สารต้านจุลชีพที่ก่อโรคในข้าวจากชะพลู *Piper sarmentosum* Roxb.

และผักแพว *Polygonum odoratum* Lour.

นายประกรรษวัต จันทร์ประไพ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ANTI-PATHOGENIC MICROBIAL COMPOUNDS AGAINST RICE DISEASES FROM WILDBETAL LEAFBUSH *Piper sarmentosum* Roxb. AND VIETNAMESE CORIANDER *Polygonum odoratum* Lour.

Mr. Pragatsawat Chanprapai

A Dissertation Submitted in Partial Fulfillment of the Requirements
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Thesis Title	ANTI-PATHOGENIC MICROBIAL COMPOUNDS AGAINST RICE DISEASES FROM WILDBETAL LEAFBUSH *Piper sarmentosum* Roxb. AND VIETNAMESE CORIANDER *Polygonum odoratum* Lour.
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ประกรรษวัต จันทร์ประไพ : สารต้านจุลชีพที่ก่อโรคในข้าวจากชะพลู *Piper sarmentosum* Roxb. และผักแพว *Polygonum odoratum* Lour.. (ANTI-PATHOGENIC MICROBIAL COMPOUNDS AGAINST RICE DISEASES FROM WILDBETAL LEAFBUSH *Piper sarmentosum* Roxb. AND VIETNAMESE CORIANDER *Polygonum odoratum* Lour.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. วรินทร ชวศิริ , 132 หน้า.

ได้นำสิ่งสกัด CH_2Cl_2 และ CH_3OH ของพืช 17 ชนิด และน้ำมันหอมระเหยของพืช 10 ชนิด มาใช้คัดกรองเบื้องต้นเพื่อศึกษาการยับยั้งการเจริญของจุลินทรีย์สาเหตุโรคในข้าว พบว่า ชะพลู *Piper sarmentosum* Roxb. และผักแพว *Polygonum odoratum* Lour. มีแนวโน้มที่ดีในการยับยั้งการเจริญของจุลินทรีย์ สิ่งสกัด CH_2Cl_2 และน้ำมันหอมระเหยจากใบและผลของชะพลู และน้ำมันหอมระเหยจากผักแพวแสดงฤทธิ์ต้านจุลชีพที่มีศักยภาพสูง การใช้ชีววิธีในการติดตามฤทธิ์ในการแยกสิ่งสกัด CH_2Cl_2 ของใบชะพลูพบสารออกฤทธิ์คือ myristicin (21) ในขณะที่ผลชะพลูสามารถแยก brachystamide B (7), sarmentine (8), brachyamide B (10) และ piperonal (29) ค่า IC_{50} ของ myristicin (21) และ brachyamide B (10) แสดงให้เห็นถึงการต้านการเจริญของเชื้อราสาเหตุโรคในข้าว (*Rhizoctonia solani* และ *Bipolaris oryzae*) ที่มีศักยภาพสูง ในขณะที่ brachyamide B (10) และ piperonal (29) แสดงฤทธิ์ต้านเชื้อแบคทีเรียสาเหตุโรคในข้าวทั้งสองสายพันธุ์ (*Xanthomonas oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*) ได้สูงสุด องค์ประกอบหลักของน้ำมันหอมระเหยของชะพลูคือ myristicin (21) น้ำมันหอมระเหยของผักแพวประกอบด้วยสารกลุ่มอัลดีไฮด์เป็นองค์ประกอบหลัก (dodecanal (2) และ decanal (1)) น้ำมันหอมระเหยและ dodecanal แสดงประสิทธิภาพในการยับยั้งการเจริญของจุลินทรีย์สาเหตุโรคในข้าวที่ใช้ทดสอบได้ดี จากการศึกษาความสัมพันธ์เชิงโครงสร้างของ dodecanal พบว่า 2-dodecanone และ 2-dodecanol ให้ผลในการยับยั้งการเจริญของเชื้อรา *R. solani* ได้ดีกว่าสารตัวอื่นอย่างมีนัยสำคัญยิ่ง ในขณะที่ 1-dodecanol และ 2-dodecanol มีประสิทธิภาพในการยับยั้งการเจริญของเชื้อแบคทีเรียทดสอบได้ดีกว่าสารตัวอื่นอย่างมีนัยสำคัญ

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2556 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

5173829523 : MAJOR BIOTECHNOLOGY
KEYWORDS: ANTI-PATHOGENIC MICROBIAL COMPOUNDS / RICE DISEASES / PIPER SARMENTOSUM ROXB. / POLYGONUM ODORATUM LOURS.

PRAGATSAWAT CHANPRAPAI: ANTI-PATHOGENIC MICROBIAL COMPOUNDS AGAINST RICE DISEASES FROM WILDBETAL LEAFBUSH *Piper sarmentosum* Roxb. AND VIETNAMESE CORIANDER *Polygonum odoratum* Lour.. ADVISOR: ASST. PROF. WARINTHORN CHAVASIRI, Ph.D., 132 pp.

The CH_2Cl_2 and CH_3OH extracts from seventeen plants, and the essential oils of ten plants were conducted for preliminary anti-rice pathogenic microorganism screening. Among them, *Piper sarmentosum* Roxb. and *Polygonum odoratum* Lour. exhibited promising antimicrobial activity. The CH_2Cl_2 extract and the essential oil of *P. sarmentosum* leaves and fruits, and the essential oil from *P. odoratum* displayed high potential antimicrobial activity. Using bioassay-guided, the separation of the CH_2Cl_2 fraction of *P. sarmentosum* leaves furnished an active compound: myristicin (21), whereas that of the fruits led to the isolation of brachystamide B (7), sarmentine (8), brachyamide B (10) and piperonal (29). The IC_{50} against two rice pathogenic fungi (*Rhizoctonia solani* and *Bipolaris oryzae*) of myristicin (21) and brachyamide B (10) exhibited the most potent antifungal activity while brachyamide B (10) and piperonal (29) displayed the most activity against two rice pathogenic bacteria (*Xanthomonas oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*). The major constituent of *P. sarmentosum* oil was myristicin (21). The essential oil of *Polygonum odoratum* contained aldehydes (dodecanal (2) and decanal (1)) as major constituents. The essential oil and dodecanal showed potential activity against all tested rice pathogenic microorganisms. According to structure anti-microbial activity relationship study, 2-dodecanone and 2-dodecanol displayed high significantly anti-*R. solani*, while 1-dodecanol and 2-dodecanol revealed significant antibacterial activity.

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

Academic Year: 2013 Advisor's Signature .....

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°C	=	degree Celsius
$CDCl_3$	=	deuterated chloroform
CH_2Cl_2	=	methylene chloride
CH_3OH	=	methanola
δ	=	chemical shift
d	=	day
d	=	doublet (for NMR spectral data)
dd	=	doublet of doublet (for NMR spectral data)
DMSO	=	dimethyl sulfoxide
DNA	=	deoxyribonucleic acid
et al.	=	and other
EtOAc	=	ethyl acetate
g	=	gram
GC	=	gas chromatography
GC/MS	=	gas chromatography-mass spectrometry
h	=	hour
^{1}H NMR	=	proton nuclear magnetic resonance
H	=	hydrogen
Hz	=	Hertz
IC_{50}	=	inhibitory concentration require for 50% inhibitory of growth
J	=	coupling constant
L	=	liter
μL	=	microliter
M	=	molar
m	=	multiplet (for NMR spectral data)
mg	=	milligram
MBC	=	minimum bacteriocidal concentration
MIC	=	minimum inhibitory concentration
min	=	minute
mL	=	milliliter

mM	=	millimolar
MS	=	mass spectroscopy
m/z	=	mass to change ratio
NA	=	nutrient agar
NaCl	=	sodium chloride
NB	=	nutrient broth
NMR	=	nuclear magnetic resonance
PDA	=	potato dextrose agar
PDB	=	potato dextrose broth
ppm	=	part per million
psi	=	pound per square inch
R_t	=	retention time
s	=	singlet (for NMR spectral data)
sp.	=	species
TLC	=	thin layer chromatography
wt	=	weight

