สารยับยั้งเชื้อก่อโรคในกล้วยไม้ ‘Pompadour’ ด้วยสารสกัดจากพืช

นางสาวภัทรานิษฐ์ เจริญศรี



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2557

# EFFECTS OF ANTIPHYTOPATHOGENIC AGENTS FROM PLANT EXTRACTS ON *Dendrobium* 'Pompadour'

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biotechnology
Faculty of Science
Chulalongkorn University
Academic Year 2014

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|---|---|
| Thesis Title | EFFECTS OF ANTIPHYTOPATHOGENIC AGENTS FROM PLANT EXTRACTS ON *Dendrobium* 'Pompadour' |
| By | Miss Patranit Charoensri |
| Field of Study | Biotechnology |
| Thesis 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

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ภัทรานิษฐ์ เจริญศรี : สารยับยั้งเชื้อก่อโรคในกล้วยไม้ ‘Pompadour’ ด้วยสารสกัดจากพืช (EFFECTS OF ANTIPHYTOPATHOGENIC AGENTS FROM PLANT EXTRACTS ON *Dendrobium* ‘Pompadour’) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. วรินทร ชวศิริ, 74 หน้า.

นำสิ่งสกัดเฮกเซน ไดคลอโรมีเทน เอทิลแอซีเทต เมทานอลและน้ำมันหอมระเหยของพืช 19 ชนิด และสารที่เลือกมา 3 สาร มาคัดกรองเบื้องต้นเพื่อศึกษาการยับยั้งการเจริญของจุลินทรีย์โรคกล้วยไม้ Pompadour พบว่าสิ่งสกัดของมังคุด *Garcinia mangostana* , น้ำมันหอมระเหยจากเทพทาโร *Cinnamomum porrectum* และสิ่งสกัดของหญ้าข้าวนก *Echinochloa colona* มีแนวโน้มที่ดีในการยับยั้งการเจริญของแบคทีเรียก่อโรคในกล้วยไม้ สารบริสุทธิ์จากมังคุด (**γ** – mangostin) และน้ำมันหอมระเหยของเทพทาโรแสดงศักยภาพที่ดีในการต้านฤทธิ์จุลชีพ *Burkholderia gladioli* เมื่อใช้ชีววิธีในการติดตามฤทธิ์ในการแยกสิ่งสกัดไดคลอโรมีเทนของหญ้าข้าวนกพบสารออกฤทธิ์คือ undecanoic acid นอกจากนี้น้ำมันหอมระเหยของไพล *Zingiber cassumunar* และ **γ** – mangostin ยังแสดงศักยภาพในการยับยั้งการเจริญของ *Phytophthora palmivora* ในขณะที่ไม่มีสิ่งสกัด น้ำมันหอมระเหย หรือสารบริสุทธิ์จากพืชทดลองแสดงศักยภาพในการยับยั้งการเจริญของ *Fusarium oxysporum* ได้ดี เมื่อทำการทดลองใช้สิ่งสกัดจากพืชที่มีแนวโน้มที่ดีมายับยั้งการเจริญของจุลินทรีย์กับการเพาะเลี้ยงเนื้อเยื่อกล้วยไม้ Pompadour พบว่าสิ่งสกัดไดคลอโรมีเทนของหญ้าข้าวนกและสารบริสุทธิ์จากมังคุดมีประสิทธิภาพในการยับยั้งการเจริญของ *B. gladioli* และ *P. palmivora* ได้ดีตามลำดับ



สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

# # 5372552623 : MAJOR BIOTECHNOLOGY

KEYWORDS: ANTIPHYTOPATHOGENIC / DENDROBIUM ‘POMPADOUR’ / PLANT EXTRACTS

PATRANIT CHAROENSRI: EFFECTS OF ANTIPHYTOPATHOGENIC AGENTS FROM PLANT EXTRACTS ON *Dendrobium* ‘Pompadour’. ADVISOR: ASST. PROF. WARINTHORN CHAVASIRI, Ph.D., 74 pp.

The hexane, $CH_2Cl_2$, EtOAc, $CH_3OH$ extracts, and the essential oils from nineteen plants, and 3 selected compounds were tested for preliminary anti-orchid pathogenic microorganism screening on *Dendrobium* ‘Pompadour’. The extracts from *Garcinia mangostana, Cinnamomum porrectum* and *Echinochloa colona* showed good tendency to inhibit bacterial disease in orchids. γ -Mangostin from *G. mangostana* and the essential oil from *C. porrectum* revealed the best inhibition against *Burkholderia gladioli*. Using bioassay-guide, the separation of the $CH_2Cl_2$ extract from *E. colona* yielded an active compound: undecanoic acid. In addition, the essential oil from *Zingiber cassumunar* and γ –mangostin showed high potential antifungal activity against *Phytophthora palmivora*, but showing not good results against *Fusarium oxysporum*. The *in vitro* antipathogenic tests in orchid *Dendrobium* sp. tissue culture revealed that the $CH_2Cl_2$ extract from *E. colona* and γ –mangostin had good potential to inhibit *B. gladioli* and *P. palmivora*, respectively.



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

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

# ACKNOWLEDGEMENTS

I would like to express gratitude to my thesis advisor, Assistant Professor Dr. Warinthorn Chavasiri for invaluable suggestion, guidance, encouragement and kindness throughout the course of this work. In addition, sincere appreciation is also extended to Associate Professor Dr. Sirirat Rengpipat, Associate Professor Dr. Kanoktip Packdibamrung and Associate Professor Dr. Kanchit Thammasiri for acting as the chairman and examiner of my thesis committee respectively, and for their valuable constructive comments and suggestions.

I gratefully acknowledge the financial support provided by CU.Graduate School Thesis Grant, Chulalongkorn University. Furthermore, I would like to thank all members of Natural Product Research Unit (NPRU), and all my friends, for their friendliness, helpful discussions, cheerful attitude and encouragements during my thesis work. Finally, I also wish to especially thank my family members for their love, kindness and support throughout my entire study.

**CONTENTS**

Page

Page

Page

# LIST OF TABLES

# LIST OF FIGURES

# LIST OF SCHEMES

# LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| °C | = | degree Celsius |
| $Ca_3(PO_4)_2$ | = | calcium phosphate |
| DMSO | = | dimethyl sulfoxide |
| *et al.* | = | et alli (Latin), and others |
| EtOAc | = | ethyl acetate |
| $FeSO_4.7H_2O$ | = | ferrous sulphate |
| g | = | gram |
| GC | = | gas chromatography |
| GC/MS | = | gas chromatography-mass spectrometry |
| HCl | = | hydrochloric acid |
| $^1$H NMR | = | proton nuclear magnetic resonance |
| $KH_2PO_4$ | = | Monopotassium acid phosphate |
| $KNO_3$ | = | Potassium nitrate |
| L | = | liter |
| μL | = | microliter |
| M | = | Molar |
| mg | = | milligram |
| $MgSO_4.7H_2O$ | = | magnesium sulphate |
| $MnSO_4.H_2O$ | = | manganese sulphate |
| MIC | = | minimum inhibitory concentration |
| min | = | minute |
| mL | = | milliliter |

| | | |
|---|---|---|
| mM | = | millimolar |
| N | = | Normality |
| NA | = | nutrient agar |
| $Na_2EDTA$ | = | sodium EDTA |
| NaCl | = | sodium chloride |
| NB | = | nutrient broth |
| $(NH_4)_2SO_4$ | = | ammonium sulphate |
| NMR | = | nuclear magnetic resonance |
| PDA | = | potato dextrose agar |
| PDB | = | potato dextrose broth |
| ppm | = | part per million |
| $R_t$ | = | retention time |
| sp. | = | species |
| TLC | = | thin layer chromatography |
| wt | = | weight |

# CHAPTER I
# INTRODUCTION

## 1.1 Orchids

Thailand is one of the richest countries in the world in terms of biodiversity of flora, fauna and ecosystems. There are approximately 15,000 different species of vascular and non-vascular plants in Thailand. Orchidaceae is the largest of the vascular plant families comprising 176 genera and 1,157 species found in all types of habitats. Presently in Thailand, the natural habitat of orchid has been heavily disturbed; forest degradation and over-exploitation for commercial and agricultural practices have had a severe impact on the orchid population, especially the terrestrial species.

Orchid populations in conserved areas, which historically have been safe places for orchids, are being harvested at an alarming rate. These beautiful and economically important species are becoming difficult to find in the wild where they used to be abundant. Species of the genera *Aerides, Cymbidium*, *Dendrobium*, *Paphiopedilum*, *Pholidota*, *Rhynchostylis* and *Vanda* have become increasingly vulnerable.



According to data from the Department of Agriculture in 2013, orchids are an important economic product for Thailand. These orchids are exported primarily as cut flowers, but the seedlings grown from tissue cultures are also highly desired on the international market. More than 47,000 tons of cut orchid flowers are exported each year from Thailand at an estimated value of more than 69 million United States dollar or 2,400 million baht. The future of Thai orchids should be considered and managed to ensure a balance between conservation and demand. Scientific study, the breeding of new hybrids, propagation and transfer of new technology should be properly investigated to align global marketing and large investment with the conservation of orchids in Thailand. (Nanakorn, 2008)

### 1.1.1 History and morphology

Orchids are grown for the astonishing beauty and variety of their flowers. The most beautiful are the gorgeous South American Cattleyas, with their often huge, sparkling, rosy-purple and mauve blooms; these are the aristocrats of the orchid world. Others have an altogether different appeal; these are the strangely weird, sometimes evil-smelling species, which are found among the bulbophyllums in particular. In between these two extremes are numerous delightful orchids of unlimited charm and desirability. It is from among this multitude of varieties that the vast number of collections is made up. (Hurley, 2013)

In the past, growing orchid was a common practice by local people, villagers saw the beautiful flowers in the wild and brought the plants back to their village without any large scale commercial production. It was not until 1837 that a large orchid nursery belonging to Mr. Henry Alabaster was established in Bangkok. Since then, many exotic species have been introduced into Thailand and many hybrids between native and exotic species have been produced.

After 1934, commercial orchid nurseries for cut flowers opened and rapidly expanded to cope with the large international demand. At present, there are many large orchid nurseries producing cut orchid flowers for export. Derived from their beautifulness, uniqueness and attractive characters, native orchids have been over collected from their natural habitats leading to some species of *Paphiopedilum*, *Vanda*, and *Rhynchostylis* to become rare or on the verge of extinction in the wild.

Orchids are plants that have been grown popular by worldwide. This is because orchids have colorful multi-ridge and found naturally of about 25,000 species. Flower as a pin head. Early large as sugar cane. Tree is growing on the island or rocks or trees growing on the dating of wood with a single root islands stretching some twigs, a ground orchid. Important source of wild orchids of the world's two major parties is also Latin America, Asia and the Pacific orchid genus grown wood is sacred.

### 1.1.2 Diseases

Orchid diseases can be fungi, oomycetes, bacteria, viruses, viroids, virus-like organisms, phytoplasmas, protozoa, nematodes and parasitic plants. Almost pathogens has a small size, cannot detect by eyes and rapid propagate there species. The common fungal diseases can see on orchids are sooty mold, petal blight, rust and black rot. They are usually caused by high humidity without sufficient air movement. Insects and pests also spread the fungus. The honeydew produced by aphids also promotes fungal growth.

Bacterial orchid diseases: A typical problem are soft rots caused by different pathogens. The bacteria enter the plant through cuts or similar and quickly infect the whole orchid. The orchid disease can quickly spread to other plants if there is some water dripping from one plant to the other somehow. For example:-

Bacterial Soft - *Burkholderia gladioli* (it was formerly known as *Pseudomonas gladioli) B. gladioli*, as a gram-negative rod, is a species of aerobic bacteria (Coenye and Vandamme, 2007) that causes disease in both humans and plants and is mainly found in soil, environmental water, and in many animals (Murray *et al*., 2007).

Symptoms: Small water-soaked spots appear on the leaves and often are surrounded by yellow halos. If unchecked, the infection will rapidly rot the leaves and roots and spread more slowly into the rhizomes or pseudobulbs. This wet rot may have a foul odor and has a water soaked appearance. (Figure 1.1)

- *Phalaenopsis*. Disease spreads so rapidly that plants may be completely rotted in 2 to 3 days. The bacteria are opportunistic organisms that can enter through wounds.

- *Dendrobium*. Leaves appear yellow and water-soaked and become black and sunken.

- *Vanda*. Leaves develop translucent patches which become black and sunken.

- *Paphiopedilum*. Leaves develop small, round spots often near the middle of the leaf. The spots are initially yellow and water-soaked but eventually become reddish brown and sunken. The spot enlarges in all directions and may reach the growing crown before the leaf tip is affected. If untreated, the disease quickly spreads throughout the plant, leaving it a dark, shriveled mass.

- *Grammatophyllum*. Leaves have water-soaked, browning spots which become black and sunken.

