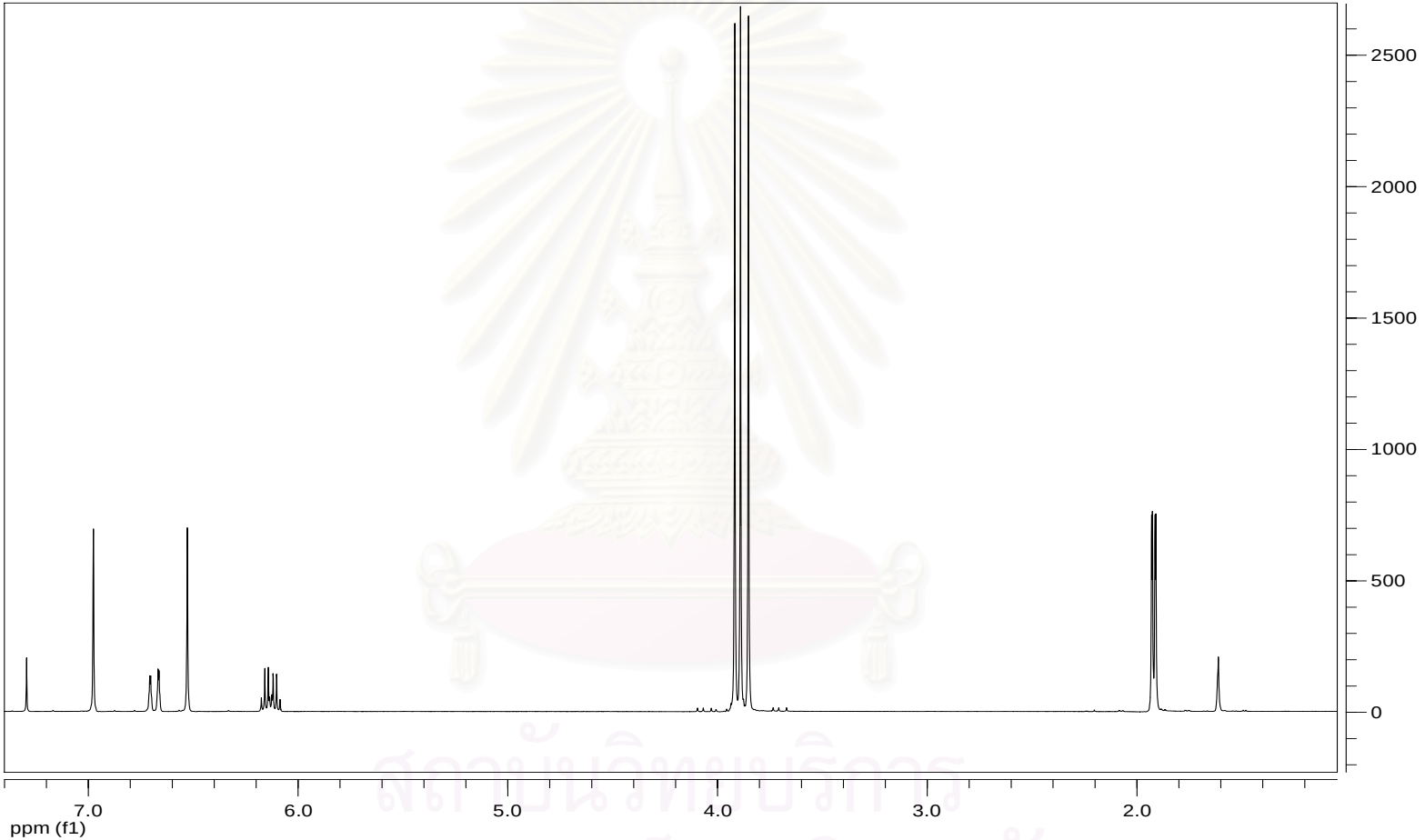


Fig. 3.18 ^{1}H-NMR spectrum of Compound **2**

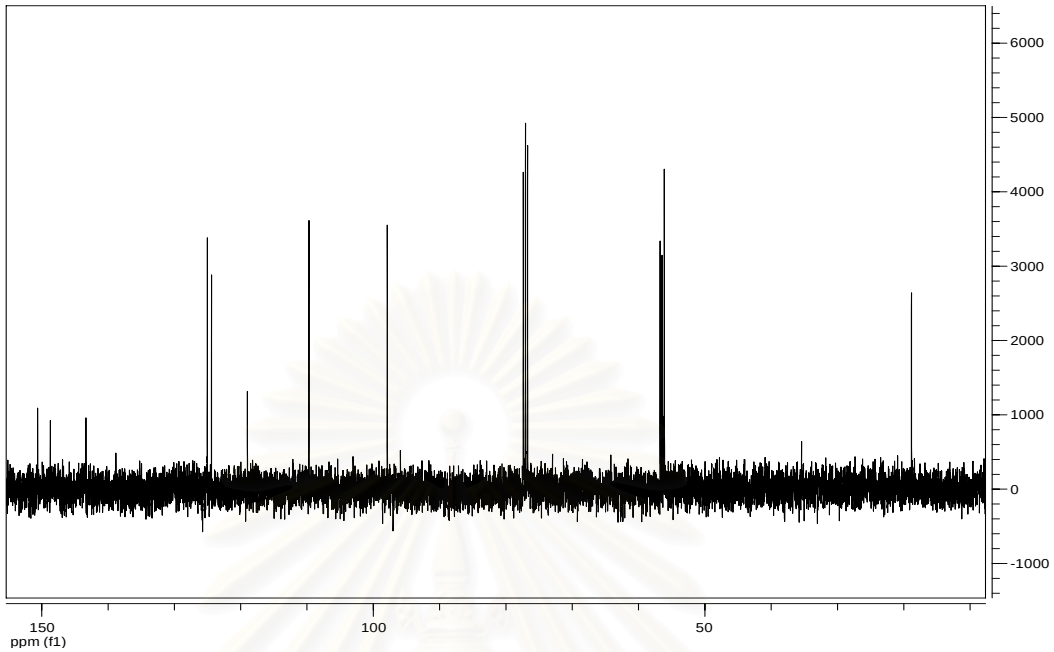