CHAPTER I

INTRODUCTION

Rice *Oryza sativa* L. exists a universally both traded agricultural products and essential nutrition for nearly half of the world's population. Especially, Thailand is the most exporting country and even though a number of private corporations conduct the exports of Thai rice, the government relieves exercises some control over rice exports because of considerable role that rice plays in the Thai frugality (Rakotoarisoa et al., 2006). The reduction in agrarian land, as well as the trouble of low rice production per raid caused by many factors such as non-tolerance to inappropriate environmental circumstances, pest diseases and rice variety rendering insufficient yields, come about its inadequate problems in different areas. Mostly, the extensive loss quality of Thai rice have especially caused by diseases. Rice diseases were the major barriers of high and sustaining rice productivity. The most effective and economical way to control disease were deploying resistant varieties and it had presented an essential position in world rice productivity. During 1970s to 1980s, when epidemics of rice tungro were recurrent in the Philippines and Indonesia, farmers expressed more confidence in using resistant varieties than in other control measures. There are restrictions; nevertheless, in effecting resistant varieties alone to control. Most varieties are resistant only to a few major diseases that are the subjects of intensive breeding actions. Numerous rice diseases, caused by fungal, bacterial, viral, and nematode occur in Thailand. Those induced by fungi including sheath blight (*Rhizoctonia solani* Khun.) and brown spot (*Bipolaris oryzae* Breda de Haan); concurrently, bacterial leaf blight is caused by *Xanthomonas oryzae* pv. *oryzae* and bacterial leaf streak is caused by *Xanthomonas oryzae* pv. *oryzicola* have been the bacterial diseases recorded (Hanson 2003, Seneviratne 2004) as well as these diseases are mainly rice infections in Thailand and also microorganisms for this research. Natural products from plants remain a vastly underutilized resource for the discovery of novel antimicrobial compounds. The majority of higher plant species are yet to be explored as potential sources of antimicrobial agents and also have undergone research as possible ways to control rice diseases. Currently, organic farming which aims at healthy crop production without usage of chemical agents and also recourse to agrochemicals is one of common strategies adopted to control diseases. However, their protection by agrochemicals present hazards to human health and environment, and farmers in Thailand are already rejecting them in favor

of more sustainable approaches. Thus, most researchers have looked into alternative, non–chemical microcides.

Based on the literature search, a few scientific reports have been published on the potential anti-microbial activities of Thai plants. Hence, this work addresses that aims at screening these plants that can perform equally efficiently under laboratory and greenhouse conditions. In addition, the regarded investigation was focused on isolation and purification of the active compounds from the solvent extract of dried, powdered and essential oil of Thai plants.

1.1 Phytochemistry

Photochemistry, natural products, is the organic compounds that are produced from living plants. There are related to the chemistry of plant metabolites and derivatives. Plant metabolites can be classified as primary and secondary metabolites. In case of primary metabolites, the compounds occur in plant cells and there are only assorted with photosynthesis, respiration, growth and development such as phytosterols, acyllipid, nucleotides, amino acids, organic acid and sugar. On the other hand, secondary metabolites display the biological effects on the other organisms and there are derived from primary metabolites (**Figure 1.1**). The secondary metabolite have been interested and studied from medicinal, commercial, poisonous and edible plants.

The function of secondary metabolites can be used for defense, attraction and protection. Their function appears to have a dominant role in protecting plants from herbivores, microbes (bacteria and fungi) and by other plants competing for light, space and nutrients. The abiotic stresses such as radiation and UV light are also protected by plant SMs. Moreover, plant SMs appear to attraction, for instant; pollinating insects, seed dispersing animals, root nodule bacteria and induced volatiles attract predatory organisms (Robbins 2000). The classes of secondary metabolites are classified by the particular structural characteristics rising from the way of biosynthesis. The SMs classes are polyketides and fatty acids, terpenoids and steroids, phenylpropanoids, alkaloids, others (specialized amino acids, peptides and carbohydrates) (Hanson 2003).

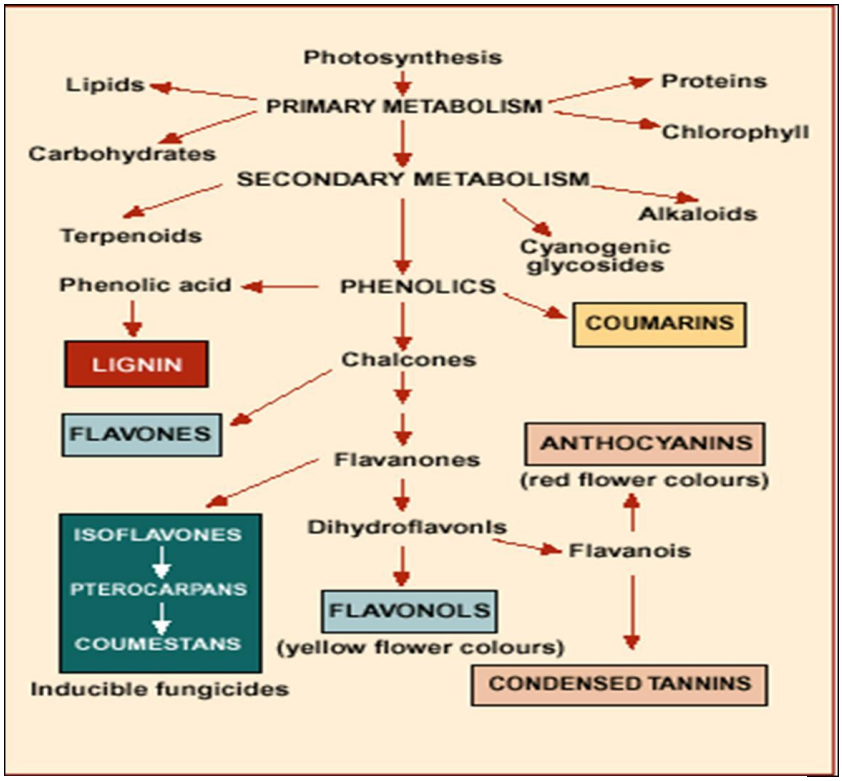


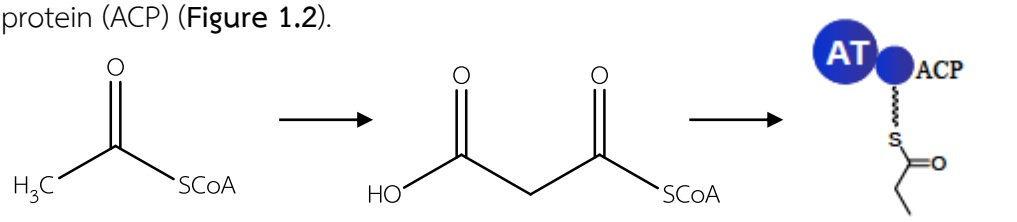
Figure 1.1 Primary and secondary metabolites from plants

(Source: http://science.marshall.edu/valluri/HFB.htm)

1.1.1 Polyketides and fatty acids

Polyketides (PKs) are the secondary metabolite oxygenation and derived from acetate units (acetyl co-enzyme A) using polyketide synthase enzymes (Kao et al., 1996). They can be found in bacteria, fungi, clinical important drugs and plants and this research only focused on bioactive polyketide compounds possessing antimicrobial activities from plants. The polyketide synthesis is possessed with three stages (Cheng 2003) following;

a) Acetyl-CoA as the first stage units is initially synthesized and led to malonyl co-enzyme bounding to a 5′-phosphopantetheinyl SH group of the acyl carrier protein (ACP) (**Figure 1.2**).



Acetyl-CoA Malonyl-CoA Initiation stage

Figure 1.2 The initial stage of polyketide synthesis

b) Second stage, the carbon chain length of C_4 and $C_{16\text{-}18}$ units from the final products of the first stage are passed from the ACP to the ketosynthase (KS) of the followed module. These intermediate carbons have been led to the optimal domains by using multi-enzyme complex as polyketide synthase (PKS). The current ACP from the acyltransferase (AT) catalyzes is derived from malonyl-CoA or its derivatives (**Figure 1.3**)

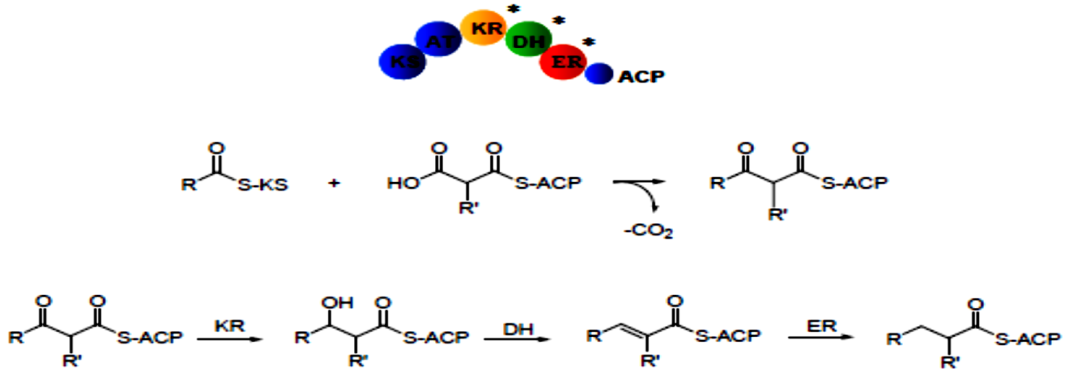


Figure 1.3 The second stage of polyketide synthesis

c) Final stage, the completed PKS chain from the ACP of the final product of the second stage is hydrolyzed from TE domain (**Figure 1.4**).