**Figure 1.1** Soft rot on *Oncidium* orchid, Source: farm8.staticflickr.

Fungal orchid diseases: Orchid fungal diseases appear when the moisture level is too high and/or the environment is too cold. For example:-

Black Rot - *Phytophthora palmivora,* this fungi can releases zoospores (Figure 1.2) from germinating sporangia. Symptoms: The infections usually starts on the leaves, new leads or roots, though all plant parts are susceptible. The disease spreads rapidly, particularly when the temperature and humidity are high. Pseudobulbs, roots or rhizomes show infections as purplish-black, often sharply delineated, discolored area in the center of the plant. The infection often starts in the roots and may spread upward to the base of the pseudobulb or leaf, which can cause the leaf to fall from the plant with a slight jarring. In seedlings and community pots, small water soaked spots may start on the seedling, and plant after plant rots and dies. (Figure 1.3)

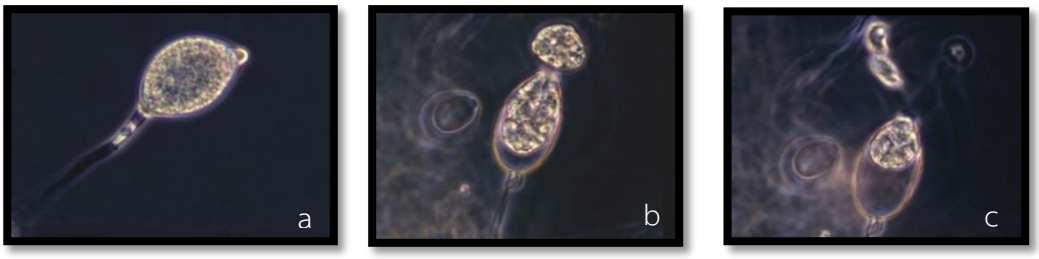



**Figure 1.2** Zoospores of *Phytopthora palmivora*, Source: botany.hawaii.edu.

a) Intact zoosporangium of *Phytophthora palmivora* before release of zoospore.

b) Zoosporangium of *Phytophthora palmivora* with zoospores clustered together and oozing out of operculum.

c) Zoospores of *Phytopthora palmivora* coming apart. Two zoospores swimming away can be seen at the top of the screen.

**Figure 1.3** Black rot on Cattleya leaf and pesudobulb, Source: farm8.staticflickr.

Fusarium Wilt - Fusarium oxysporum Symptoms: Fusarium can infect a plant through orchid roots or by invading the rhizome of recently divided plants. Fusarium blocks the flow of moisture through the plant's vascular system plugging the phloem. The pathogen is spread through improper hygiene, generally as a result of using non-sterile cutting tools, which transfers the fungus from plant to plant.

Infected leaves are yellow, thin, shriveled, wrinkled or wilted and eventually die. Severely infected plants may die in 3-9 weeks, while mildly infected plants gradually decline over a year or so. The diagnostic symptom in the plant is a circle or band of purple or pinkish-purple discoloration on the outer layers of the rhizome evident when the rhizome is cut. If the disease is extensive, the entire rhizome may turn purple, and the discoloration may extend to the pseudobulbs. (Figure 1.4)

**Figure 1.4** *Fusarium* Wilt on orchid leaf, Source: staugorchidsociety.org.

### 1.1.3 Control of disease

The demand for agricultural productivity and the expansion of industry caused a rapid increase in the use of chemicals. Most chemicals, organic and inorganic, are imported from approximately 40 countries including developed and industrialized countries. The importation of chemicals, as reported, increased every year. Pesticides from one of the major toxic groups of chemicals to be imported increasingly. Most of the imported pesticides were organochlorine pesticides, such as DDT, toxaphene, drins, heptachlor and others.

In case of orchids, pesticides are usually used to get rid of microorganisms. For example Physan 20, Phyton 27, Diconal, Thioml, Metalaxyl, Aliette, and Agrimycin. The use of pesticides may cause hazards to human health and may directly increase environmental pollution. From the database of the office of Agriculture Regulation, Department of Agriculture found that Thailand has used pesticides more than other countries in South-East Asia. Furthermore, some

pesticides may not readily be biodegradable and tend to persist for years in environment and increase day by day from over abundantly or take amiss of use.

## 1.2 Literature search on the anti-orchid pathogen activity from natural sources

The *Orchidaceae* is the largest family of flowering plants (angiosperms) with 700–800 genera and 20,000–30,000 described species, or approximately 10% of all known angiosperms, and is the most highly evolved family amongst the monocotyledons (Arditti, 1979; Dressler, 1981, 1993). Orchids are a major floral crop worldwide and represent over 8% of the global floriculture trade (Griesbach, 2002). Globally, *Dendrobium* hybrids are considered to be one of the most popular orchids.

Since the traditional asexual propagation of orchids is extremely slow, tissue culture has been adopted as a useful technique for their micropropagation. Tissue culture was first introduced and applied to *Cymbidium* orchids by Morel in 1960. After that, tissue culture methods, culture media and various explants for orchid tissue culture were introduced and developed by several workers. Media devised by Vacin and Went (1949) and Murashige and Skoog (1962) are usually employed for *in vitro* orchid culture (Lakshmanan *et al*. 1995; Ket *et al*. 2004; Ravindra *et al*. 2004). Half strength MS medium is also used successfully for orchid culture (Chen and Chang 2000). Carbon sources are critical components in these culture media (Islam and Ichihashi 1999) and sucrose is the most frequently used.

Black rot of orchids can be caused by several pathogens. Frequently, black rot is caused by *Phytophthora palmivora* (Hine, 1962; Uchida, 1994; Orlikowski and Szkuta, 2006).

Although the use of synthetic pesticides in plant protection had made a great contribution to plant protection, many are no longer used because of economic, environmental or health concerns, or due to development of resistant strains. Fungicides that are primarily used for controlling the diseases have recently come under special scrutiny as posing a potential oncogenic risk. Therefore, the scientific community at international level is looking for safer alternative products from plants

for effective control of pests during storage. Naturally occurring biologically active compounds from plants are generally assumed to be more acceptable and less hazardous than synthetic compounds and represent a rich source of potential disease-control agents.

Biologically active essential oils and plants extracts represent a rich potential source of an alternative and perhaps environmentally more acceptable disease management compounds. The general antifungal activity of essential oils is well documented (Alankararao *et al*., 1991; Baruah *et al*., 1996; Gogoi *et al*., 1997; Pitarokili *et al*., 1999; Meepagala *et al*., 2002; Mayachiew and Devahastin., 2008) and there have also been studied on the effects of plant extracts. (Kotan *et al*., 2013; Zhang *et al*., 2013)

Plant oils and extracts have been used for a wide variety of purposes for many thousands of years. (Jones *et al*., 1996) Recently, the essential oils and various extracts of plants have provoked interest as sources of natural products. Plants were screened for a treatment of many infectious diseases. The antimicrobial activities of plant oils and extracts have formed the basis of many application (Reynolds *et al*., 1996 and Lis *et al*., 1997), including prevention, alternative pesticide and natural therapies.

Some plant extracts were evaluated for control of disease in crop such as cassumunar *Zingiber cassumunar* Roxb. and galangal *Alpinia galanga* (Linn.) Swartz. for chili anthracnose (Sonthiha and Leksomboon., 2010). The extracts from *Eupatorium* spp. and *Curcuma* spp. were used to control rice blast and bacterial leaf blight diseases (Klinmanee and Sribua *et al*., 2008) and canker disease (*Xanthomonas campestris* pv. *citri*) in pummelo was controlling by medicinal plants (Siwakorn *et al*., 2008).

Furthermore, some reports involving anti-phytopathogenic fungal activity were addressed from some Thai medicinal herb extracts (Kruasanit, 2004). For example, *Acorus* calamus (mytle grass) inhibited phytopathogenic fungi, especially *P. palmivora* by potato dextrose agar (PDA) mixture with herb powder at concentration

50,000 ppm. The $CH_2Cl_2$ extract of *A. calamus* could inhibit *P. palmivora* 100% at concentration 10,000 ppm while pure compounds ($\beta$- and $\alpha$-asarones, and 2,4,5-trimethoxybenzaldehyde) from mytle grass could resist the growth of *F. oxysporum* using bioautographic technique.

## 1.3 Objectives of this research

The objective of this work was to search for anti-phytopathogenic agents for orchids from natural sources, their compound and the effect of them in orchid tissue culture.

# CHAPTER II
# MATERIAL AND METHODS

## 2.1 Materials

### 2.1.1 Plant materials

Nineteen plant samples from 15 families were selected. The part of studied plants included leaves, branches, flowers, fruits, barks, stems, heartwood, rhizomes, aerial plants and whole plants. All plants were identified by botanist as collected in Table 2.1.

**Table 2.1** Plants used for antimicrobial activity screening.

| List | Species | Common name | Plant part |
|---|---|---|---|
| | **Agavaceae** | | |
| 1 | *Dracaena loureiri* Gagnep (Chan-daeng) | mountain dracaena | heartwood |
| | **Asteraceae** (Compositae) | | |
| 2 | *Eclipta prostrata* (L.) L. (Ka-meng) | false daisy | whole plant |
| 3 | *Eupatorium odorata* L. (Sab-sua) | bitter bush, Siam weed | leave, stem |
| | **Bignoniaceae** | | |
| 4 | *Oroxylum indicum* (L.) Vent. (Pae-ka) | broken bones tree, Indian trumpet flower | bark, stem |

**Table 2.1** (continued).

| List | Species | Common name | Plant part |
|---|---|---|---|
| 5 | **Clusiaceae** *Garcinia mangostana* (Mung-kud) | mangosteen | peel |
| 6 | **Fabaceae** *Xylia xylocarpa* Roxb. (Daeng) | iron wood | heartwood |
| 7 | **Lauraceae** *Cinnamomum porrectum* (Roxb.) Kosterm. (Thep-tha-row) | citronella laurel, hardy cinnamon | stem |
| 8 | **Loganiaceae** *Fagraea fragrans* Roxb. (Kun-Krao) | tembusu | flower, bark, stem |
| 9 | **Piperaceae** *Piper sarmentosum* Roxb. (Cha-plu) | wildbetal leafbush | leave |
| 10 | **Poaceae** (Gramineae) *Echinochloa crus-galli* (L.) P.Beauv. (Ya-khao-nok) | barnyard grass, jungle rice | aerial |
| 11 | **Polygonaceae** *Polygonum odoratum* Lour. (Preaw) | Vietnamese coriander | whole plant |

**Table 2.1** (continued).

| List | Species | Common name | Plant part |
|---|---|---|---|
| | **Rubiaceae** | | |
| 12 | *Gardenia angusta* (L.) Merr. (Pud-soun) | cape jasmine | flower, leave, branch, fruit |
| 13 | *Gardenia jasminoides* J. Ellis (Pud) | gardenia | flower |
| | **Rutaceae** | | |
| 14 | Zanthozylum limonella Alston. (Ma-khan) | Indian ivy-rue | flower |
| | **Saururaceae** | | |
| 15 | *Houttuynia cordata* Thunb. (Plu-kao) | Chinese lizard tail, heart-leaved houttuynia | whole plant |
| | **Scorphulariaceae** | | |
| 16 | *Limnophila aromatica* (Lam.) Merr. (Ka-yang) | balloon vine | whole plant |
| 17 | *Otacanthus coeruleus* A. Rose. (Blue hawaii) | blue hawaii, Brazilian snapdragon | whole plant |
| | **Zingiberaceae** | | |
| 18 | *Amomum xanthioides* Wall. (Rew) | bustard cardamom, tavoy cardamom | rhizome |
| 19 | *Zingiber cassumunar* Roxb. (Plai) | Bengal ginger, cassumunar ginger | rhizome |

Three pure compounds (as presented in Table 2.2) were kindly obtained, *i.e.*, safrole hydrodistilled from the roots of *C. porrectum* by Ms. Suekanya Jarupinthusophon, and terpinen-4-ol and sabinene isolated from the essential oil from the rhizomes of *Z. cassumunar*.

**Table 2.2** Pure compounds for antimicrobial activity screening.

| List | Species | Common name | Pure compound | Part |
|---|---|---|---|---|
| 1 | **Lauraceae** | | | |
| | *Cinnamomum porrectum* (Thep-tha-row) | hardy cinnamon | safrole | stem |
| 2 | **Zingiberaceae** | | | |
| | *Zingiber cassumunar* (Plai) | Bengal ginger, cassumunar ginger | terpinen-4-ol<br>sabinene | rhizome |

### 2.1.2 Microbial materials

Microbes including bacteria Burkholderia gladioli, and fungi Phytophthora palmivora and Fusarium oxysporum were purchased from plant protection research and development office, Department of agriculture, Bangkok, Thailand. B. gladioli was incubated in NA and NB while P. palmivora and F.oxysporum were on carrot agar and on PDA, respectively.