Fig. 3.19 ^{13}C-NMR spectrum of Compound **2**

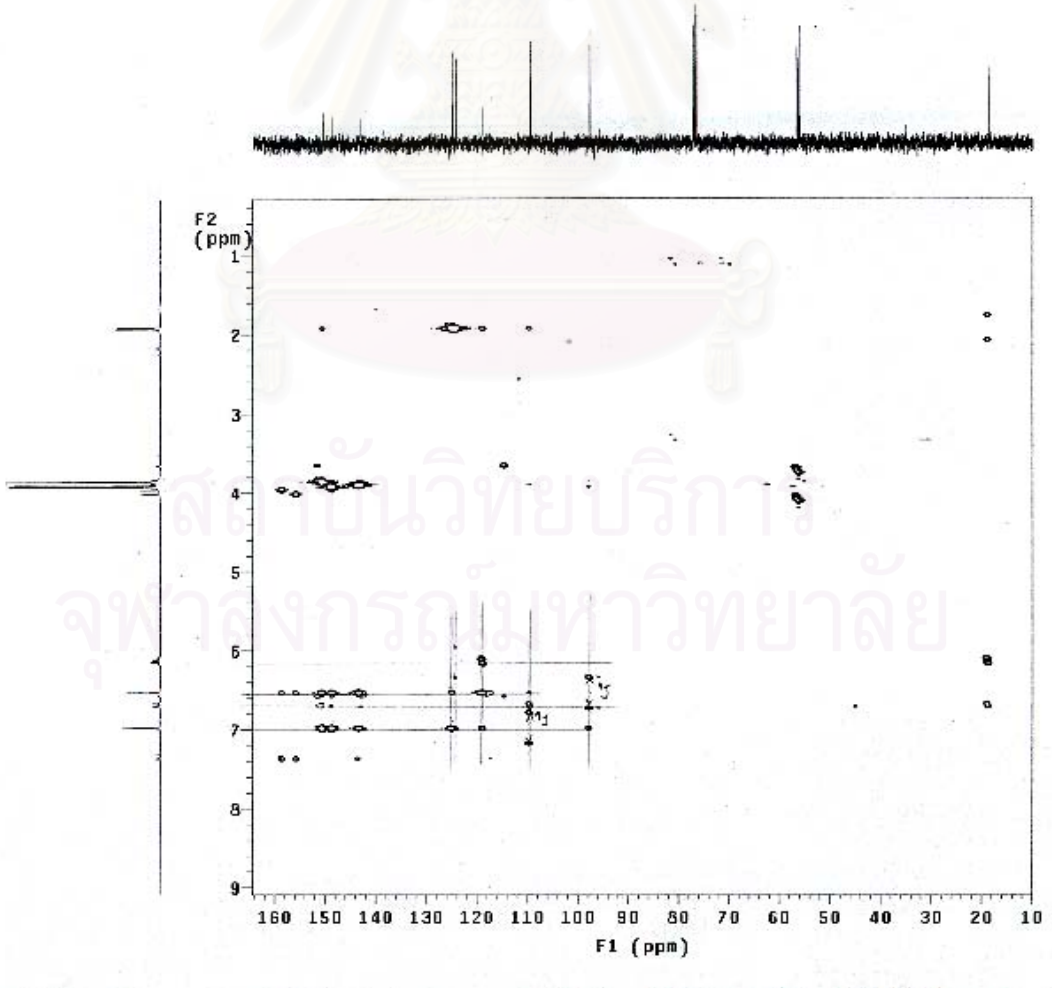


Fig. 3.20 HMBC spectrum of Compound **2**

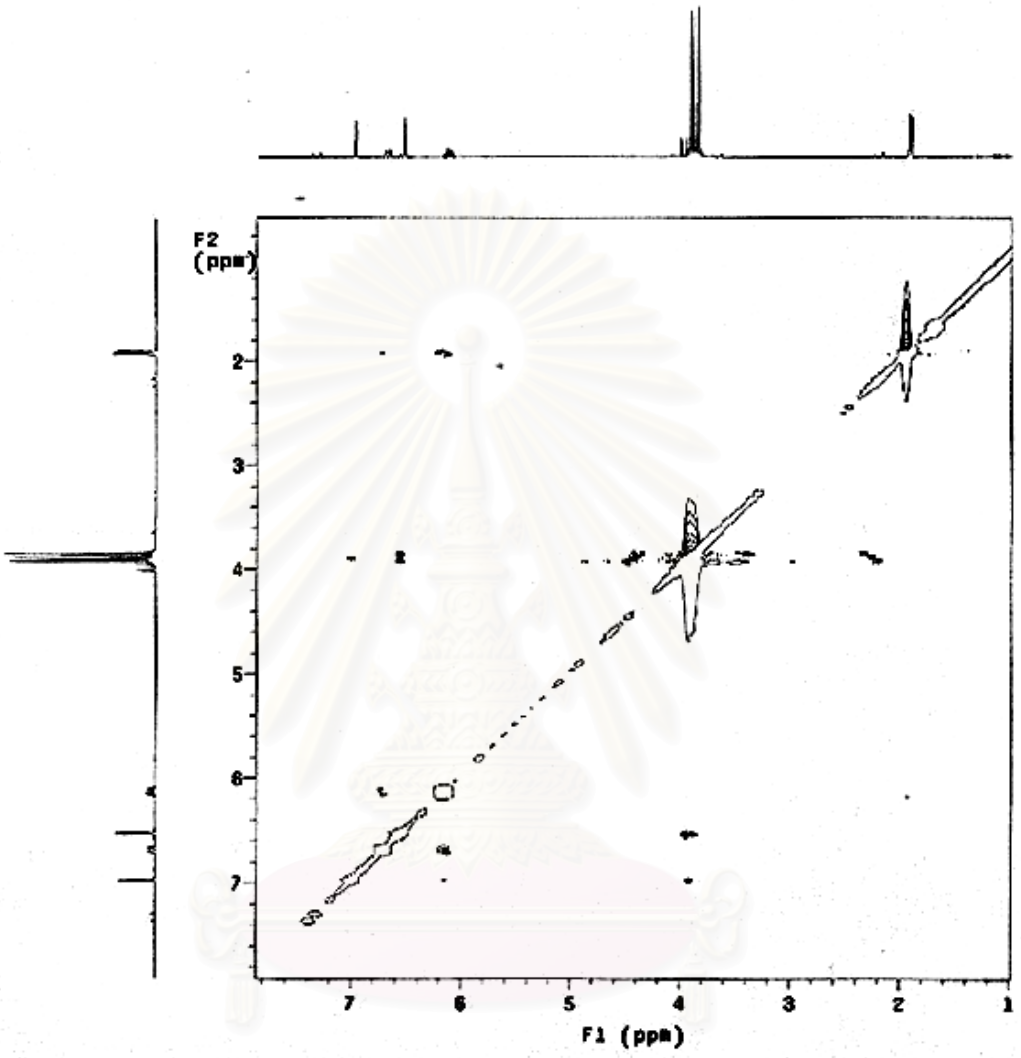