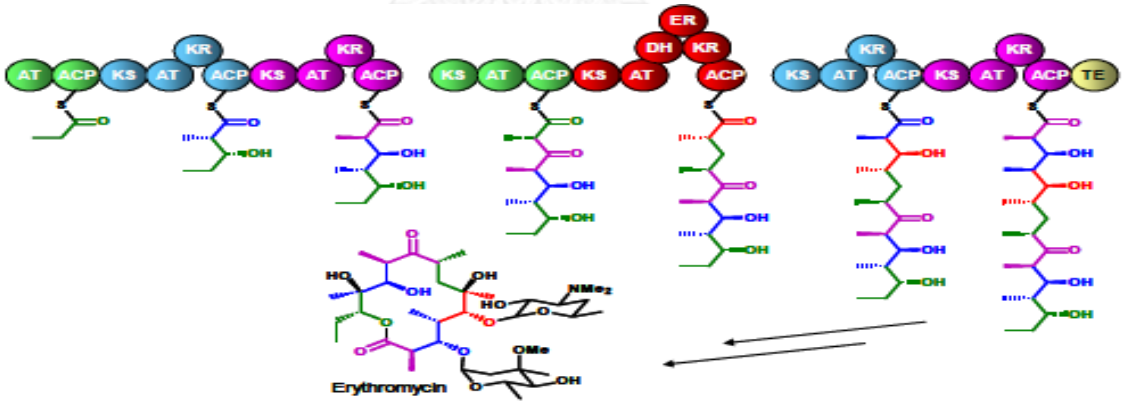


Figure 1.4 The final biosynthetic pathway of erythromycin as a polyketide.

In case of fatty acid, this acids display in both free acids and mixed as esters with alcohols, for instance; glycerol and cholesterol. The acids have been linked by numerous carbon atoms as $C_{12\text{-}20}$ and together with double bonds of carbons. The natural fatty acids are found in leave waxes and seed coatings such as myristic acid (C_{14}) (in nutmeg seeds), palmitic acid (C_{16}) (in all plant oils) and stearic acid (C_{18}) (in

animal fat). Fatty acids in plants have been identified as unsaturated carbons such as polyacetylenes (family Dahlia) and wyerone (*Vicia fabae*).

1.1.2 Terpenes and steroids

The terpene compounds are derived from isoprene units. The structures of these units are the C_5 isoprene units and linked with a head-to-tail manner. The classes of terpenes, for instance; monoterpenoids (C_{10}), sesquiterpenes (C_{15}), diterpenoids (C_{20}), sesterterpenoids (C_{25}), triterpenoids (C_{30}) and carotenoids (C_{40}) have been classified by the units. The tetracyclic triterpenoids are descended to steroids, additionally; sesquiterpenes and sesquiterpene lactones have been mainly reported for antimicrobial activities (Gallucci 2010). The functions of plant terpenoids are related with plant growth processing. Some compounds have been used for insect attractants purposing of plant reproduction, expelled and protected on agricultural pests.

Monoterpenes are the major constituents in the essential oils. These oils from plants were extracted with hydro-distillation and then liquid extraction was used by ether or diethyl ethers. The volatile plant oil products have been usually studied from many researchers. These terpenes are distributed in many plant families such as *Compositae*, *Labiatae*, *Rosaceae*, *Rutaceae*, *Piperaceae*, *Myrtaceae*, *Polygonaceae*, *Scorphulariaceae*, *Zingiberaceae* and *Umbelliferae*. There are isolated from all plant parts: flowers, leaves, roots, rhizomes, woods, barks, fruits and seeds (Hanson 2003). The major constituents of many essential oils have been identified as geraniol (geranium oil), linalool (coriander, English and continental lavender), citral (lemon grass oil), menthol (field mint), terpineol and **α**-pinene (pine oil) and camphor (camphor tree; *Cinnamomum camphora*).

Sesquiterpenes (C_{15}) are derived from farensyl pyrophosphate and found in the higher boiling portions (>200 °C). Some compounds of this terpene have been functioned about plant and animal growth developments such as abscisic acid and xanthinin. The majority of essential oil compounds was identified and included as caryophyllene (clove oil), humulene (hop oil), cedrene (cedar wood oil), longifolene (Indian turpentine oil). Saponin (wormwood) displayed biological active constituent.

Diterpenes (C_{20}) mostly display the wood resin. Some compounds are performed about plant growth controlling such as gibberellic acid and vitamin A. According to Barnes *et. al.* (2013), the diterpene constituents from the Australian native plant *Eremophila microtheca* were identified as 3-acetoxy-7,8-

dihydroxyserrulat-14-en-19-oic acid, 3,7,8-trihydroxyserrulat-14-en-19-oic acid, 3,19-diacetoxy-8-hydroxyserrulat-14-ene, verbascoside and jaceosidin. These compounds revealed moderate anti-bacterial activity against *Streptococcus pyogenes* (ATCC12344) with MICs of 64-128 μg/mL. The 3-acetoxy-7,8-dihydroxyserrulat-14-en-19-oic acid showed antibacterial activity all gram positive bacteria but it was excepted on *Enterococcus faecalis* and *E. faecium*. Diterpenes from *Viguiera arenaria* and semi-synthetic derivatives including *ent*-pimara-8(14),15-dien-19-oic acid its sodium salt and *ent*-8(14),15-pimaradien-3β-ol showed the most anti-bacterial activity against endodontic bacteria with MICs (Carvalho et al., 2011).

Sesterterpenoids (C_{25}) can be mostly produced from marine natural products such as sponges; for instance, *Sarcotragus* sp. (Wang et al., 2008), *Psammocinia* (Shin 2001), *Smenospongia* and *Spongia* genus from Korean waters (Choi et al., 2004, Liu et al., 2003, Nam et al., 2006, Rho 2004). They have usual function with furan (furannosesterterpenoids) or tetracyclic structures. The compounds of these terpenes are furanosesterterpene, scalarin and ophiobolin A. According to antimicrobial activity, the bioactive sesterterpenoids such as hyrtiosal (*Hyrtios erectus*, anti-HIV virus), 1,4-benzoquinone moiety (antimicrobial and antiviral activities), puupehanol, puupehenone and chloropuupehenone (*Hyrtios* sp., antifungal activity), and nakijiquinones G-I (Okinawan marine sponges) were summarized (Abad 2011).

Triterpenoids (C_{30}) and sterols can be extracted and isolated from oils of plants and animals. The first triterpenoid compound was extracted from fish liver oils. The majorities of this terpenoid was found as α- and β-amyrins, triterpene lactone (abietospiran and crystallizes), glycyrrhetinic acid. Moreover, triterpenes could be founded from fruiting body of mushrooms such as *Ganoderma applanatum* (ganoderic acid F and A, lucidenic acid DI and applanoxidic acid A) (Boh 2000).

1.1.3 Phenylpropanoids

Phenylpropanoids display the large group of plant secondary metabolites containing with an aromatic ring and attaching to a three-carbon propane side chain (C_6-C_3) in their structure such as cinnamic acid and various coumarins (Hanson 2003). The biosynthesis pathway leads to shikimic acid pathway falling into two sections included shikimic acid formation and the conversion of it to aromatic C_6-C_3 compounds. The natural phenylpropanoid compounds were found in essential oil plants. Curcumin and its derivatives from rhizomes of *Curcuma longa* have been

exhibited with anti-bacterial and fungi activities (Patra et al., 2012). 1′-S-1′-Acetoxychavicol acetate, *trans*-p-coumaryl diacetate and 1′-S-1′-acetoxyeugenol acetate were found from *Alpinia galaga* and could be inhibited about the efflux of EtBr in *Mycobacterium smegmatis* (Roy et al., 2012). The CH_2Cl_2 extract of *Aster flaccidus* roots – Chinese traditional medicine- found the two new phenylpropanoids including (7′R, 8S)-9′-lariciresinol-(α-methyl)-butanoate and 5,9-dimethoxyl-7-(α-methyl)-butanoxyl-phenyl-2E-propenol-(α-methyl)-butanoate that displayed strong antitumor activity against BEL7402 (human liver carcinoma) and anti-HIV-1 activity (Liu et al., 2010). The MeOH extract of *Parastrephia lucida* (Meyen) displayed antibacterial activity against *Staphylococcus aureus* and *Enterococcus faecalis* and it was identified as the mixtures of prenyl and phenethyl esters of caffeic and cinnamic acids (D'Almeida et al., 2012). The chloroform stem bark extract of *Tecoma mollis* was identified as seven active phenylpropanoid glycosides including verbascoside, 6'-*O*-acetylverbascoside, verpectoside B, tocomoloside, luteoside A, isoverbascoside, luteoside B and lxoside that were showed antiparasitic antioxidant (Abdel-Mageed et al., 2012).