### 2.1.3 Orchid materials

Orchid tissue culture *Dendrobium* 'Pompadour' obtained from *in vitro* seed germination, was kindly sponsored from Associate Professor Dr. Kanchit Thammasiri, Department of plant science, Faculty of Science, Mahidol University. Chemicals and reagents for media that used in this study were analytical grade and molecular grade purchased from Fluka (Switzerland), Mallinckrodt (USA), Merck (Germany) and Sigma (USA).

## 2.2 Equipments and instruments

Laminar air flow cabinet

Laminar air flow cabinet used for preventing contamination of semiconductor wafers, biological samples, or any particle sensitive device. In this study, it was used for antimicrobial and pathogenic activities testing with orchid tissue culture.

Plant growth chamber

Plant growth chamber model MLR-350/T/H/HT was used to control suitable temperature and moisture for orchid tissue culture (no light or less than 100 foot candle until 2,000 foot candle, average of light intensity around 150 μw/cm$^2$ or 2,000-3,000 lux for 12-18 h per day and temperature around 22-29°C).

## 2.3 Methods

### 2.3.1 Plant extraction

All plant materials were milled into coarse powder and then soaked with appropriate solvent (s) as presented in Table 2.3. The extracts were filtered and evaporated under reduced pressure. In addition, *C. porrectum* (Thep-tha-row), *P. sarmentosum* (Cha-plu), *P. odoratum* (Preaw), *H. cordata* (Plu-kao), *L. aromatica* (Ka-yang), *O. caeruleus* (Blue hawaii) and *Z. cassumunar* (Plai) were hydrodistilled for essential oil.

**Table 2.3** The part and solvent or extraction of studied plants.

| Plant | Part | Solvent / Extraction |
|---|---|---|
| *Dracaena loureiri* (Chan-daeng) | heart wood | dichloromethane |
| *Eclipta prostrata* (Ka-meng) | whole | methanol |
| *Eupatorium odorata* (Sab-sua) | leave | - hexane<br>- dichloromethane<br>- methanol |
| | stem | hexane |

**Table 2.3** (continued).

| Plant | Part | Solvent / Extraction |
|---|---|---|
| *Oroxylum indicum* (Pae-ka) | bark | - dichloromethane<br>- methanol |
| | stem | methanol |
| *Garcinia mangostana* (Mung-kud) | bark | ethyl acetate |
| *Xylia xylocarpa* (Daeng) | heartwood | - dichloromethane<br>- ethyl acetate |
| *Cinnamomum porrectum* (Thep-tha-row) | stem | hydrodistillation |
| *Fagraea fragrans* (Kun-Krao) | flower | - dichloromethane<br>- methanol |
| | bark | - dichloromethane<br>- methanol |
| | stem | - dichloromethane<br>- methanol |
| *Piper sarmentosum* (Cha-plu) | leave | - dichloromethane<br>- methanol<br>- hydrodistillation |

**Table 2.3** (continued).

| Plant | Part | Solvent / Extraction |
|---|---|---|
| *Echinochloa colona* (Ya-khao-nok) | aerial | - hexane<br>- dichloromethane<br>- ethyl acetate<br>- methanol |
| *Polygonum odoratum* (Preaw) | whole | hydrodistillation |
| *Gardenia angusta* (Pud-soun) | flower | - dichloromethane<br>- methanol |
| | leave | - dichloromethane<br>- methanol |
| | branch | - dichloromethane<br>- methanol |
| | fruit | - dichloromethane<br>- methanol |
| *Gardenia jasminoides* (Pud) | flower | - dichloromethane<br>- methanol |
| *Zanthoxylum limonella* (Ma-khan) | fruit | - dichloromethane |
| *Houttuynia cordata* (Plu-kao) | aerial part | hydrodistillation |
| *Limnophila aromatica* (Ka-yang) | aerial part | hydrodistillation |

**Table 2.3** (continued).

| Plant | Part | Solvent / Extraction |
|---|---|---|
| *Otacanthus caeruleus* (Blue hawaii) | aerial part | hydrodistillation |
| *Amomum xanthioides* (Rew) | rhizome | - hexane<br>- dichloromethane<br>- methanol |
| *Zingiber cassumunar* (Plai) | rhizome | hydrodistillation |

### 2.3.2 Antimicrobial activity tests

Antibacterial activity test

*B. gladioli* was preliminary tested using disc diffusion method as following: prepare *B. gladioli* in NB at 37°C for 24 h. Use 100 µL of prepared NB with concentration of $10^5$-$10^7$ cells per mL or 0.5 McFarlane, mix with 9.9 mL of NA and pour plate. Place paper disc after agar was dried and drop 25 µL of 10,000 ppm plant extract per paper disc. DMSO was used as control in each plate. Measure clear zone (in mm) after 24 h. The experiment was done in triplicate and average the data. In case of pure compound, the concentration of 1 mM was prepared and then followed the assay.

Antifungal activity test

*P. palmivora* and *F. oxysporum* were preliminary tested using agar poison method as following: mix 100 µL of plant extract (100,000 ppm) in 9.9 mL of media to make the final concentration of plant extract as 1,000 ppm and cultured fungi. The records were collected after the full fungi growth on control media. The experiment was done in triplicate and average the data. In case of pure compound, 1 mM of sample was prepared and assayed.

The antifungal inhibited were calculated follow formula of antifungal inhibit as shown in Eq 2.1 (Suwanmanee et al., 2014).

$$\text{Percentage inhibition} = \frac{(DC - DE)}{DC} \times 100 \quad \% \qquad \textbf{(Eq 2.1)}$$

Where, DC = the diameter of the growth zone in the control plate (cm)

DE = the diameter of growth zone in experimental plate (cm)

### 2.3.3 Pathogenicity *in vitro* tests

The five month-orchid tissue cultures were on basal solid media including modified Vacin and Went medium (VW) (Vacin and Went 1949) contained with plant extract or pure compound. The condition was maintained at 25±2ºC under white fluorescent light at intensity of 37 µmol $m^{-2}$ $s^{-1}$ for 16 h per day (Sopalun *et al*., 2010).

The experiment was separated into 5 groups:

Group 1: The orchid was transferred into media contained agrimycin (positive control for bacteria) or aliette (positive control for fungi) and infected later with *B. gladioli*.

Group 2: The orchid was transferred into media contained distilled water or DMSO as the negative control and then infected with bacteria or fungi.

Group 3: The orchid was transferred into media and treated by oil or plant extract or pure compounds. The date were recorded seven day later.

Group 4: The orchid was transferred into media contained oil or plant extract or pure compounds. The orchid was infected with bacteria or fungi two days later and the data were recorded after four days of infection treatment. For fungal treatment after the orchid was infected with fungal the data were recorded seven days later.

Group 5: The orchid was transferred and infected by bacteria or fungal. The orchid was treated by oil or plant extract or pure compounds after two days. The data were recorded four days later for bacteria and seven days later for fungi.

The results were compared with positive and negative controls for its antimicrobial activities. For bacterial infection treatment the body part of orchid was wounded and soaked in bacteria suspension for 90 seconds while for fungal infection the mycerial of mature fungi was placed at orchid wounded part.

# CHAPTER III
# RESULTS AND DISCUSSIONS

Thailand produces and exports orchids more than 2,400 million baht annually. The exportation of orchids is increasing value in forms of bottle of tissue culture which is ready to cultivate, plantlets or cut flowers. Microorganisms are always technical barrier to trade the exportation. Based on Agricultural Research Development Agency (Public Organization) (ARDA), agricultural extension about technical matter and export promotion was important to consider to protect orchids from infected microorganisms. This study focuses on using Thai plant extracts as natural antimicrobial agents for orchids to increase the quality and lighten the cost of using pesticide.

## 3.1 Preliminary screening test

### 3.1.1 Preliminary screening of antibacterial activity

One of the aims of this study is to search for antibacterial agents for orchids that could inhibit soft rot from *Burkholderia gladioli*. From previous studies, the extracts from many Thai plants were reported to be of antibacterial activities. Therefore, in this study seventeen plant extracts were selected and tested for antibacterial activity by disk diffusion assay using the dose of 10,000 ppm (Barry and Thornsberry, 1999). The results are shown in Table 3.1.

**Table 3.1** Antibacterial activity against *B. gladioli* at 10,000 ppm from selected

| List | Species | Plant part | Extraction | Inhibition zone |
|---|---|---|---|---|
| 1 | **Agavaceae** *Dracaena loureiri* (Chan-daeng) | heartwood | $CH_2Cl_2$ | +++ |
| 2 | **Asteraceae** (Compositae) *Eclipta prostrata* (Ka-meng) | whole plant | MeOH | +++ |
| 3 | **Asteraceae** (Compositae) *Eupatorium odorata* (Sab-sua) | leave | Hexane | ++ |
| | | | $CH_2Cl_2$ | +++ |
| | | | MeOH | +++ |
| | | stem | Hexane | +++ |
| 4 | **Bignoniaceae** *Oroxylum indicum* (Pae-ka) | bark | $CH_2Cl_2$ | ++ |
| | | | MeOH | ++ |
| | | stem | MeOH | +++ |
| 5 | **Clusiaceae** *Garcinia mangostana* (Mung-kud) | bark | EtOAc | +++ |

plants.

**Table 3.1** (Continued).

| List | Species | Plant part | Extraction | Inhibition zone |
|---|---|---|---|---|
| 6 | **Fabaceae** *Xylia xylocarpa* (Daeng) | heartwood | $CH_2Cl_2$ | +++ |
| | | | EtOAc | + |
| 7 | **Lauraceae** *Cinnamomum porrectum* (Thep-tha-row) | stem | Hydrodistillation | ++++ |
| 8 | **Loganiaceae** *Fagraea fragrans* (Kun-Krao) | flower | $CH_2Cl_2$ | ++ |
| | | | MeOH | ++ |
| | | bark | $CH_2Cl_2$ | ++ |
| | | | MeOH | ++ |
| | | stem | $CH_2Cl_2$ | +++ |
| | | | MeOH | +++ |
| 9 | **Piperaceae** *Piper sarmentosum* (Cha-plu) | leave | $CH_2Cl_2$ | +++ |
| | | | MeOH | ++ |
| | | | Hydrodistillation | ++ |
| 10 | **Poaceae** (Gramineae) *Echinochloa colona* (Ya-khao-nok) | aerial | Hexane | ++ |
| | | | $CH_2Cl_2$ | +++ |
| | | | EtOAc | ++ |
| | | | MeOH | ++ |

**Table 3.1** (Continued).

| List | Species | Plant part | Extraction | Inhibition zone |
|---|---|---|---|---|
| | **Polygonaceae** | | | |
| 11 | *Polygonum odoratum* (Preaw) | whole plant | Hydrodistillation | ++ |
| 12 | **Rubiaceae** *Gardenia angusta* (Pud-soun) | flower | $CH_2Cl_2$ | +++ |
| | | | MeOH | +++ |
| | | leave | $CH_2Cl_2$ | +++ |
| | | | MeOH | +++ |
| | | branch | $CH_2Cl_2$ | +++ |
| | | | MeOH | +++ |
| | | fruit | $CH_2Cl_2$ | +++ |
| | | | MeOH | +++ |
| 13 | *Gardenia jasminoides* (Pud) | flower | $CH_2Cl_2$ | +++ |
| | | | MeOH | +++ |
| | **Rutaceae** | | | |
| 14 | *Zanthozylum limonella* (Ma-khan) | flower | $CH_2Cl_2$ | +++ |
| | **Saururaceae** | | | |
| 15 | *Houttuynia cordata* (Plu-kao) | whole plant | Hydrodistillation | +++ |

**Table 3.1** (Continued).

| List | Species | Plant part | Extraction | Inhibition zone |
|---|---|---|---|---|
| | **Scorphulariaceae** | | | |
| 16 | *Limnophila aromatica* (Ka-yang) | whole plant | Hydrodistillation | ++ |
| 17 | *Otacanthus coeruleus* (Blue Hawaii) | whole plant | Hydrodistillation | ++ |
| | **Zingiberaceae** | | Hexane | +++ |
| 18 | *Amomum xanthioides* (Rew) | rhizome | $CH_2Cl_2$ | ++++ |
| | | | MeOH | ++++ |
| | **Zingiberaceae** | | | |
| 19 | *Zingiber cassumunar* (Plai) | rhizome | Hydrodistillation | ++ |

Inhibition zone was measured and compared with control (DMSO). The data was categorized and noted as + (diameter 0.7 mm or less), ++ (diameter 0.8-1.0 mm), +++ (diameter 1.1-1.3 mm) and ++++ (diameter 1.4 mm or more).

Three best extracts displaying large clear zones against B. gladioli by disk diffusion method were a) the essential oil of C. porrectum, b) the crude extract of *A. xanthioides* and c) the CH2Cl2 extract of *X. xylocarpa* (Figure 3.1).