Fig. 3.21 NOESY spectrum of Compound **2**

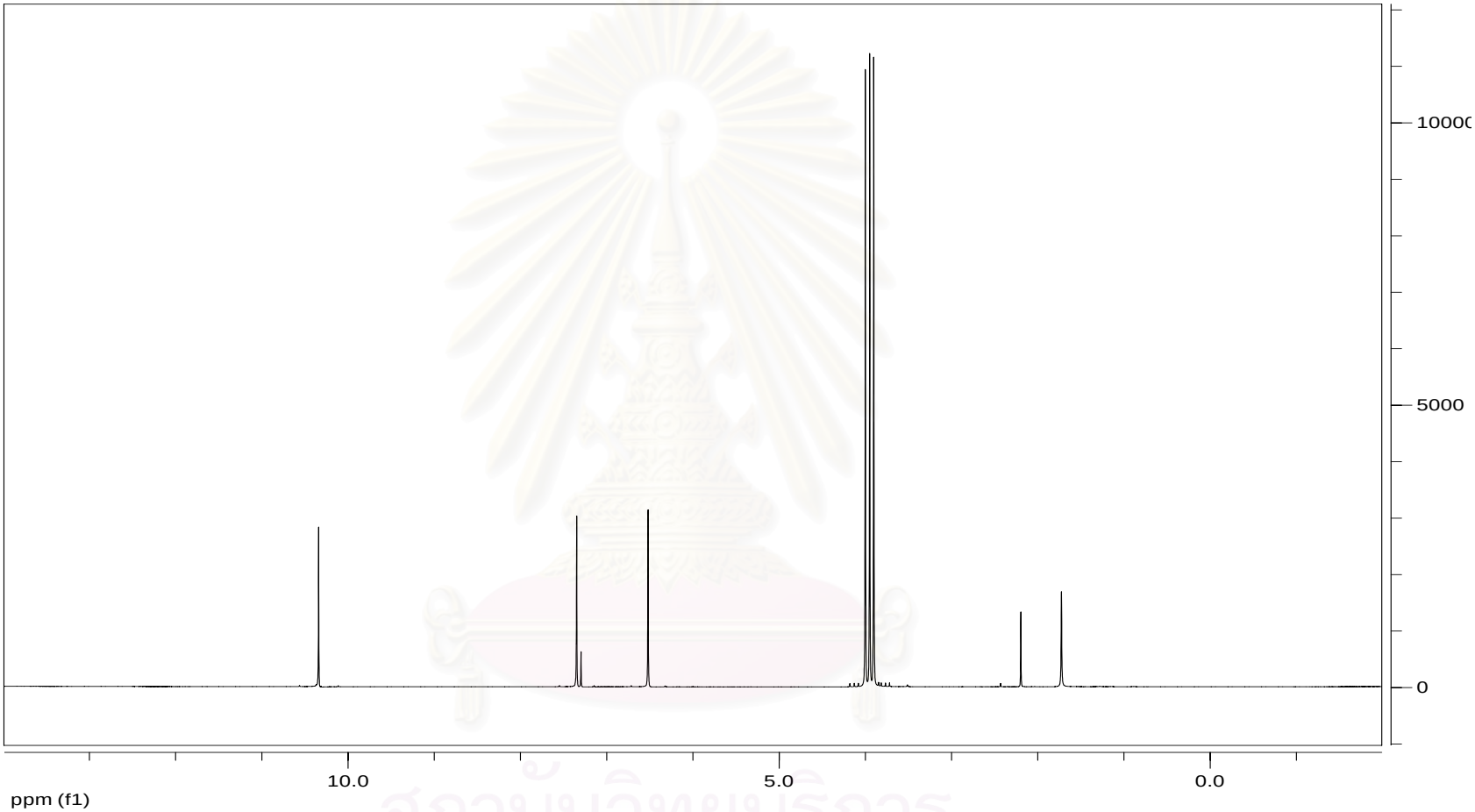


Fig. 3.22 ^{1}H-NMR spectrum of Compound **3**