From the previous studies appear that antibiotic substances could be found from Thai plants and belong to the different chemical class of the active compounds. Therefore, the secondary metabolites of Thai plant varieties have been used for antimicrobial activity because they were exhibited by the various bioactive compounds.

1.2 Antimicrobial activity of plant extracts, essential oil and their active compounds

1.2.1 *Cinnamomum porrectum* (Roxb.) Kosterm.

C. porrectum belongs to Lauraceae family and distributes in all areas of Thailand. The anti-microbial activities of various parts of this plant included roots, barks, woods and leaves. The essential oils of roots indicated the strongest MIC on human pathogens such as *S. mutans* (0.01 mg/mL), *Candida albicans* (0.5 – 1.0 mg/mL), *B. subtilis* (2 mg/mL) and *S. aureus* (4 – 16 mg/mL) (Phongpaichit 2007). The EtOH extract of barks and woods displayed antibacterial activities against *S. pyrogenes* with the MIC/MBC range of 62.50-1000/62.50-1000 and 250-1000/250-1000 µg/mL, respectively (Limsuwan 2013). Moreover, the 50 and 95% EtOH extract of

woods displayed antibacterial activity against *S. aureus* ATCC 25923, *Salmonella typhi* ATCC 14028, *B. subtilis* ATCC 6633 and *Escherichia coli* ATCC 25922 with inhibition zone range of 8-10 mm and related the range of total phenolic content values of 118.18-189.88 mg GAE/g (Pukdeekumjorn 2012).

In view of the chemical composition, the 3 h distillate essential oil of *C. porrectum* roots showed the highest safrole content 99.69% w/w (Chaisaeng 2009). The essential oil of leaves was also analyzed as safrole (93.19%) using GC-MS and GC-FID analysis (Subki et al., 2013). In addition, the callus culture on half-strength MS basal medium comprised of 0.5 mg/L NAA and 0.5 mg/L BA provided the highest callus formation yield. These calli produced high safrole (Nathalang 2011).

1.2.2 *Echinochloa crus-galli* (L.) P.Beauv.

E. crus-galli is the rice weed distributed widely in 42 countries including Thailand. It belongs to Poaceae family and can be reduced rice production by 2,000–4,000 kg/ha (Macias et al., 2005). This weed liberates allelochemicals to inhibit the growth of rice. *p*-Hydroxybenzoic acid as phenolic acid from leave extract displayed allelopathic activity on seedling properties of rice genotypes (Esmaeili 2012).

1.2.3 *Eclipta prostrata* (L.) L.

E. prostrata (syn: *E. alba* (L.) Hassk.), as local name as Ka-Meng, belongs to *Asteraceae* family. It has been used in traditional cuisine and in folk medicine. According to antimicrobial activity, the EtOAc extract revealed anti-*B. cereus* with zones of inhibition in the range of 11 to 22 mm following by the EtOH extract against *E. coli* (Borkataky 2013). Additionally, the EtOAc fraction inhibited all tested microorganisms *viz. B. subtilis*, *C. albicans*, *Erwinia carotovora*, *E. coli*, *Klebseilla pneumonia*, *Pseudomonas aeroginosa* and *S. aureus* (Bakht 2011). For phytochemical analysis by GC-MS, naphthoquinone, hydrazine carboxamide (Chauhan 2012), wedelolactone and demethylwedelolactone (Sharma 2010) were reported. Moreover, the volatile oil was analyzed to 55 compounds and the major compounds included heptadecane (14.78%) and 6,10,14-trimethyl-2-pentadecanone (12.80%) were the main components (Lin et al., 2010).

1.2.4 *Eupotorium odortum* L.

E. odortum (Sabsua) belongs to *Asteraceae* family and widely distributing in the middle of America, the northern of Argentina and tropical country. The EtOH extract of *E. odortum* leaves revealed low anti-*Campylobacter jejuni* activity with inhibition zone of 7.3 mm (Dholvitayakhun 2012).

1.2.5 *Tabernaemontana pandacaqui* Poir.

T. pandacaqui belongs to family *Apocynaceae* and it is distributed in Thailand, Taiwan, Philippines, Borneo, Sulawesi, Java, to New Guinea, N and E Australia, to the Pacific. The alcoholic extract of stems displayed anti-inflammatory, antipyretic and anti-nociceptive activities, additionally; this extract was separated as alkaloidal components (Taesotikul 2003).

1.2.6 *Gardenia angusta* (L.) Merr.

Gardenia angusta (L.) Merr. belongs to *Rubiaceae* family and also Thailand called Pudson. It is widely found in Eastern Asia (Koo 2006). Three iridoids including Gardenal-I, Gardenal-II and Gardenal-III, along with nine comprehended iridoid glycosides; geniposide, 6-β-hydroxy geniposide, 6-α-hydroxy geniposide, 6-α-methoxy geniposide, feretoside, genipin-1-β-gentiobioside, shanzhiside, lamalbidic acid and picrocrocinic acid were identified and isolated from ethanol extract (Rao 2013). The ethanol extract of dried fruits investigated antiviral activity on influenza virus and active compounds were identified as same as the previous research (Yang 2012).

1.2.7 *Hedychium coronarium* Koenig.

H. coronarium or Mahahong in Thai, belonging to *Zingiberaceae* family cultivates in Japan, Phillippines and Thailand. It releases the volatile monoterpenes and sesquiterpenes, and benzenoid from flowers (Lan et al., 2013). The chemical constituents from its rhizomes were identified as three new labdane diterpenes and coronarins G-1, coronarin D, coronarin D methyl ether, hedyforrestin C, (*E*)-nerolidol, β-sitosterol, daucosterol and stigmasterol (Kiem 2011, Suresh et al., 2010). For antibacterial activity, methanol and dichloromethane extracts of rhizomes represented antibacterial activity against gram positive bacteria (*S. aureus*, *B. subtilis*,

B. megaterium and *Sarcina lutea*) and gram negative bacteria including *E. coli*, *Shigella sonnei*, *S. shiga*, *P. aeruginosa* and *S. typhi* (Aziz 2009).

1.2.8 *Houttuynia cordata* Thunb.

H. cordata, as local name as Phu-kraw, is a traditional and medicine Chinese medicinal herb and distributed in Japan, Korea, southern China and Southeast Asia. Moreover, it has exhibited in anti-allergy, antioxidant, antiviral and antibacterial activities (Lai et al., 2010). The major compounds of its volatile oil were identified as pentadecanol (13.96%) and *n*-decanoic acid (11.09%) using FGC-MS analysis (Qi 2004). The secondary metabolite compounds were released as strigone, strigol, sorgomol and 5-deoxystrigol (Kisugi et al., 2013); norcepharadione B, 4, 5-dioxodehydroasimilobine, cepharadione B, aristololactam B II, aristololactam A II, sauristolactam, piperolactam A, splendidin and aristololactam F II (Wei 2011).

1.2.9 *Limnophila aromatica* (Lam.) Merr.

L. aromatica is a member with *Scorphulariaceae* family and widely distributed in Southeast Asia. It has been used as Vietnamese cuisine and looks like an aquarium plants. The major chemical constituents of volatile oil were identified as z-ocimene (39.21%), terpinolene (17.24%) and camphor (12.89%) (Bhuiyan 2010), and uncommon 8-oxygenated flavonoids (Bui 2004) using GC-MS spectrometry. Moreover, the 100% EtOH extract displayed the highest antioxidant activity and it exhibited the highest both phenolic and flavonoid contents (Do 2013).

1.2.10 *Melodorum fruticosum* L.

M. fruticosum belongs to *Annonaceae* family as local name as Lum-Duan and it has widely distributed in Indo-China and Thailand. The characteristics of its flower are sole, canary yellow, and fragrances**.** Additionally, the aroma oil from flowers is applied as traditional medicines of Thailand (Rujjanawate 2008). However, the chemical constituents of volatile oil were analyzed as phenyl butanone, linalool, benzyl alcohol, **α**-cadinol, globulol and viridiflorol using GC-MS analysis and the extracts viz. hexane extract, essential oil and methanol extract revealed antifungal activities with range of MIC values of 125 – 1,000 µg/mL. Moreover, dichloromethane extract showed only high activity with IC_{50} of 87.6 µg/mL.

1.2.11 *Myristica fragrans* Houtt.

M. fragrans belongs to *Myristicaceae* family and have been used to treat many diseases. Nutmeg and mace of 85% methanol extracts displayed anti-food-borne bacterial activities against *S. aureus* and *B. cereus* with MIC of 50 µg/mL and nutmeg had been found phenolic compounds (Sulaiman 2012). Acetone extract revealed the strongest antimicrobial activity against *S. aureus* and *Aspergillus niger*, moreover; this extract was identified as sabinene (28.61%) as the major compound using GC-MS (Gupta 2013).