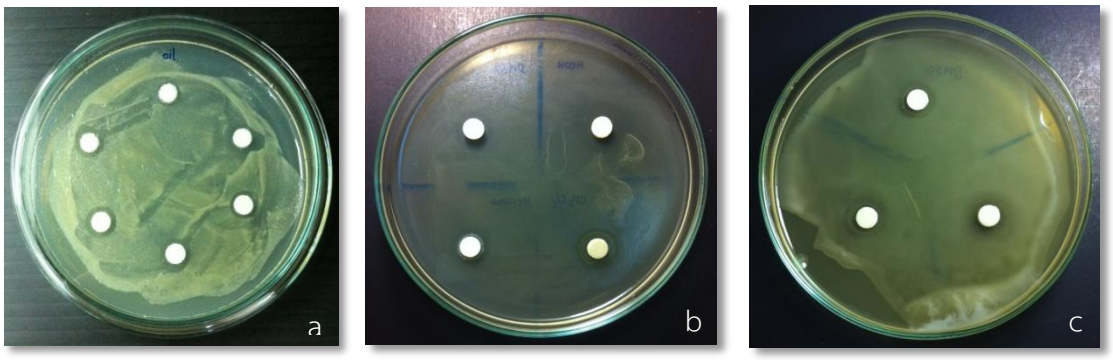



**Figure 3.1** The clear zone against *B. gladioli* of a) the essential oil of *C. porrectum*, b) the crude extract of *A. xanthioides* and c) the $CH_2Cl_2$ extract of *X. xylocarpa*.

*In vitro* anti-gram negative bacterial activity against *B. gladioli* of nineteen plant extracts (Table 3.1) demonstrated that the hydrodistillation of the stems of *C. porrectum* (Thep-tha-row) was the most potent. According to the reports of Phongpaichit et al. and Uthairatsamee et al., the essential oil from *C. porrectum* could inhibit many bacteria such as *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa* (Phongpaichit, S., et al., 2006), but no report against *B. gladioli*. Another positive results could be seen from the $CH_2Cl_2$ and MeOH extracts of the rhizomes of *Amomum xanthioides* Wall. (Rew). Natta et al., 2008. addressed that the essential oil of *A. xanthioides* could inhibit the growth of some gram positive and gram negative bacteria. The third is the $CH_2Cl_2$ extract from the heartwood of *X. xylocarpa* (Daeng). Sumthong and Verpoorte (2011) reported the result of chloroform-methanol extract from *X. xylocarpa* to inhibit gram negative bacteria. All data was analyzed the differences between group means and data associated procedures by using statistical model analysis of variance (ANOVA) at significant activities (<0.05).

### 3.1.2 Preliminary screening of antifungal activity

Many previous studies confirmed that Thai plants constituted of antifungal agents (Pithayanukul *et al.*, 2007, Suwanmanee *et al.*, 2013). Twelve plant samples were selected for antifungal screening to inhibit black rot from *Phytophthora palmivora* and wilt from *Fusarium oxysporum* using poisoned food technique (Mohana 2007) at 1,000 ppm. The results are shown in Table 3.2 and Figures 3.2-3.3.

**Table 3.2** Antifungal activity from screening plants.

| List | Species | Plant part | Extraction | %Inhibition | |
|---|---|---|---|---|---|
| | | | | *P. palmivora* | *F.oxysporum* |
| 1 | **Agavaceae** *Dracaena loureiri* (Chan-daeng) | heartwood | $CH_2Cl_2$ | 81.05 ± 0.32 | 15.17 ± 0.00 |
| 2 | **Asteraceae** (Compositae) *Eclipta prostrata* (Ka-meng) | whole plant | MeOH | 10.81 ± 1.62 | 0.00 ± 0.00 |
| 3 | **Clusiaceae** *Garcinia mangostana* (Mung-kud) | peel | EtOAc | 52.55 ± 0.25 | 0.00 ± 0.00 |
| 4 | **Lauraceae** *Cinnamomum porrectum* (Thep-tha-row) | stem | Hydrodistillation | 11.11 ± 0.92 | 41.44 ± 0.12 |

**Table 3.2** (Continued).

| List | Species | Plant part | Extraction | %Inhibition | |
|---|---|---|---|---|---|
| | | | | *Phytophthora palmivora* | *Fusarium oxysporum* |
| 5 | **Piperaceae** *Piper sarmentosum* (Cha-plu) | leave | $CH_2Cl_2$ | 73.00 ± 0.06 | 42.32 ± 0.06 |
| | | | MeOH | 4.44 ± 0.69 | 4.11 ± 0.12 |
| 6 | **Polygonaceae** *Polygonum odoratum* (Preaw) | whole plant | Hydrodistillation | 35.70 ± 1.00 | 0.00 ± 0.00 |
| 7 | **Rubiaceae** *Gardenia angusta* (Pud-soun) | flower | $CH_2Cl_2$ | 38.37 ± 0.42 | 40.54 ± 0.06 |
| | | | MeOH | 0.00 ± 0.00 | 5.36 ± 0.30 |
| | | leave | $CH_2Cl_2$ | 16.28 ± 0.85 | 11.96 ± 0.06 |
| | | | MeOH | 0.00 ± 0.00 | 2.32 ± 0.15 |
| | | branch | $CH_2Cl_2$ | 44.19 ± 0.28 | 38.75 ± 0.06 |
| | | | MeOH | 0.00 ± 0.00 | -0.54 ± 0.93 |
| | | fruit | $CH_2Cl_2$ | 14.77 ± 2.19 | 0.00 ± 0.00 |
| | | | MeOH | 0.00 ± 0.00 | 0.00 ± 0.00 |
| 8 | *Gardenia jasminoides* (Pud) | flower | $CH_2Cl_2$ | 0.00 ± 0.00 | 0.00 ± 0.00 |
| | | | MeOH | 0.00 ± 0.00 | 0.00 ± 0.00 |
| 9 | **Rutaceae** *Zanthozylum limonella* (Ma-khan) | flower | $CH_2Cl_2$ | 48.49 ± 1.24 | 0.00 ± 0.00 |

**Table 3.2** (Continued).

| List | Species | Plant part | Extraction | %Inhibition | |
|---|---|---|---|---|---|
| | | | | *Phytophthora palmivora* | *Fusarium oxysporum* |
| | **Saururaceae** | | | | |
| 10 | *Houttuynia cordata* (Plu-kao) | whole plant | Hydrodistillation | 41.17 ± 0.06 | 0.00 ± 0.00 |
| | **Scorphulariaceae** | | | | |
| 11 | *Limnophila aromatica* (Ka-yang) | whole plant | Hydrodistillation | 43.84 ± 0.45 | 0.00 ± 0.00 |
| 12 | *Otacanthus coeruleus* (Blue Hawaii) | whole plant | Hydrodistillation | 94.19 ± 0.87 | 41.86 ± 0.00 |
| | **Zingiberaceae** | | | | |
| 13 | *Zingiber cassumunar* (Plai) | Rhizome | Hydrodistillation | 100.00 ± 0.00 | 10.47 ± 0.00 |

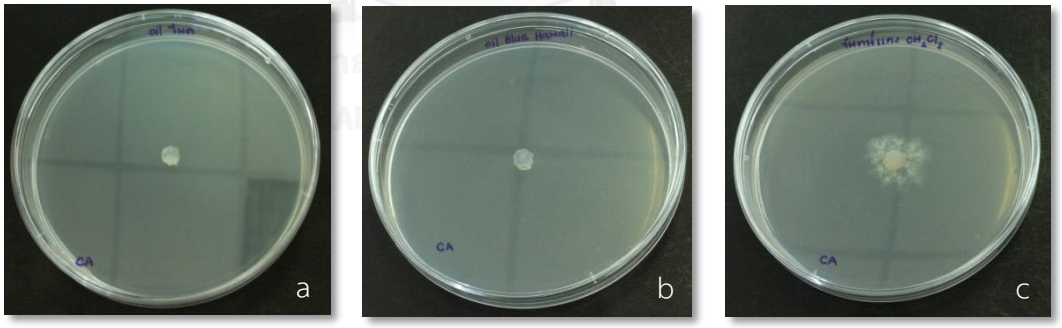



**Figure 3.2** *P. palmivora* mycelial growth on agar dilution method a) the essential oil of *Z. cassumunar* (Plai), b) the essential oil of *O. coeruleus* (Blue Hawaii) and c) the $CH_2Cl_2$ extract of *D. loureiri* (Chan-daeng)

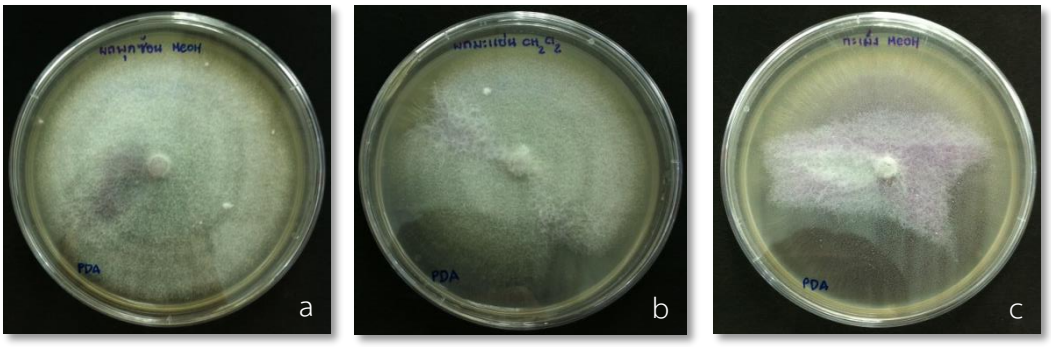



**Figure 3.3** *F. oxysporum* mycelial growth on agar dilution method: a) the MeOH extract of *G. angusta* (Pud-soun), b) the $CH_2Cl_2$ extract of *Z. limonella* (Ma-khan) and c) the MeOH extract of *E. prostrata* (Ka-meng).

The strongest inhibition was found from the essential oil of *Z. cassumunar* (Plai) with complete growth inhibition of *P. palmivora*. The second was the essential oil of *O. coeruleus* (Blue Hawaii) and the $CH_2Cl_2$ extracts from *D. loureiri* (Chan-daeng), respectively. On the other hand, no twelve selected plant extracts could inhibit the growth of *F. oxysporum* more than 50%.

### 3.2 Extraction and isolation of *Echinochloa colona* (L.) Link

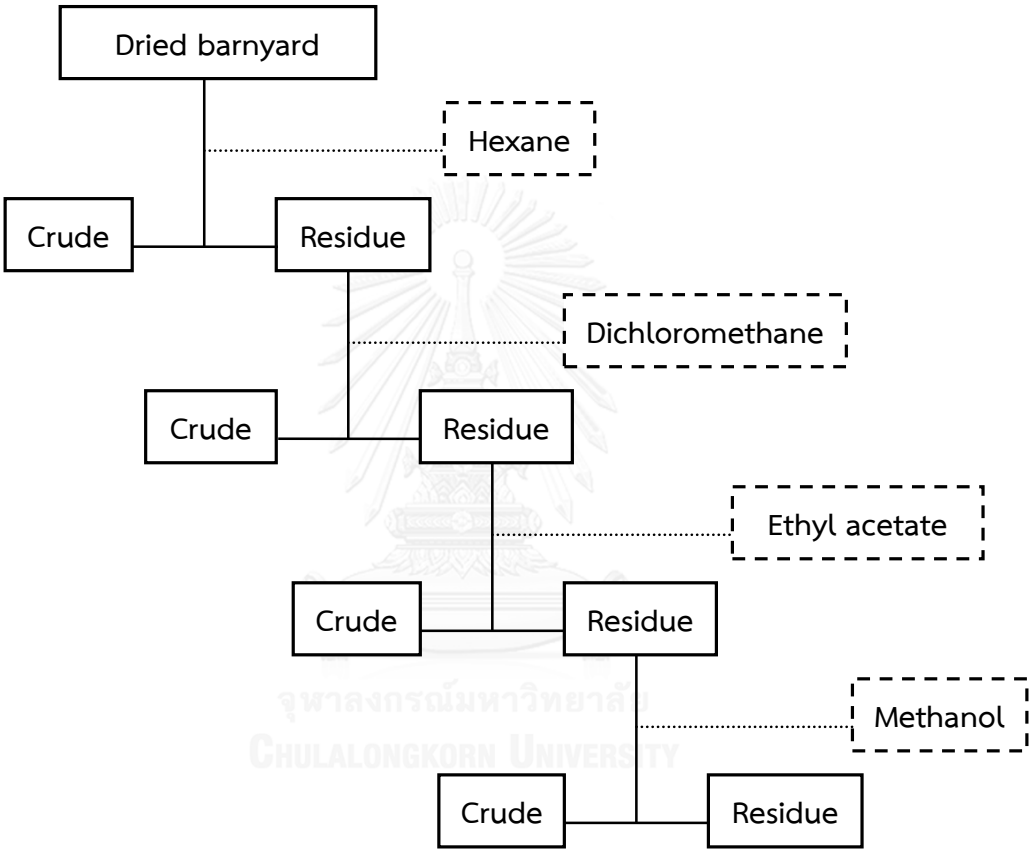
*Echinochloa colona* (L.) Link or barnyard grass belonging to Poaceae (Gramineae) (Figure 3.4) is wild grass in tropical Asia. The collected plants were confirmed by Assist. Prof. Paweena Traiperm, Ph.D (biological science), department of plant science, faculty of science, Mahidol University.