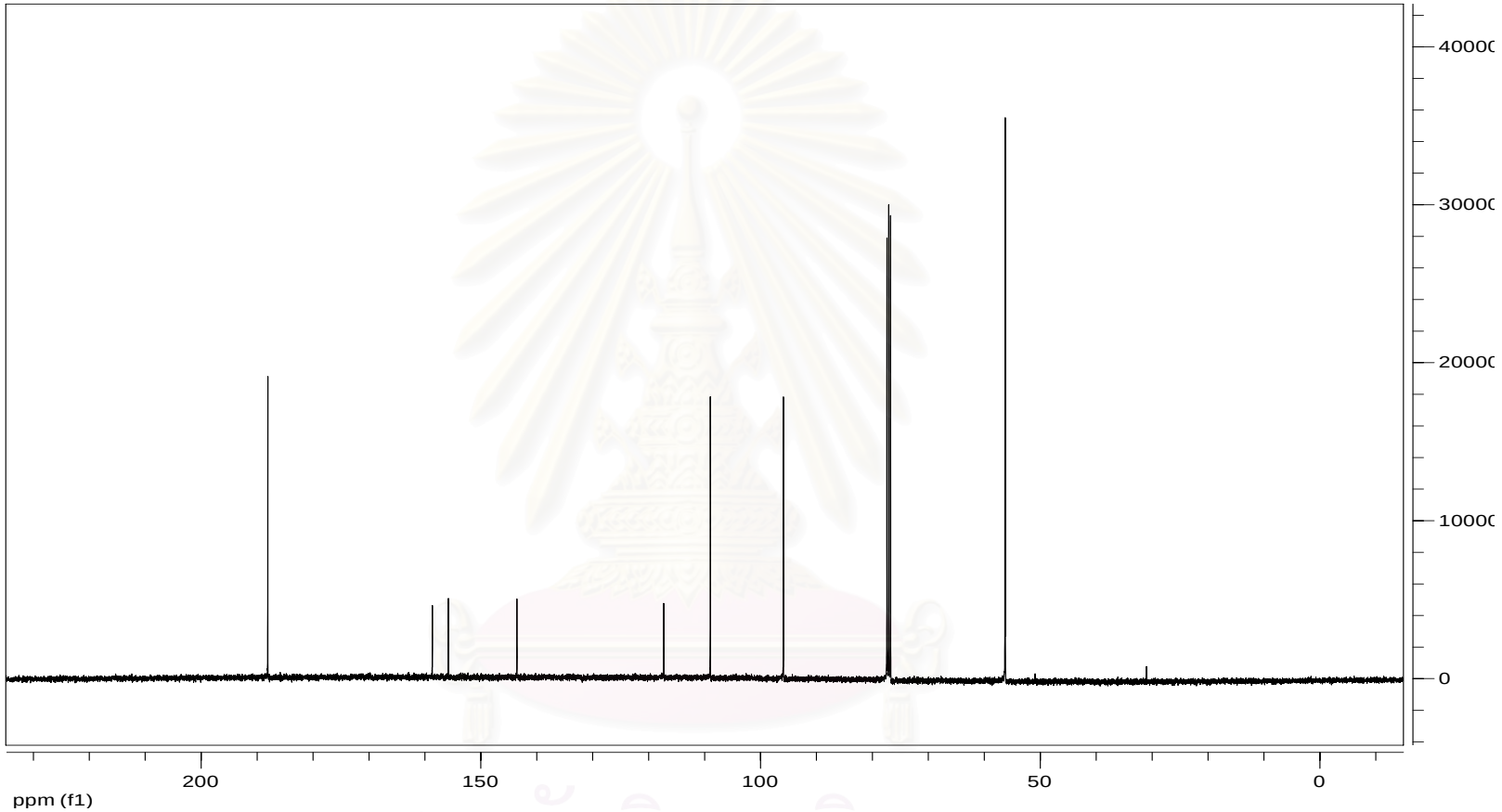


Fig. 3.23 ^{13}C-NMR spectrum of Compound **3**

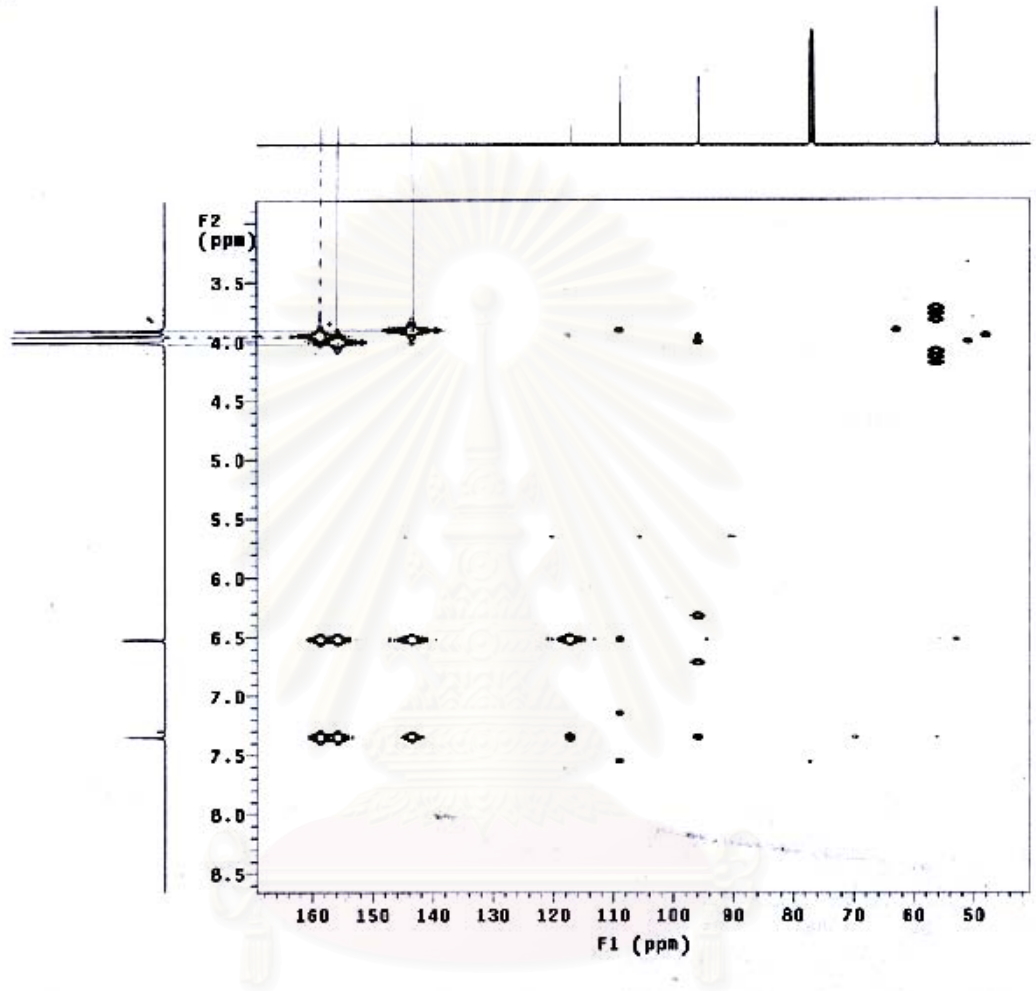


Fig. 3.24 HMBC spectrum of Compound **3**