1.2.12 *Oryza sativa* L. (Allelopathic rice)

Allelopathic rice *Oryza sativa* L. can potentially be used to adjust weed and pathogen management in rice production (Kim et al., 2010). Expansive examinations of allelochemicals in rice plants revealed the detection of a level of phenolic compounds, including *p*-hydroxybenzoic, vanillic, *p*-coumaric and ferulic acids (Chung 2001, Chung 2002, Mattice 1998, Olofsdotter et al., 2002, Rimando et al., 2001). Additionally, an increasing number of studies showed that some flavones, diterpenes and other types of compounds are influential allelochemicals in rice (Kato-Noguchi 2002, Kato-Noguchi 2003, Kong 2002, Lee et al., 1999). It exists that there are undisturbed other allelochemicals in rice plants, particularly in a scanty allelopathic rice acceptances of many rice germplasm collections (Dilday,Lin,Yan 1994, Olofsdotter 1995).

1.2.13 *Otacanthus coeruleus* A. Rose.

O. coeruleus (Scrophulariaceae) have common named as Blue Hawaii and it is a shrub plant. The origin of this plant is from Brazil. The components were analyzed about 46 compounds using GC-MS analysis, especially; main compounds were identified as dehydroaromadendrene, α-humulene, *trans*-verbenol, benihinal, and α-copaene (Ronse 1997).

1.2.14 *Piper sarmentosum* Roxb.

Piper sarmentosum Roxb. is a member of the family Piperaceae used in many Southeast Asia cuisines as far as the Andaman Islands. It, locally known as “Cha–plu”, is a glabrous, creeping terrestrial herb about 20 cm tall. The plant and fruits are

used in Thailand as an expectorant (Rukachaisirikul et al., 2004). According to antimicrobial activity, 1-allyl-2,6-dimethylenedioxybenzene, 1-allyl-2,4,5-trimethoxybenzene, 1-(1-*E*-propenyl)-2,4,5-trimethoxybenzine and 1-allyl-2-methoxy-4,5-methylenedioxybenzene were isolated from the leaves and expressing activities on *E. coli* and *B. subtilis* (Masuda 1991). In addition, isolated amides such as pellitorine, guineensine, brachyamide B and sarmentosine were discovered to possess antituberculosis activity (Rukachaisirikul et al., 2004). Two amides including 3-(3',4',5'-trimethoxy-phenylpropanoyl) pyrrolidine, *N*-(3-phenylpropanoyl) pyrrole and β-sitosterol revealed an activity towards gram-positive bacteria (Atiax et al., 2011). Rahman *et. al.* (1999) reported the antimalarial activity of *P. sarmentosum* from the chloroform extract (Rahman 1999). The methanol extract of the leaves also exhibited potential antibacterial activity against both Gram positive *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) and Gram negative *P. aeruginosa* (Zaidan 2005).

1.2.15 *Polygonum odoratum* Lour.

P. odoratum (syn. *Persicaria odorata*) is classified in *Polygonaceae* family and the common name is Vietnamese mint or Vietnamese coriander (Thai: Pak-Preaw). The previous studies reported the compositions of *P. odoratum* were identified as dodecanal (44.05%), decanal (27.73%) and decanol (10.88%) which are the major compounds (Hunter 1997, Starkenmann et al., 2006).

1.2.16 *Polygonum tomentosum* Willd.

P. tomentosum is local name as aung ped marh and classified as *Polygonaceae* family. It has been distributed in Africa, tropical country and Thailand.

1.2.17 *Psidium guajava* Linn.

P. guajava has known as an important fruit tree and belongs to family *Myrtaceae*. It distributes in tropical Central America and around the tropical countries. The major compositions of essential oil of *P. guajava* stems were identified as α-humulene (10.93%), germacrene D (16.79%) and valerenol (10.62%), besides leaf oil identified veridiflorol (36.4%) and *trans*-caryophyllene (5.9%) (Khadhri 2014). In addition, water, methanol and chloroform extracts of dry guava leaves were inhibited on *S. aureus* and β-streptococcus group A ($P<0.01$) (Jaiarj et al., 1999).

1.2.18 *Solanum torvum* Sw.

S. torvum belongs to Solanaceae family, the *Solanum* genus, distributed in India, Malaysia, China, Philippines, tropical America and Thailand. The green fruits of *S. torvum* are edible and applied in Thai consumption, existence one of the crucial elements of Thai yellow curry; similarly, they are also used in Lao cuisine. It is usually known by several different names depending on the local tribe such as Turkey Berry, Devil's Fig, Prickly Nightshade, Shoo – shoo Bush, Wild Eggplant, Pea Aubergine and Makhua Phuang (Thai: มะเขือพวง) (Arthan et al., 2002). In addition, promising antibacterial and antifungal effects on all organisms are exhibited by methanolic root extracts (Bari 2010) as well as highly polyphenol of acetone leave extracts is effective to gram positive and gram negative bacteria (Jimoh 2010). Moreover, some important seed borne pathogens of paddy viz (such as *Pyricularia oryzae, Alternaria alternata, Bipolaris oryzae* and *Xanthomonas oryzae*) can be eliminated by methanolic leave extracts (Lalitha 2010) and the amounts of polyphenols and flavonoids from fruit coat extracts have depressed antibacterial activity using MIC (Sivapriya 2011). Detailed separation work has been done obtaining alkaloids and other compounds. These compounds include tannin, steroids, torvanol A, torvoside H, polyphenol, saponin, phytate and flavonoids.

1.2.19 *Zanthozylum limonella* Alston.

Z. limonella belongs to *Rutaceae* family and it is widely distributed in the northern part of Thailand. The chemical constituents of stems of it were identified as a novel quinolone alkaloid, 4-methoxy-3-(3-methyl-2-oxobut-3-enyl)quinolin-2(1H)-one, limonellone, together with five known compounds using spectroscopic and MS analysis (Tungjitjaroenkun 2012). Additionally, the crude chloroform extract revealed antimalarial activity against *Plasmodium falciparum* and antituberculous activity against *Mycobacterium tuberculosis* H37 Ra (Charoenying 2008).

1.3 Rice diseases

The numerous things can cause plants to become diseased including living agents, other factors (nonliving), or a combination of the two (Trigiano 2008). This study focuses only on living agents including fungi and bacteria. The fungal and

bacterial pathogens differ in their ability to survive, spread, and reproduce. Fungi are the largest group of plant pathogens. The mycelium and fruiting bodies and spores have been used for identify and diagnose fungal problems. Fungi cause plant diseases in the following ways: making toxins that kill plant cells, growing within a plant's vascular system and plugging up, rotting roots and sending robot-like structures into plant cells (Pscheidt 2013). Bacteria are single-celled organisms and they cause plant diseases by forming toxins or by producing enzymes that break down plants' cell walls. Rice diseases are the important in limiting profitable rice production in Thailand such as sheath blight (*Rhizoctonia solani* Kuhn., DOAC1406), brown spot (*Bipolaris oryzae* Breda de Hann Shoem, DOAC1760), bacterial leaf blight (*Xanthomonas oryzae* pv. *oryzae,* TB0006) and bacterial leaf streak (*Xanthomonas oryzae* pv. *oryzicola*, TS8203) (Seneviratne 2004); so, these diseases were used for this study.

1.3.1 Fungal rice diseases

1.3.1.1 Sheath blight disease

Sheath blight disease is caused by *R. solani*. This disease can be decreased rice production distributing in Thailand and other countries of the tropical areas. It produces a phytotoxin that could reproduce the most of symptoms of the disease (Qin,Zhang 2005)(**Figure 1.5**).

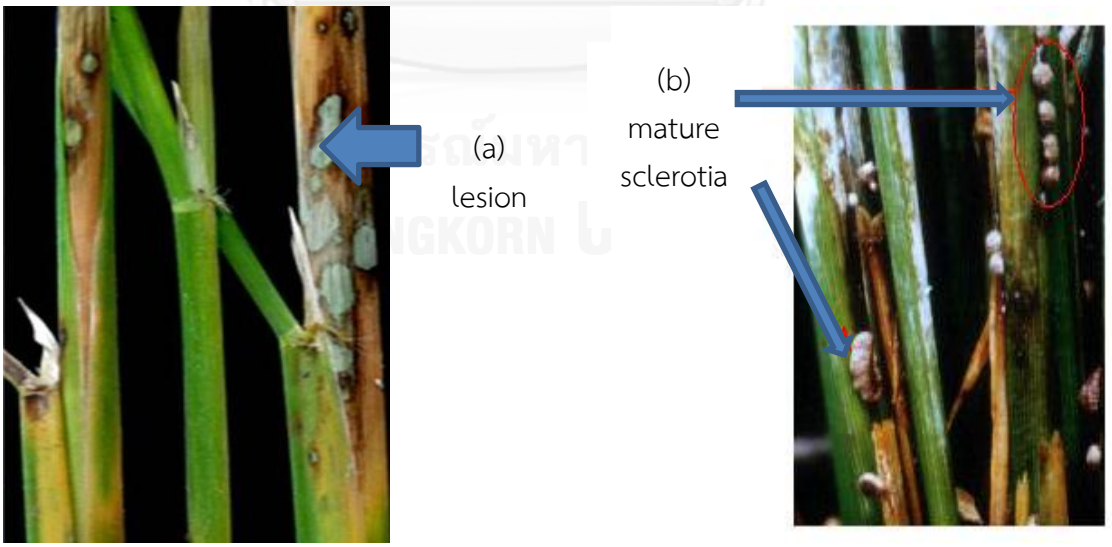


Figure 1.5 Lesion symptoms of sheath blight disease (a) and the major sclerotia (b).