Almost of previous studies on *E. colona* was the use of this plant as a basic weed to study how to eliminate by various treatments. There was no study concerning the antimicrobial activity of this plant. In this study, barnyard grass was extracted by hexane, $CH_2Cl_2$, EtOAc and MeOH and tested against *B. gladioli* and *P. palmivora*.

**Figure 3.4** *Echinochloa colona* from Pak Chong district, Nakhon Ratchasima Province.

### 3.2.1 Extraction

The aerial part of *E. colona* was dried, milled and extracted for three times with hexane, $CH_2Cl_2$, EtOAc and MeOH (Scheme 3.1). The extracts were filled and then evaporated under reduced pressure by rotatory evaporator. The extracts were subjected to antibacterial and antifungal tests.




**Scheme 3.1** The extraction of *E. colona.*

### 3.2.2 Preliminary screening tests

Antibacterial activity of all extracts from *E. colona* was tested against *B. gladioli*, gram negative bacteria of orchid pathogen. The agar disk diffusion method was used for antibacterial activity of *E. colona* extracts. Disk paper were placed equate with sample and drop 25 ml of *E. colona* extracts and DMSO (control) per each. The results of antibacterial activity of all extracts against *B. gladioli* are shown in Figure 3.5.

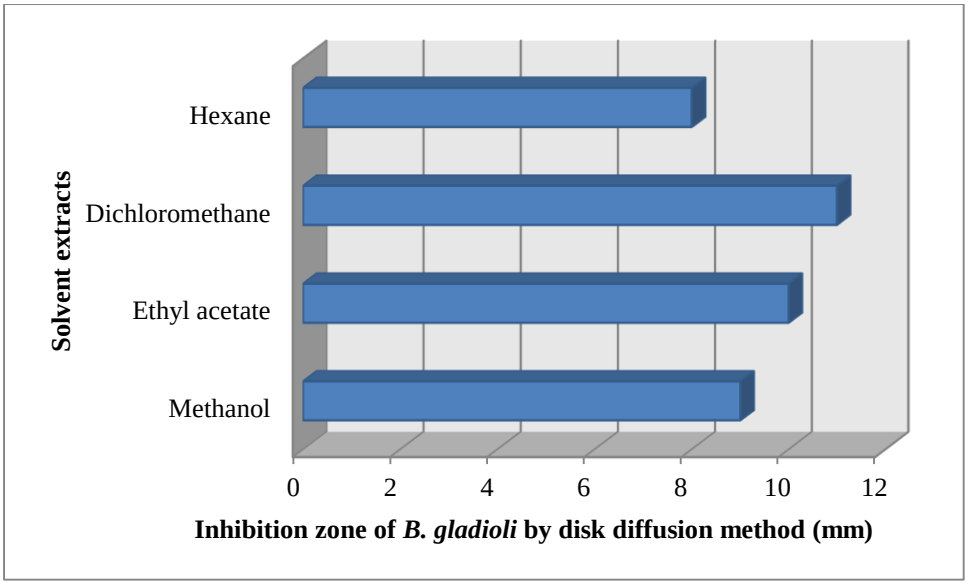





**Figure 3.5** Antibacterial activity of *Echinochloa colona* extracts against *Burkholderia gladioli*.

The antifungal activity of E. colona extracts was evaluated against orchid fungal pathogen; black rot from P. palmivora. Dissolved 100 µL of the extracts or DMSO in PDA to execute a final concentration of 1,000 mg/L. 1% DMSO was used as negative control. The results of antifungal activity from E. colona extracts against P. palmivora are presented in Figure 3.6.

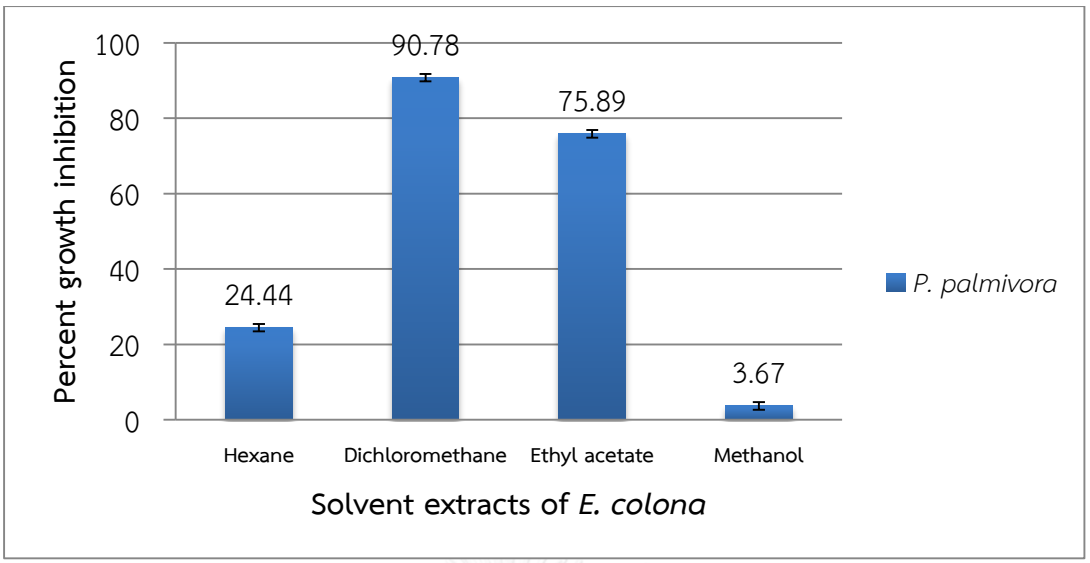



**Figure 3.6** Antifungal activity of *Echinochloa colona* extracts against *Phytophthora palmivora*.

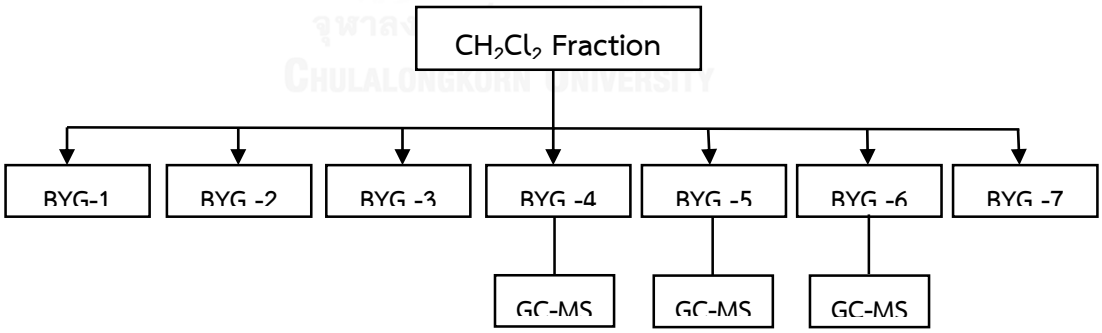
### 3.2.3 Isolation of the $CH_2Cl_2$ extract from *Echinochloa colona*

The $CH_2Cl_2$ extract revealed high inhibitory activity compared with other extracts. This extract was thus separated by column chromatography eluting with increasing solvent polarity of hexane, hexane-EtOAc, EtOAc, EtOAc-MeOH and MeOH. Each fraction was monitored by TLC and combine to seven fractions according to the TLC pattern (Table 3.3 and Scheme 3.2).

**Table 3.3** The separation of the $CH_2Cl_2$ extract of *E. colona*

| Fraction | Elution system | Weight (gram) | Physical |
|---|---|---|---|
| BYG-1 | 100% Hexane - 10% EtOAc | 1.08 | pink-orange powder |
| BYG-2 | 10% EtOAc - 15% EtOAc | 0.23 | pink powder |
| BYG-3 | 15% EtOAc - 20% EtOAc | 0.97 | light yellow powder |
| BYG-4 | 40% EtOAc | 0.82 | yellow powder |
| BYG-5 | 60% EtOAc | 1.03 | yellow-brown viscosity |
| BYG-6 | 60% EtOAc - 5% MeOH | 1.14 | dark yellow powder |
| BYG-7 | 20% MeOH | 1.02 | dark brown viscosity |

Among seven fractions, fractions BYG-6 and -1 gave the highest yield as 1.14 and 1.08 grams, respectively. From TLC result of all fraction revealed that BYG-4, -5 and -6 has interested spots so the three fraction were continued isolated by GC-MS.




**Scheme 3.2** The separation of the $CH_2Cl_2$ extract of *E. colona*.

### 3.2.4 Gas Chromatography - Mass Spectrometry (GC-MS) of *Echinochloa colona* (L.) Link

Gas chromatography–mass spectrometry (GC-MS) is an analytical method that combines the features of gas-liquid chromatography and mass spectrometry to identify different substances within a test sample. Applications of GC-MS include drug detection, fire investigation, environmental analysis, explosives investigation, and identification of unknown samples.

GC analysis of BYG-4 was performed in Figure 3.7. BYG-4 displayed peak at $R_t$ 14.03 min, area% 28.44. It could be observed that the peak at $R_t$ 14.03 min was Undecanoic acid ($C_{11}H_{22}O_2$). BYG-5 and -6 the data not match with any compound.

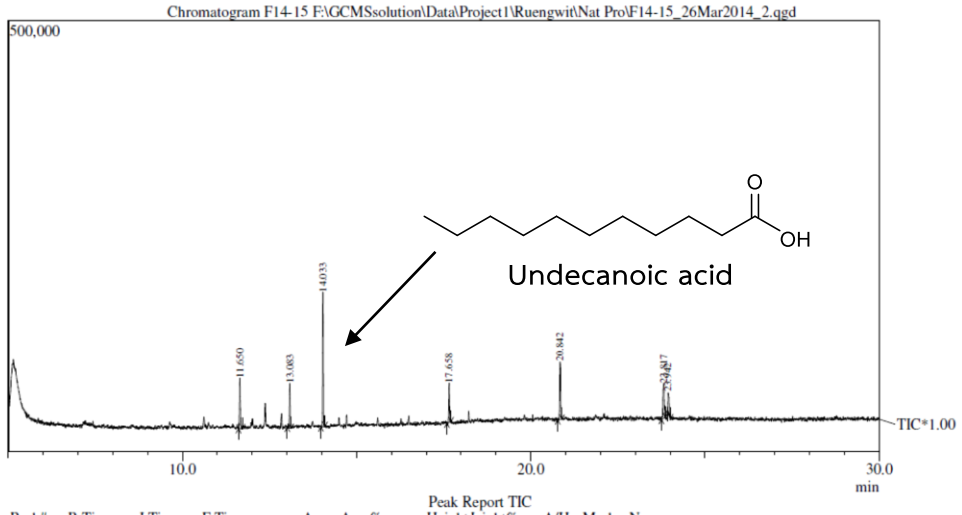



**Figure 3.7** GC-MS analysis $CH_2Cl_2$ extract of *E. colona* fraction 4 (BYG-4).

Peak Report TIC

| Peak# | R.Time | LTime | F.Time | Area | Area% | Height | Height% | A/H | Mark | Name |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 11.650 | 11.608 | 11.725 | 108918 | 14.08 | 58583 | 13.29 | 1.86 | MI | |
| 2 | 13.083 | 13.008 | 13.125 | 80847 | 10.45 | 51421 | 11.66 | 1.57 | MI | |
| 3 | 14.033 | 13.975 | 14.092 | 219937 | 28.44 | 156111 | 35.40 | 1.41 | MI | |
| 4 | 17.658 | 17.592 | 17.708 | 68608 | 8.87 | 45507 | 10.32 | 1.51 | MI | |
| 5 | 20.842 | 20.775 | 20.892 | 141393 | 18.28 | 66817 | 15.15 | 2.12 | MI | |
| 6 | 23.817 | 23.750 | 23.867 | 105191 | 13.60 | 40421 | 9.17 | 2.60 | MI | |
| 7 | 23.942 | 23.925 | 24.017 | 48513 | 6.27 | 22109 | 5.01 | 2.19 | MI | |
| | | | | 773406 | 100.00 | 440969 | 100.00 | | | |

### 3.3 Extraction of *Garcinia mangostana* Linn.

*Garcinia mangostana* Linn. or known simply as mangosteen belongs to the family of Clusiaceae or Guttiferae (Figure 3.8). Mangosteen is a tropical evergreen tree, grows mainly in Southeast Asia, and also in tropical South American countries. *G. mangostana* is named ‘‘the queen of fruits”. Many reports concerning the activity of mangosteen were addressed such as antioxidant, anti-inflammatory, antiallergy, antibacterial, antifungal and antiviral properties (José Pedraza-Chaverri et al., 2008). Nevertheless, there was no study about antimicrobial activity in orchid (against *B. gladioli*, *P. palmivora* and *F. oxysporum*). This study was thus focused on the extraction of mangosteen, the separation and isolation of active constituents against orchid pathogens.