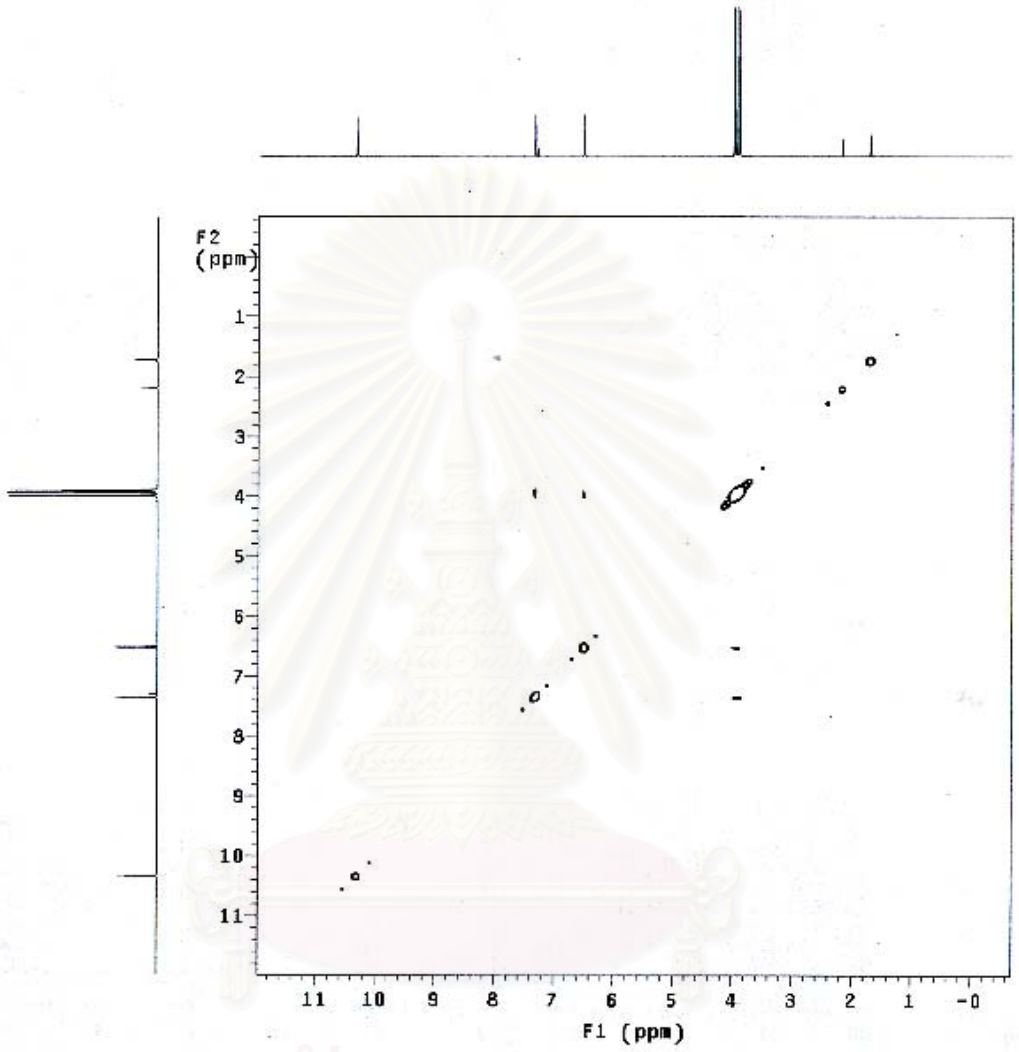


Fig. 3.25 The COSY spectrum of Compound **3**

VITA

Nongnoot Khruasanit was born on July 31, 1979 in Krabi province, Thailand. She obtained a Bachelor Degree of Science (Agricultural Technology) from Faculty of Science and Technology, Prince of Songkhla University, Thailand in 2000. During she studying in Master's degree, she was obtained a scholarship support form the Graduate School of Chulalongkorn University.