The **Figure 1.5** shows the sheath blight lesion and the major sclerotia are the specific characteristics of *R. solani*. The disease lesions are oval gray spots with black brown margins that later enlarge on the leaf sheath, beside leave symptoms display gray irregular lesions banded with green brown coloration and grayish center; panicle exertion affected when flag leaf is infected. These sclerotia are floated and infected on sheathes near water line, and spread by irrigation water and soil movement (Williamson 1998).

Disease cycle of sheath blight was clarified by Kanjanamaneesathian (1994) (**Figure 1.6**). *R. solani* remains alive in the soil forming the sclerotia that floats to the water surface pending soil preparation. The sclerotia will then be germinated and initiated for mycelia infection into the rice plant tissues when free sclerotia contacted with the rice plants. The stomatal slit and cuticular penetration are infected and grown by the fungal mycelia. The mycelium often grows externally from the outer sheath to the inner surface. After the primary lesions are formed, mycelia grow swiftly on both the surface of rice plant and inside its tissue, process upwards to the secondary lesions. The sclerotia forms are developed on this lesion and then they are later germinated and infected on the other plants.

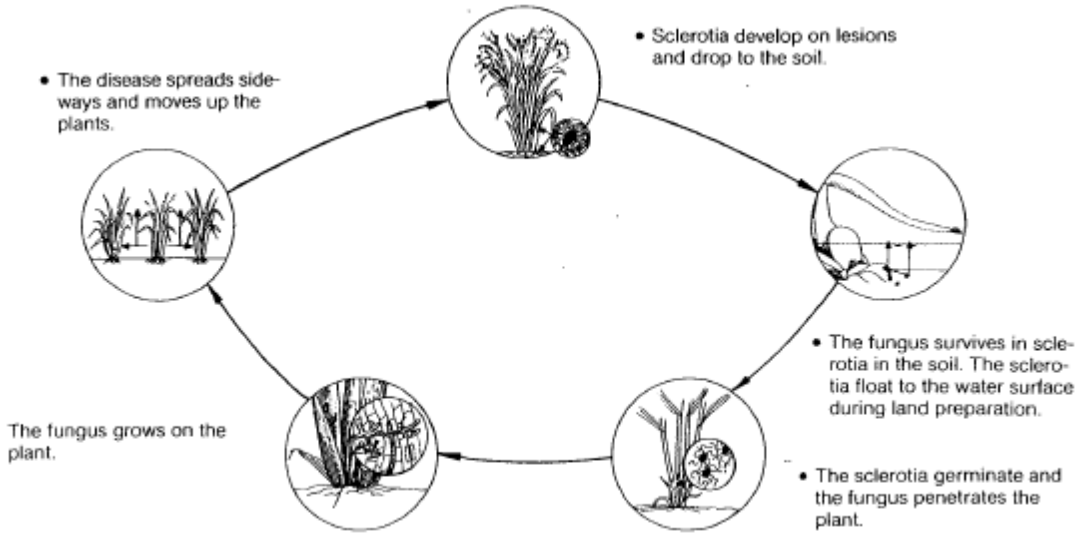
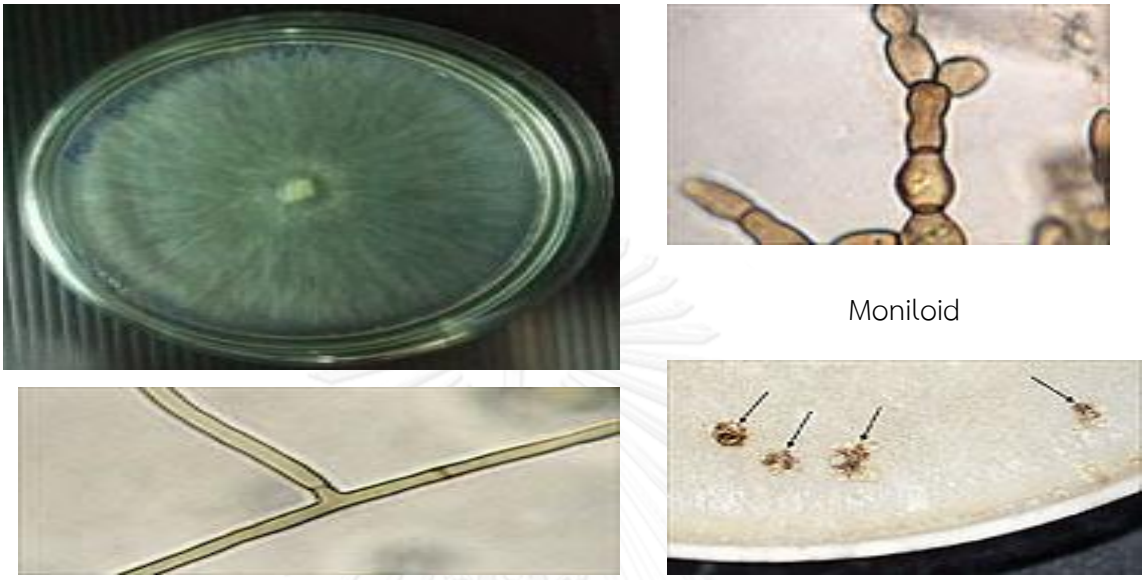


Figure 1.6 Disease cycle of sheath blight disease of rice pathogenic fungus

(Kanjanamaneesathian 1994)

R. solani belongs to phylum Basidiomycota, class Agaricomycetes, order Cantharellales and family Ceratobasidiaceae. The growth of the mycelium is confined

to the surface layer on agar and all sclerotial forms only produced larger sclerotia on fresh agar medium. In addition, the characteristics of the hyphae are septate hyphae and moniloid structures (**Figure 1.7**).



Moniloid

Hyphae

Sclerotia

Figure 1.7 *R. solani* on PDA, hyphae, moniloid and sclerotia

1.3.1.2 Brown spot

Brown spot is caused by the fungus *Bipolaris oryzae* as anamorph stage and represented in the main rice cultivating countries of the world (Khalili 2012). It is spread on leaves and grains. Damage to plants is seedling mortality and quality and number of grains affected. The symptoms of brown spot usually are circular to oval spots on the first seedling leaves from tiny dark spots to oval up to one-half inch in diameter. The small spots may appear on reddish-brown to brown, even though the large spots have a gray in the center of a dark brown perimeter. Additionally, these spots will also emerge on rice gains (**Figure 1.8**).

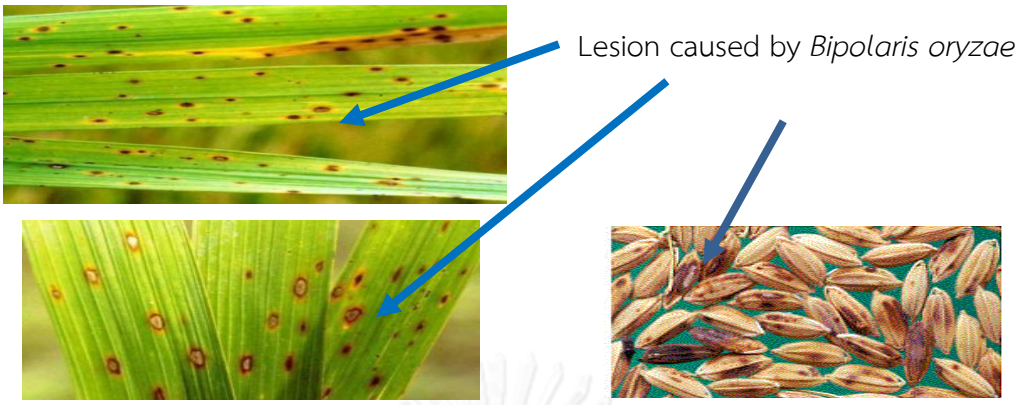


Figure 1.8 The lesions of rice brown spots on leaves and grains by *B. oryzae*

The optimal conditions of *B. oryzae* were grown in 25-30 °C with relative humidity above 80% and excessed of nitrogen aggravates the disease severity. Disease cycle of brown spot disease is initially infected and developed of the fungus in the grains that are in the panicles. This disease will be transmitted by infected seeds. After that these fungal spores can be germinated and entered the seedling roots or coleoptiles. For infecting a plant, conidiospores form an appressorium and penetrate the epidermal cells with an infection peg. Once penetrated, the mycelium spreads throughout the mesophyll. The rice kernels are infected with brown spot when the fungus reaches the panicle (Elsacker 2013) (**Figure 1.9**).