**Figure 3.8** *Garcinia mangostana* Linn.

Source : cocktailprofessor.com.

### 3.3.1 Extraction

The pericarps of *G. mangostana* were dried, milled and extracted three times with EtOAc. The extracts were filtered and then evaporated under reduced pressure by rotatory evaporator. The crude extract was preliminarily tested for antibacterial and antifungal activities.

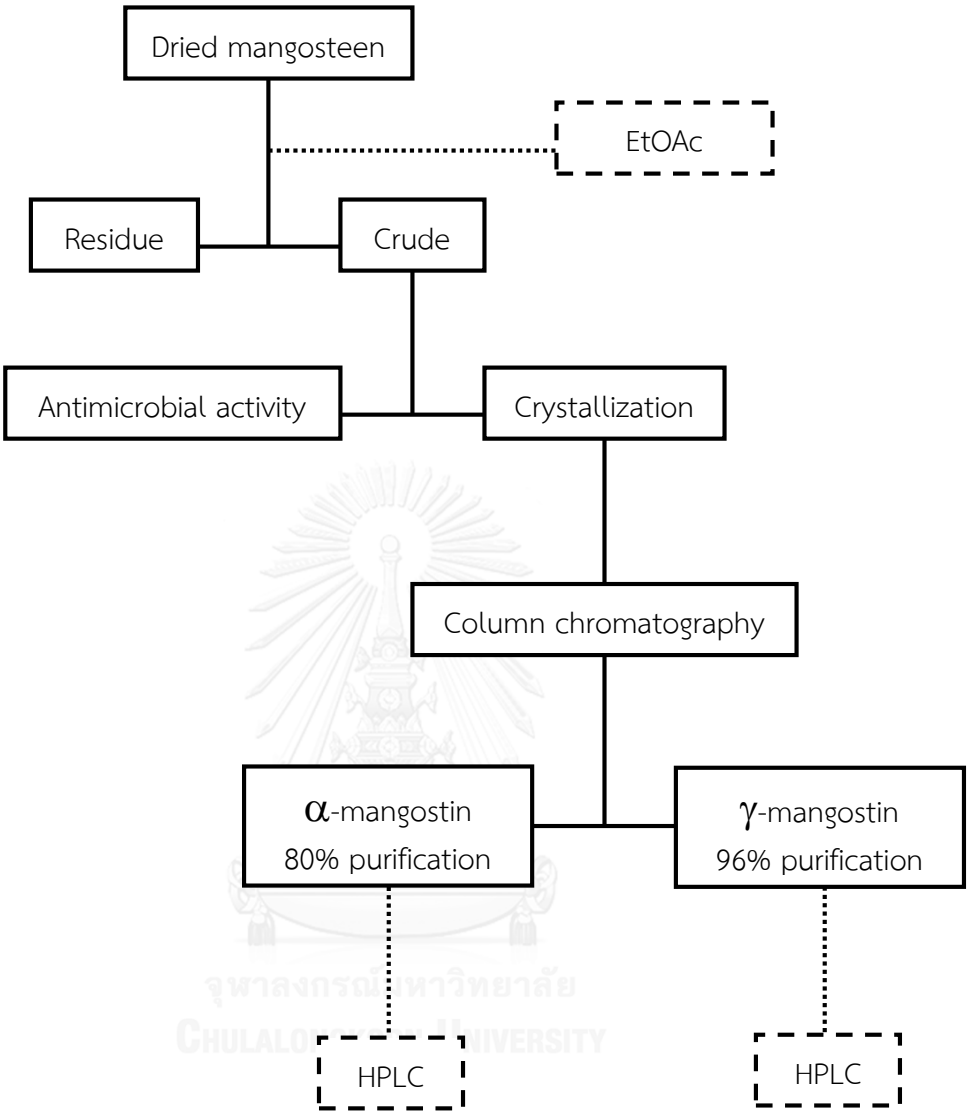
### 3.3.2 Preliminary screening tests

Antibacterial activity of the EtOAc extract of *G. mangostana* was conducted against *B. gladioli* using agar disk diffusion method. The result as displayed in Table 3.1 (entry 5) revealed that the EtOAc extract could inhibit *B. gladioli* with good activity (+++).

The antifungal activity against two fungi: black rot from *P. palmivora* and wilt from *F. oxysporum* was conducted using agar poison method on suitable media. The EtOAc extract inhibited *P. palmivora* more than 50%, but could not inhibit *F. oxysporum* (Table 3.2, entry 3).

### 3.3.3 Separation of the EtOAc extract of *G. mangostana*

The pericarps of mangosteen were dried by hot air oven and then ground into small size. Dried ground pericarps were macerated with EtOAc for 5 days. The EtOAc extract was filtered and concentrated by rotatory evaporator until dry. Once it dried, the yellow precipitate was formed which was then filtered and washed with 20%EtOAc in hexane, followed by hexane. Finally, faint yellow powder was formed. Silica gel column was used to separate $\alpha$- and $\gamma$-mangostins with increasing polarity of eluents from 100%hexane to 100%EtOAc. A reverse-phase high performance liquid chromatography (RP-HPLC) was carried out on a Hypersil BDS C-18 column (4.6 x 250 mm, 5 $\mu$m) at RT using a gradient mobile phase consisting of 70-80% $CH_3CN$ in 0.1% v/v *o*-phosphoric acid at the flow rate of 1 mL $min^{-1}$ with 320 nm UV detection. The separation of the EtOAc extract is summarized in Scheme 3.3.




**Scheme 3.3** The separation of the EtOAc extract of *G. mangostana.*

### 3.3.4 Antimicrobial activity of isolated compounds

The antibacterial activity of α- and γ-mangostins was conducted against *B. gladioli*. The agar disk diffusion method was used with the pure compounds isolated. Disk paper was placed equating with sample and drop 25 mL of α- and γ-mangostins with the concentration of 1 mM and DMSO (control). The results of antibacterial activity of isolated mangostins are shown in Table 3.4.

**Table 3.4** The antibacterial activity of isolated mangostins from *G. mangostana*.

| **Compound (1,000 ppm)** | **Clear zone (cm) (*B. gladioli*)** |
|---|---|
| α -mangostin | 0.9 ± 0.00 |
| γ-mangostin | 1.0 ± 0.08 |

Two isolated compounds from *G. mangostana* with the concentration of 1,000 ppm exhibited the inhibition zone approximately 1 cm (Table 3.4).

The antifungal activity of α – and γ-mangostins was tested against *P. palmivora* and *F. oxysporum* at 1,000 ppm final concentration on agar poison method. *P. palmivora* was cultured on carrot agar while *F. oxysporum* was cultured on PDA. The results are presented in Table 3.5.

**Table 3.5** The antifungal activity of isolated mangostins from *G. mangostana*.

| Compound (1,000 ppm) | %Inhibition | |
|---|---|---|
| | *P. palmivora* | *F. oxysporum* |
| α-mangostin | 62.35 ± 0.23 | 60.00 ± 0.10 |
| γ-mangostin | 100.00 ± 0.00 | 56.47 ± 0.36 |

α-Mangostin could inhibit *P. palmivora* 62.4% and *F. oxysporum* 60%, while γ-mangostin completely inhibited the mycelia growth of *P. palmivora* 100%, but inhibited the mycelia growth of *F. oxysporum* 56%.

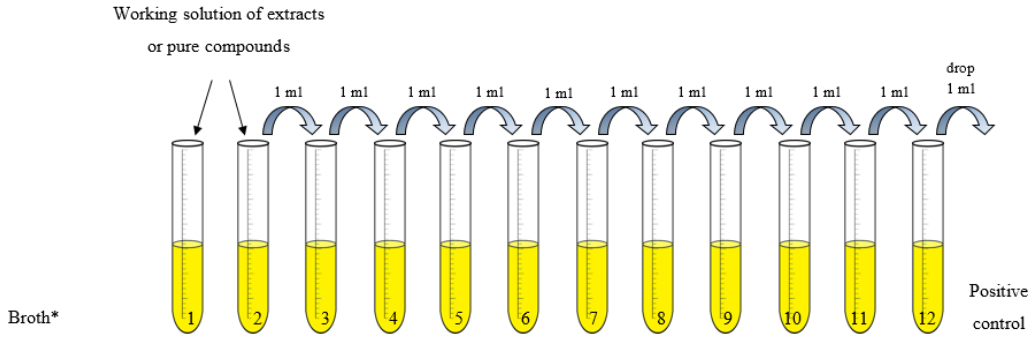
γ-mangostin from *G. mangostana* exhibited the highest activity than α-mangostin towards *B. gladioli* and *P. palmivora*. This compound could inhibit the disease caused of soft rot in orchid for 100%. On the other hand, α– and γ-mangostins could not inhibit the mycelia growth of *F. oxysporum* more than 80%.

### 3.4 The antimicrobial activity of isolated and selected compounds

The preliminary of antimicrobial activity from five pure compounds, antibacterial activity from nineteen plant extracts, and antifungal activity from thirteen plant extracts could selecte the high inhibition that is oil from *C. porrectum* (Thep-tha-row), γ – mangostin (Mung-kud) from *G. mangostana*, $CH_2Cl_2$ extract from *E. colona* (Ya-khao-nok), and $CH_2Cl_2$ extract from *X. xylocarpa* (Daeng) for antibacterial activity. For antifungal activity for *P. palmivora* that is oil from *O. coeruleus* (Blue hawaii), $CH_2Cl_2$, EtOAc from *E. colona* (Ya-khao-nok), oil from *Z. cassumunar* (Plai) and $CH_2Cl_2$ extract from *D. loureiri* (Chan-daeng). For antifungal activity against *F. oxysporum* there were no plant extracts or pure compounds could inhibit more than fifty percent therefore this fungi will not be tested for antifungal activity on orchid tissue culture.

Minimum inhibitory concentration (MIC) is method to selected plant extracts or pure compounds that revealed the concerning clear zone or inhibit percentage to test antimicrobial activity on orchid tissue culture. MIC is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after suitable incubation for each microbial. MIC could confirmed the resistance of microorganisms to an antimicrobial agent.

MIC of an antibiotic is determined by using the following procedure (Scheme 3.4); preparation of microorganism for bacteria using few colony to culture and incubated before tested MIC. Preparation nutrient broth (NB) for bacteria 12 test tubes contain NB 1 ml per each. Drop plant extracts or pure compound 1 ml into test tube No.1 and No.2. Mixed substance with NB in test tube No.2 and pulled in the mixture 1 ml into test tube No.3, keep following this dilution until test tube No.12 and discarded the 1 ml from test tube No.12. The bacteria concentration of colony forming unit (CFU) using 105 - 107 cell/ml or 0.5 McFarland standard each dilution were pipetted with 1 ml into every test tube. The tubes were incubated at 37$^{o}$C for 24 h. The least concentration of the substance showing no visible growth was taken as the MIC. After 24h of incubation period, mean MIC values were calculated. The test was performed in triplicates. For fungi, transferred 5 mm (by cork borer) the mature mycerial from carrot agar into carrot broth.




| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Concentration** | X | X/2 | X/4 | X/8 | X/16 | X/32 | X/64 | X/128 | X/256 | X/512 | X/1024 | X/0 |
| Microorganism*** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |

Note;
* is Broth depend on microorganism, Unit = ml
** is X for plant extract = 100 mg/ml, X for pure compound = 1 mM/ml
*** is Bacteria; Concentration of colony forming unit (CFU) = $10^5$ - $10^7$ cell/ml, Unit = ml, Fungi; 5 mm agar of mycelial

**Scheme 3.4** Minimum inhibitory concentration (MIC) method

### 3.5 Antimicrobial activity test on orchid tissue culture

Both pathogens (*P. gladioli* and *P. palmivora*) were tested on orchid tissue culture using along plantlet to cultivate approximately 5 months. The orchid used in this experiment was *Dendrobium* 'Pompadour' which was propagated from seed germination by tissue culture on modified Vacin and Went (VW) (1949) media (Figure 3.9). The MIC of plant extracts was used in mg/mL and pure compounds were in mM. List of treatments for antimicrobial activity test were collected in Tables 3.6-3.7.

**Figure 3.9** Dendrobium orchid plantlets on modified VW medium.

**Table 3.6** List of treatments for antibacterial activity test with orchid tissue culture.

| Treatment | Clear zone (cm) | MIC |
|---|---|---|
| | *B. gladioli* | |
| Agrimycin<br>(Positive control for bacteria) | 3.6 ± 0.32 | 6.25 mg/mL |
| *C. porrectum* (Thep-tha-row)<br>- Oil | 1.5 ± 0.05 | 6.25 mg/mL |
| *E. colona* (Ya-khao-nok)<br>- Dichloromethane | 1.1 ± 0.22 | 3.12 mg/mL |
| *G. mangostana* (Mung-kud)<br>- γ-mangostin (1 mM) | 1.0 ± 0.06 | 0.125 mM |

**Table 3.7** List of treatments for antifungal activity tested with orchid tissue culture.

| Treatment | % inhibition | MIC |
|---|---|---|
| | *P.palmivora* | |
| Aliette<br>(Positive control for fungi) | 47.06 ± 0.64 | 6.25 mg/mL |
| *Z. cassumunar* (plai)<br>- Oil | 100.00 ± 0.00 | 0.5 mg/mL |
| *O. caeruleus* (blue Hawaii)<br>- Oil | 94.19 ± 0.87 | 2.0 mg/mL |
| *G. mangostana* (mung-kud)<br>- γ-mangostin (1 mM) | 100.00 ± 0.00 | 0.0625 mM |

### 3.5.1 Antibacterial activity test on orchid tissue culture

The preparation of bacterial suspension in distilled water was carried out using a few colonies to culture and incubated on NA for 48 h. Adjust optical density (OD) of bacteria at $A_{600}$ by spectrophotometer or comparison with 0.5 McFarlane, the containing bacterial suspension $10^8$ colony forming units (CFU) per mL ($1 \times 10^8$ CFU/ $mL^{-1}$) was tested in orchid tissue culture as pathogen by slashed on leaves of orchid and soaked in bacteria suspension for 90 sec. Observe and record the data every 24 h for 4 days (modified from Kaewcheed S., 2002)

The experiment was divided into five types, *i.e.*,

i) Normal orchid + infect (positive control)

ii) Normal orchid + distill water (negative control)

iii) Normal orchid + treatment

iv) Normal orchid + treatment --> 2 days --> infect --> 4 days --> record

v) Normal orchid + infect --> 2 days --> treatment --> 4 days --> record

Index: Signs of soft rot (*P. gladioli*)

0 No symptom

+1 Small water-soaked or yellow spots appear on the leaves

+2 Pale brown tissue

+3 Burned wound

+4 Sunken and leave fell

**The experiment i:** normal orchid + infect

Normal orchids were slashed on leaves and soaked in bacterial suspension for 90 sec, after 2 days the yellow spot appeared on the leaves (Figure 3.10). This condition was used as a positive control.

**Figure 3.10** Orchid plantlets after being infected with bacterial suspension.

**The experiment ii:** normal orchid + distilled water

The negative control was cultured with distilled water at the first day until finishing the record of every condition (Figure 3.11).

**Figure 3.11** Orchid plantlets with distilled water.

**The experiment iii**: normal orchid + treatment

The normal orchids were cultured for 7 days. Several treatments were examined involving DMSO (Figure 3.12), agrimycin (Figure 3.13), *C. porrectum* oil (Figure 3.14), γ- mangostin 0.125 mM (Figure 3.15) and the $CH_2Cl_2$ extract of *E. colona* (Figure 3.16).

**Figure 3.12** (Left) An orchid plantlet before culture with DMSO, (Right) after 7-day culture with DMSO.

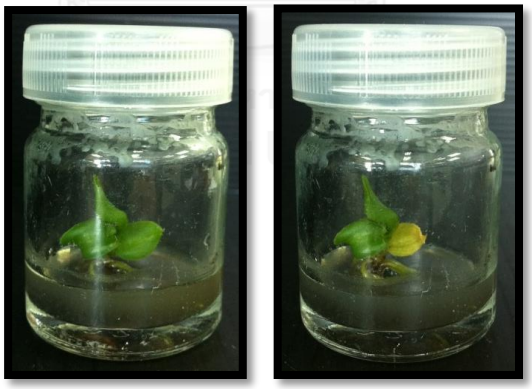


**Figure 3.13** (Left) An orchid plantlet before culture with agrimycin, (Right) after 7-day culture with agrimycin.

**Figure 3.14** (Left) An orchid plantlet before culture with *C. porrectum* oil, (Right) after 7-day culture with *C. porrectum* oil.

**Figure 3.15** (Left) An orchid plantlet before culture with γ-mangostin 0.125 mM, (Right) after 7-day culture with γ-mangostin 0.125 mM.

**Figure 3.16** (Left) An orchid plantlet before culture with the $CH_2Cl_2$ extract of *E. colona*, (Right) after 7-day culture with the $CH_2Cl_2$ extract of *E. colona*.

From the experiment (iii), the oil from *C. porrectum* turned all leaves to yellow and brown. Using DMSO, agrimycin and the $CH_2Cl_2$ extract of *E. colona*, some leaves turned yellow; however, for γ at 0.125 mM the color of the leaves still kept the same. The result of this condition revealed that the treatment has effective with orchids.

**The experiment iv:** normal orchid + treatment --> 2 days --> infect --> 4 days --> record

The normal orchids were cultured on treatment for 2 days before being infected. After 4 days, the results were recorded. All treatments were agrimycin (Figure 3.17), *C. porrectum* oil (Figure 3.18), γ-mangostin 0.125 mM (Figure 3.19) and the $CH_2Cl_2$ extract of *E. colona* (Figure 3.20).

**Figure 3.17** (Left) 2 days orchid with treatment, (Right) pre-Agrimycin orchid was infected.

**Figure 3.18** (Left) 2-day orchid with treatment, (Right) pre-*C. porrectum* oil orchid was infected.

**Figure 3.19** (Left) 2-day orchid plantlet with treatment, (Right) pre-γ-mangostin orchid was infected.



**Figure 3.20** (Left) 2-day orchid plantlet with treatment (Right) pre-E. colona CH2Cl2 orchid was infected.

The results of experiment (iv) with *B. gladioli* revealed the difference of the orchid plantlets before and after treatment. Only agrimycin could keep orchids as previously; whereas, the treatments with the oil of *C. porrectum*, γ-mangostin and the $CH_2Cl_2$ extract of *E. colona* made the green leaf of orchid turned brown.

**The experiment v:** normal orchid + infect --> 2 days --> treatment --> 4 days --> record

2 Days after the orchid plantlets were infected with *B. gladioli*, the infected orchid plantlets were cultured by mixing media for 4 days and recorded. The treatments are agrimycin (Figure 3.21), *C. porrectum* oil (Figure 3.22), γ–mangostin 0.125 mM (Figure 3.23) and the $CH_2Cl_2$ extract of *E. colona* (Figure 3.24).

**Figure 3.21** (Left) infected orchid plantlet, (Right) infected orchid plantlet with agrimycin after 4 days.

**Figure 3.22** (Left) infected orchid plantlet, (Right) infected orchid plantlet with *C. porrectum* oil after 4 days.

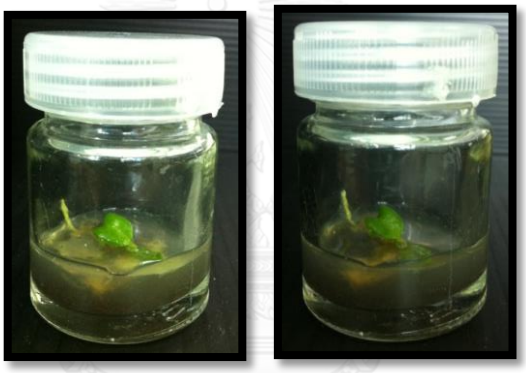


**Figure 3.23** (Left) infected orchid plantlet, (Right) infected orchid plantlet with γ-mangostin after 4 days.

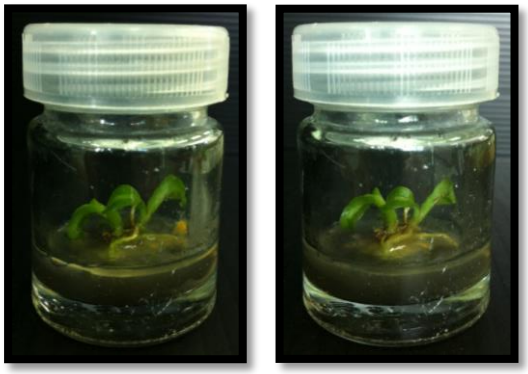


**Figure 3.24** (Left) infected orchid plantlet, (Right) infected orchid plantlet with *E. colona* $CH_2Cl_2$ after 4 days.

The results of experimental (v) revealed that the treatment with agrimycin turned some leaves to yellow and brown. For the oil of *C. porrectum*, some brown leaves were observed, while the treatment of orchids with γ–mangostin 0.125 mM and the $CH_2Cl_2$ extract of *E. colona* the leaves of orchid still existed green.

The normal orchids that cultured on mix media displayed one treatment that kept orchids still the same is γ-mangostin while another treatment turned the green leaves to yellow or brown. Under condition pre-infected, agrimycin has few effective to orchids but other treatment changed the colour to brown. The treated of infected orchid revealed only γ-mangostin and $CH_2Cl_2$ extract of *E. colona* shown the healthy orchids.

### 3.5.2 Antifungal activity on orchid tissue culture

Preparation of mycelia of growth fungal on $V_8$ or carrot agar to mycelia suspension. Scraped the mycelia and added distilled water, placed the mycelia on the wound of orchid tissue culture leaves. Observe and record the data every 24 h for 7 days.

The experiment was divided into five types that is

i) Normal orchid + infect (positive control)

ii) Normal orchid + distill water (negative control)

iii) Normal orchid + treatment

iv) Normal orchid + treatment --> 2 days --> infect --> 7 days --> record

v) Normal orchid + infect --> 2 days --> treatment --> 7 days --> record

Index : Signs of black rot (*P. pamivora*)

| | |
|---|---|
| 0 | No symptom |
| +1 | Small water-soaked spots appear on the leaves |
| +2 | Light brown or black small water-soaked spots |
| +3 | Black sunken |
| +4 | White mycelium |

**The experiment i:** normal orchid + infect

Normal orchids were slashed on leave and placed the mycelium of *P. palmivora* on wound after 7 days. The leaves of orchid changed the color from green to brown, and found the white mycelium on wound (Figure 3.25). This condition used for positive control.

**Figure 3.25** An orchid plantlets 7 days after being infected with myceliam.

**The experiment ii:** normal orchid + distill water

The negative control (Figure 3.26) was cultured with distilled water at the first day until finish the record of every condition.

**Figure 3.26** An orchid plantlet with distilled water.

**The experiment iii:** normal orchid + treatment

The normal orchid plantlet was cultured with every treatment for 7 days including DMSO (Figure 3.27), aliette (Figure 3.28), γ-mangostin 0.0625 mM (Figure 3.29), the essential oil of *Z. cassumunar* (Figure 3.30) and *O. caeruleus* (Figure 3.31).

**Figure 3.27** (Left) An orchid plantlet before culture with DMSO

(Right) orchid plantlet 7 days after being cultured with DMSO.

**Figure 3.28** (Left) An orchid plantlet before culture with aliette,

(Right) Orchid plantlet 7 days after being cultured with aliette.

**Figure 3.29** (Left) An orchid plantlet before culture with γ-mangostin 0.0625 mM,

(Right) Orchid plantlet 7 days after being cultured with γ-mangostin 0.0625 mM.

**Figure 3.30** (Left) An orchid plantlet before culture with *Z. cassumunar* oil,

(Right) Orchid plantlet 7 days after being cultured with *Z. cassumunar* oil.

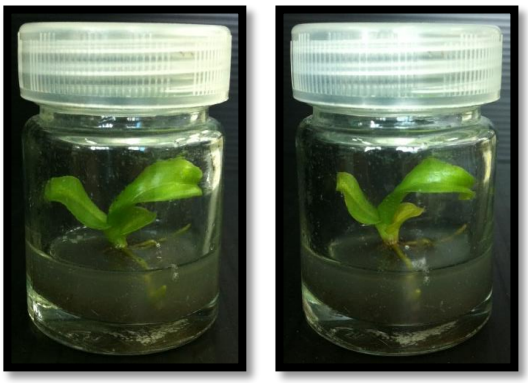


**Figure 3.31** (Left) An orchid plantlet before culture with *O. caeruleus* oil, (Right) Orchid plantlet 7 days after being cultured with *O. caeruleus* oil.

The results of experiment iii revealed that DMSO, aliette and *O. caeruleus* oil turned some leaves from green to brown, but orchids cultured with γ-mangostin and *Z. cassumunar* oil still provided the same characteristics as normal orchid.