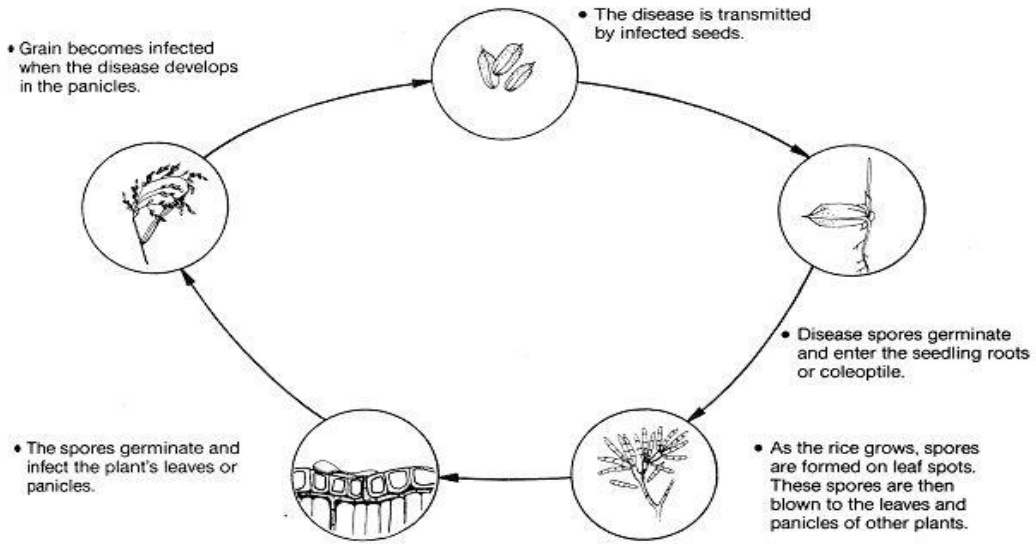


Figure 1.9 Disease cycles of rice brown spots

B. oryzae is classified in the subdivision Deuteromycotina (imperfect fungi), class Deuteromycetes, order Moniliales, family Dematiaceae (Shabana et al., 2008). The fungus colony structures are composed of black mycelia that are built up from primary mycelia and set up the sporophores. An ample, brancing and anastomosing are appeared in the fungal hypha which are dark brown and measure 8-15 µm or more in diameter. The sporophores are usually originated from the lateral branches of hyphae and 150-600 x 4-8 µm. For its conidia, they measures 35-170 x 11-17 µm and slightly curved, widest at the middle and belong to the hemispherical apex; moreover, these mature conidia are brownish and thin peripheral walls (**Figure 1.10**).

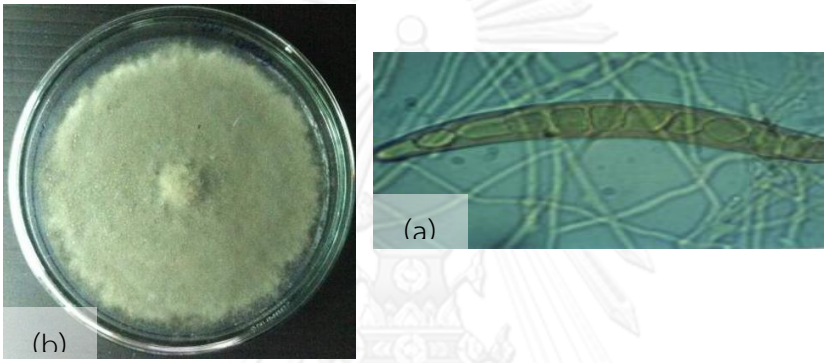


Figure 1.10 Mycelia on PDA (a) and spores (b) of *B. oryzae*

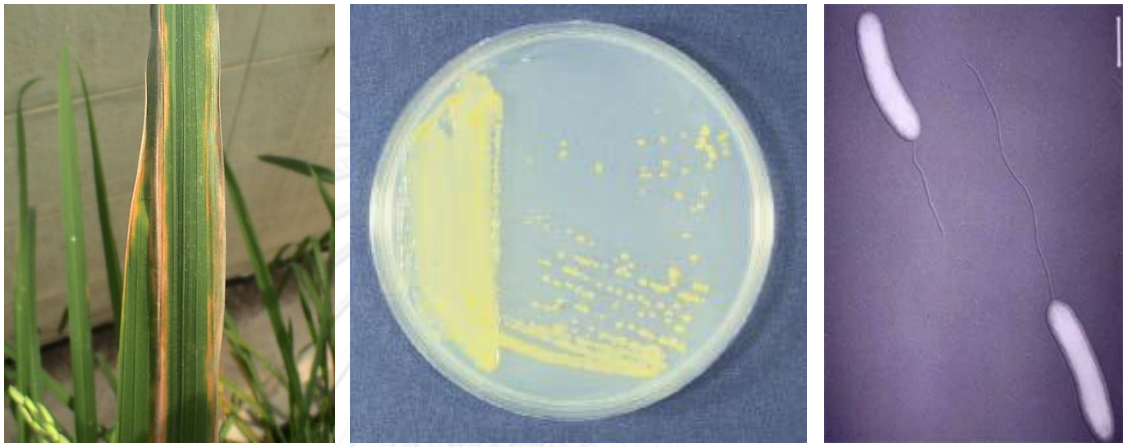
1.3.2 Bacterial rice diseases

Rice production in South East Asia have been reduced by bacterial pathogens such as *Xanthomonas oryzzae* pv. *oryae* (*Xoo*) and pv. *oryicola* (*Xoc*) – gram negative bacteria- that causes a bacterial leaf blight disease (BLB or BB) and bacterial leaf streak (BLS), respectively (Kaur 2004). The appearance colonies of these diseases are similar because the bacterial strains are originated from the same of genus and species. The physiological colonies appear in 1.3.2.1 and 1.3.2.2.

1.3.2.1 Bacterial leaf blight (BLB or BB)

Xoo is the causal agent of BLB (**Figure 1.11a**) (Adhikari et al., 1995, Ochiai et al., 2000). This disease is the most earnest pathogens of worldwide rice and is especially found around both tropical and temperate zones. The disease occurs in Australia, Africa, Latin America, Asia, and Caribbean. The rice yields are loose of 10-50% from leaf blight and it have been reported (Mew 1992). The taxonomic positions are classified in Class: Gammaproteobacteria, Order: Xanthomonodales, and Family:

Xanthomonodaceae. *Xoo* is gram-negative bacterium, non-sporeforming rod and is 0.55 to 0.75 x 1.35 to 2.17 μm in colonial size. The colonies are light yellow, circular, convex and smooth. The yellow water-soluble pigments are produced from this bacterium (**Figure 1.11b**). The xylem of rice hosts can be infected by the bacterium into the systemic stems. There is moved by flagellum (**Figure 1.11c**) and it is aerobic bacteria. The range of optimize temperature for growth reveals 25-30 °C (Nino-Liu 2006).



(a) (b) (c)

Figure 1.**11** The characteristic of BLB (a), colonies (b) and flagella (c)

(Sources: (a) http://www.brrd.in.th/rkb/disease%20and%20insect/index.php-file=content.php&id=120.htm

(b) Mew, 1992

(c) http://bacmap.wishartlab.com/organisms/303)

The disease cycle of BLB is presented in **Figure 1.12**. The rice leaf typically though the hydathodes at the leaf tip and leaf margin are entered by *Xoo*. The bacterial cells are displayed on the rice leaf surface and then suspended in guttation fluid is withdrawn into the leaf in the morning. These cells are increased, infected under the epithelial cells of leaves and spread together with xylem. The multiplied bacterium can be accessed to wounds and emerged roots at the based leaf sheathes that interacts with the xylem parenchyma cells. The pathogens can be moved through the leaf via the primary veins and progressed though commissural veins and then ooze out from hydrathodes (Nino-Liu 2006).