**The experiment iv:** normal orchid + treatment --> 2 days --> infect --> 7 days --> record

The normal orchids were cultured on treatments for 2 days before being infected. After being infected for 7 days, the results were recorded for aliette (Figure 3.32), γ-mangostin 0.0625 mM (Figure 3.33), the essential oil of *Z. cassumunar* (Figure 3.34) and *O. caeruleus* (Figure 3.35).

**Figure 3.32** (Left) 2 days orchid with treatment (Right) pre-aliette orchid was infected.

**Figure 3.33** (Left) 2 days orchid with treatment, (Right) pre-γ-mangostin orchid was infected.

**Figure 3.34** (Left) 2 days orchid with treatment (Right) Pre-*Z. cassumunar* oil orchid was infected.

**Figure 3.35** (Left) 2 days orchid with treatment (Right) pre-*O. caeruleus* oil orchid was infected.

The results of experiment iv with fungal showed the different between orchid before and after treatment. Every treatment made green leaves of orchids turned to brown.

**The experiment v:** normal orchid + infect --> 2 days --> treatment --> 7 days --> record

2 days after infected orchids with *P. palmivora*, the infected orchids were cultured by mixing media with aliette (Figure 3.36), γ-mangostin 0.0625 mM (Figure 3.37), the essential oil of *Z. cassumunar* (Figure 3.38) and *O. caeruleus* (Figure 3.39) for 7 days and recorded the results.

**Figure 3.36** (Left) An infected orchid plantlet, (Right) infected orchid plantlet with aliette after 7 days.

**Figure 3.37** (Left) An infected orchid plantlet, (Right) infected orchid plantlet with γ-mangostin 0.0625 mM after 7 days.

**Figure 3.38** (Left) An infected orchid plantlet, (Right) infected orchid plantlet with *Z. cassumunar* oil after 7 days.

**Figure 3.39** (Left) An infected orchid plantlet, (Right) infected orchid plantlet with *O. caeruleus* oil after 7 days.

The experiment v displayed that aliette, γ-mangostin 0.0625 mM and *Z.* cassumunar oil changed some orchid leaves from green to brown, while the O. caeruleus oil turned whole orchid to brown.

The orchid tissues were cultured with treatment, the experimental displayed the green as before treated are oil of *Z. cassumunar* and γ-mangostin. While the orchids tissue that treated with compound before infected shown all brown. The infected orchids were cultured on mix media revealed almost treatment changed some leave from green to brown but only oil of *O. caeruleus* turned the orchids tissue to whole brown.

# CHAPTER IV
# CONCLUSIONS

Plant extracts and pure compounds were used to evaluate for their potency as antibacterial activity against *B. gladioli*. The most effective compound was γ-mangostin (96% HPLC) from the pericarp of *G. mangostana* (Mung-kud). This compound revealed the best in vitro antibacterial activity with MIC of 0.125 mM. The $CH_2Cl_2$ extract of *E. colona* exhibited the most effectiveness as antibacterial agent in orchid tissue culture. The highest in vitro inhibition against *P. palmivora* was observed from γ-mangostin and the oil of *Z. cassumunar* (Plai). Nevertheless, there was no plant extract or pure compound that could inhibit *F. oxysporum* more than 50%. In additional, the plant extracts or compounds in this study revealed the high effective with infected orchids more than use as prevention of symptom.

# REFERENCES

# APPENDIX

# APPENDIX A

## Media for microbial culture

1. Nutrient broth and agar (NB and NA)

| | |
|---|---|
| Beef extract | 3 g |
| Peptone | 5 g |
| Agar | 15 g (for NA) |

Dissolved in distilled water up to 1 L and autoclaved at 121 °C, 15 psi for 15 min.

2. Potato dextrose broth and agar (PDA and PDB)

| | |
|---|---|
| Potato starch | 4 g |
| Dextrose | 20 g |
| Agar | 15 g (for PDA) |

Dissolved in distilled water up to 1 L and autoclaved at 121 °C, 15 psi for 15 min.

3. Carrot agar

| | |
|---|---|
| Carrot | 200 g |
| Agar | 15 g |

Dissolved in distilled water up to 1 L and autoclaved at 121 °C, 15 psi for 15 min.

**Media for orchid tissue culture**

Components of Modified Vacin and Went (VW, 1934) medium.

Stock I

| | |
|---|---|
| $KNO_3$ | 525 mg.$L^{-1}$ |
| $KH_2PO$ | 250 mg.$L^{-1}$ |
| $(NH_4)_2SO_4$ | 500 mg.$L^{-1}$ |
| $MnSO_4.H_2O$ | 5.7 mg.$L^{-1}$ |

Stock II

| | |
|---|---|
| $MgSO_4.7H_2O$ | 250 mg.$L^{-1}$ |

Stock III

| | |
|---|---|
| $Feso_4.7H_2O$ | 27.85 mg.$L^{-1}$ |
| $Na_2EDTA$ | 37.25 mg.$L^{-1}$ |
| $Ca_3(PO_4)_2$ | 200 mg.$L^{-1}$* |
| Sucrose | 10-20 g.$L^{-1}$ |
| Agar | 8 g.$L^{-1}$ |
| Coconut water | 150 mL |
| Activated charcoal | 70 mg.$L^{-1}$ |

pH 5.2

*$Ca_3(PO_4)_2$ was dissolved in 10 ml of 1N HCl.



**McFarland Turbility Standard No. 0.5**

Approximate formula per 100 mL purified water

| | |
|---|---|
| Sulfuric acid, 0.18 M | 99.5 mL |
| Barium Chloride, 0.048 M | 0.5 mL |

## APPENDIX B

### Statistical analysis

All data were analyzed with statistic analytical analysis software SPSS for windows version 20.0 and comparison of means using the Duncan's Multiple Range Test at the level $P < 0.05$. The experiment was designed in general linear model within completely randomized design with tri-replications.

### Homogeneous Subsets (*Pseudomonas gladioli*)

**MICs**

| | plants | N | Subset 1 | Subset 2 | Subset 3 |
|---|---|---|---|---|---|
| Duncan[a,b] | PL oil | 3 | .50 | | |
| | BH oil | 3 | | 2.00 | |
| | BYG $CH_2Cl_2$ | 3 | | | 3.00 |
| | JD $CH_2Cl_2$ | 3 | | | 3.00 |
| | Sig. | | 1.000 | 1.000 | 1.000 |

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square (Error) = .203.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = 0.05.

**Homogeneous Subsets (*Phytophthora palmivora*)**

**MICs**

| trt | | N | Subset 1 | Subset 2 | Subset 3 |
|---|---|---|---|---|---|
| Duncan[a,b] | BYG $CH_2Cl_2$ | 3 | 3.1200 | | |
| | MD $CH_2Cl_2$ | 3 | | 6.2500 | |
| | TTR oil | 3 | | | 12.5000 |
| | Sig. | | 1.000 | 1.000 | 1.000 |

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = .046.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Alpha = 0.05.



Where as

BH oil = oil of *Otacanthus coeruleus*

BYG CH2Cl2 = *Echinochloa colona* part $CH_2Cl_2$

JD CH2Cl2 = *Dracaena loureiri* part $CH_2Cl_2$

MD CH2Cl2 = *Xylia xylocarpa* part $CH_2Cl_2$

PL oil = oil of *Zingiber cassumunar*

TTR oil = oil of *Cinnamomum porrectum*

# APPENDIX C

## Nuclear magnetic resonance (NMR) of *Echinochloa colona* (L.) Link

1H NMR analysis of CH2Cl2 extract of E. colona fraction 2 (BYG-2) was performed in Figure C1 and fraction 3 (BYG-3) in Figure C2

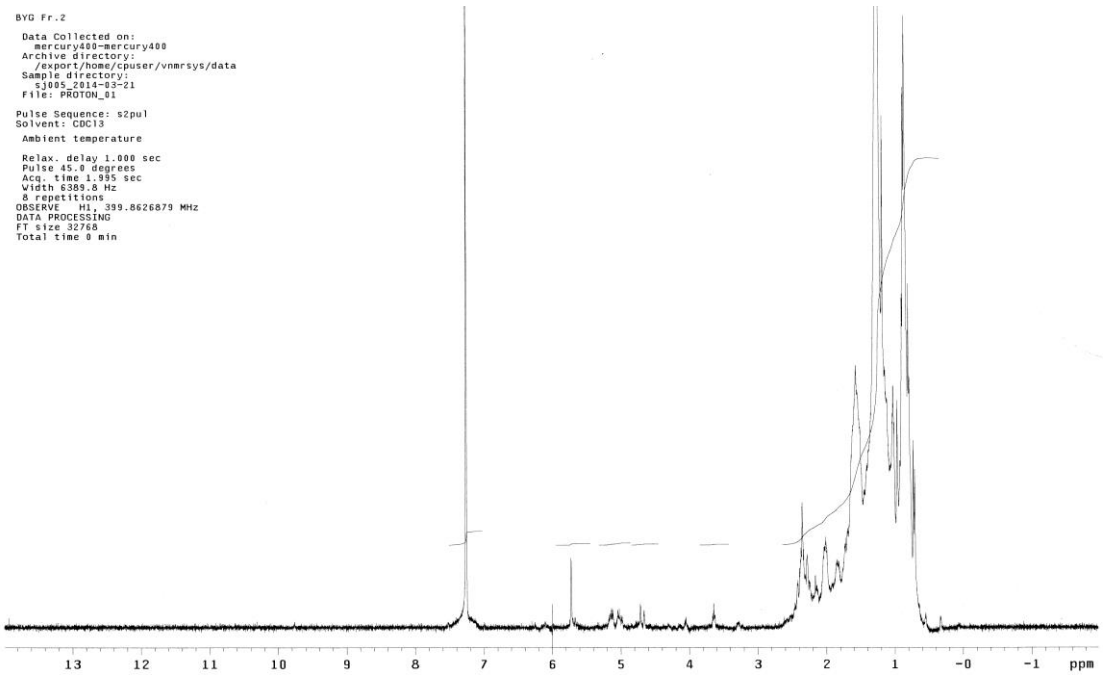



**Figure C1** NMR analysis $CH_2Cl_2$ extract of *E. colona* fraction 2 (BYG-2).

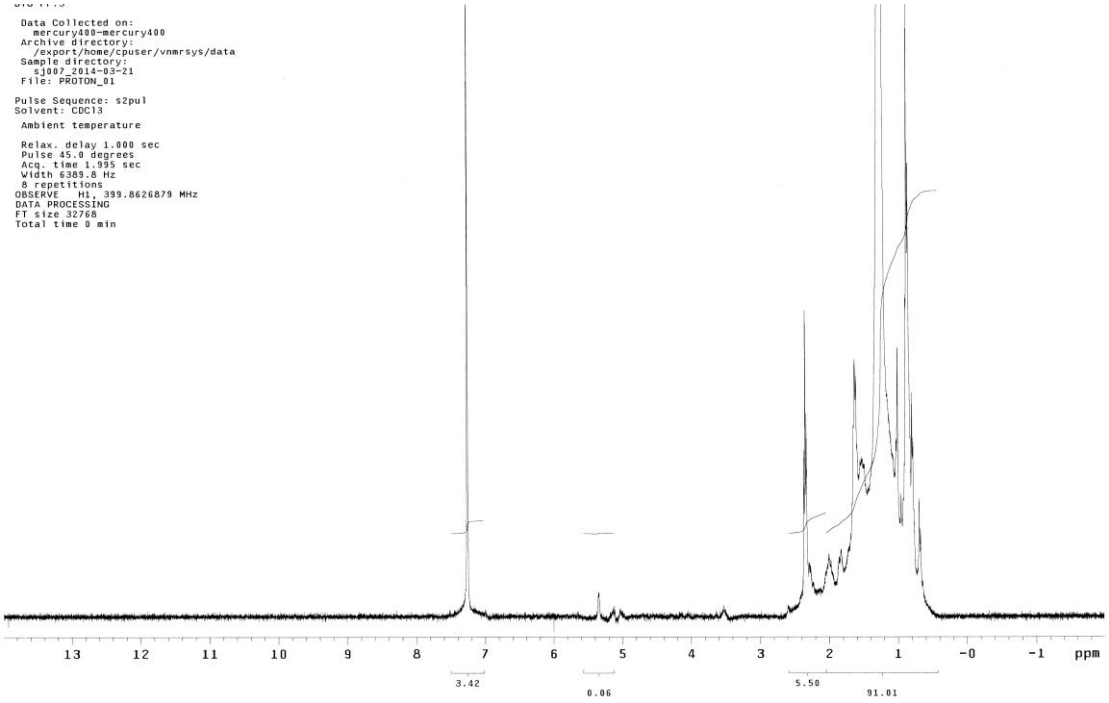



**Figure C2** NMR analysis $CH_2Cl_2$ extract of *E. colona* fraction 3 (BYG-3).

# VITA

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