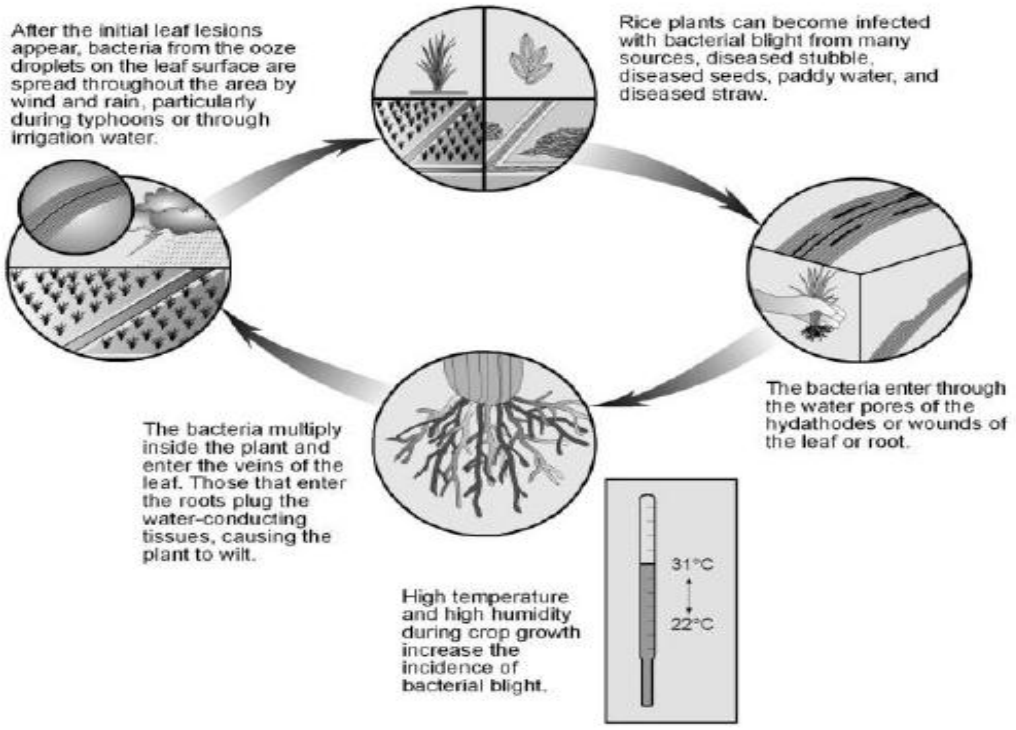


Figure 1.12 Disease cycle of BLB of rice caused by *Xoo*

1.3.2.2 Bacterial leaf streak (BLS)

BLS (**Figure 1.13a**) is caused by *Xoc* and founded in all parts of Thailand – especially distributed around rainy and high humidity area such as Asia, Africa, and Australia. The symptoms might be appearing on the rice leaf; firstly, it can be seen as scratch along with the leaf veins. Secondly, these scratches will gently be changed to yellow of orange color and then scares are thirdly combined to the big scares. This disease can be altogether expanded to leaf sheathes. *Xoc* is classified to Class: Gammaproteobacteria, Order: Xanthomonodales and Family: Xanthomonodaceae. It is a gram-negative, non-spore forming rod, 1.2 x 0.3 to 0.5 µm and a single flagellum. The colonies on NA medium are pale yellow, circular, smooth and convex (**Figure 1.13b**).

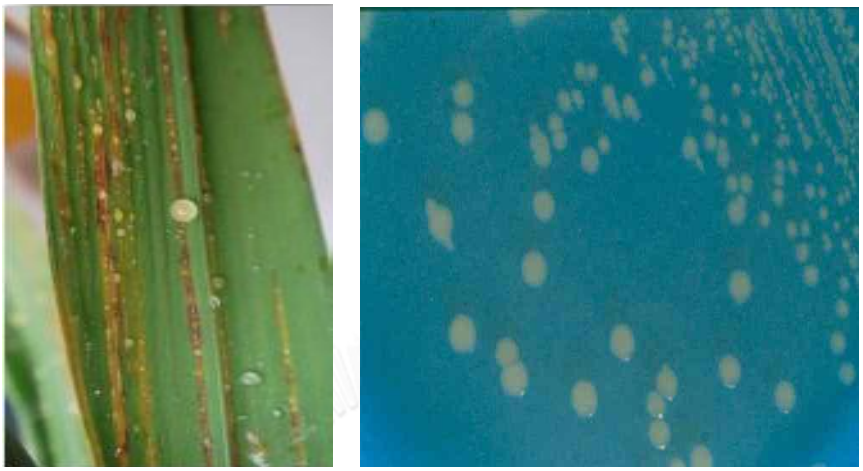


Figure 1.13 The characteristic of BLS (a), colonies (b)

(Source: (b) http://www.knowledgebank.irri.org/smta/importance-of-seed-health-in-seedgermplasm-exchange-mainmenu-84/283.html)

Disease cycle of *Xoc*, it is an intercellular pathogen that enters plants either through wounds or by invading the open stomata. Once inside the plants, *Xoc* multiplies between the mesophyll parenchyma cells, spreading up and down the leaf between the vascular bundles. *Xoc* can invade the host plant xylem tissue, but only at later stages of infection, when multiplication is limited. BLS lesions may begin anywhere on the leaf between the veins as water soaked symptom and extend generally lengthwise throughout the leaf (Vera 2013). Older lesions may extend over veins. The BLS lesion margin is characterized by fine water-soaked streaks (**Figure 1.14).**

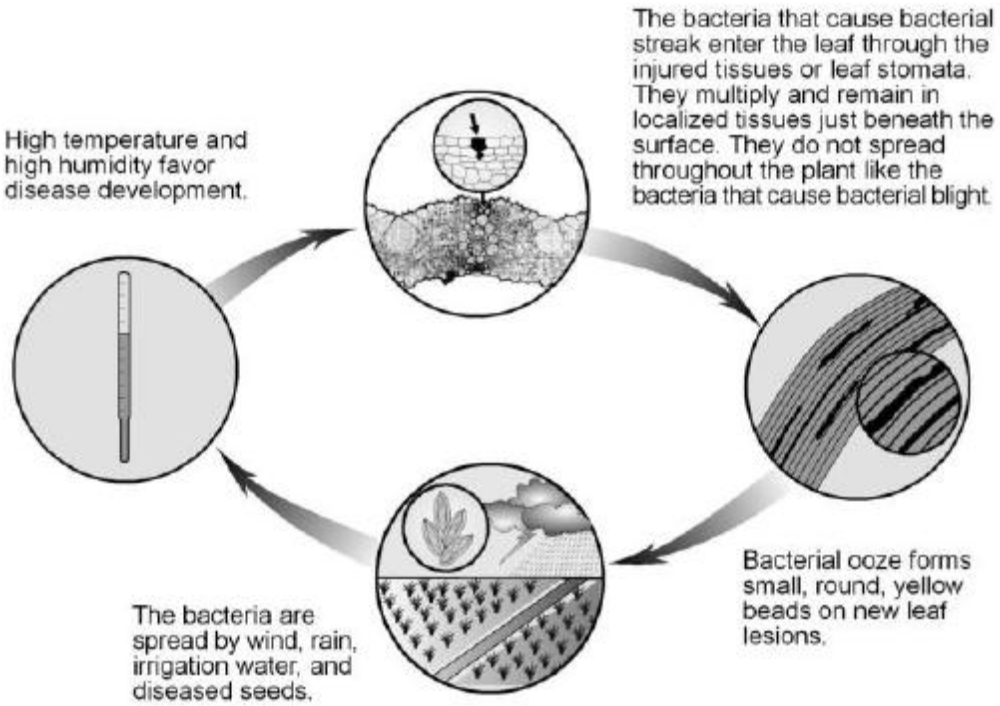


Figure 1.14 Disease cycle of BLB

These pathovar of the bacterial strains are similar characteristics. *Xanthomonas oryzae* has been usually reported about two pathovar *viz Xoo* and *Xoc*. Furutani *et. al*. was studied the different pathovar of the strains (**Table 1.1**) by bacteriological tests.

Table 1. 1 Bacteriological tests for strain identification and differentiation of two pathovars of X. oryzae

	Xoo	*Xoc*
Gram staining	-	-
Oxidase test	-[a]	-[a]
2-ketoglucontate production	-	-
Fluorescence on King's B medium	-	-
Nitrate reduction	-	-
Acetoin production	-	+
Oxidation-fermentation of glucose	O/-	O/-
Gelatin hydrolysis	-/v	-/v
Starch hydrolysis	-	+
Sensitive to 0.001% cupric nitrate (w/v)	+	-
Utilization of L-alanine as carbon source	-	+/v
Growth on 0.2% vitamin-free casamino acids	-	+
Strong peptonization of litmus milk	-	+
Phenylalanine deaminase	-	+[b]

+ = positive; - = negative; O = oxidative; v = variable

[a] Weak positive reactions can be observed. [b] Positive response in 50% of strains.

1.4 Objectives of this research

1. To evaluate anti–phytopathogenic microbial activity from wildbetal leafbush *Piper sarmentosum* Roxb. and Vietnamese coriander *Polygonum odoratum* Lour.

2. To search for bioactive compounds possessing antimicrobial activity against rice diseases

3. To isolate, purify and characterize bioactive compounds from wildbetal leafbush *Piper sarmentosum* Roxb. and Vietnamese coriander *Polygonum odoratum* Lour.

CHAPTER II

PRELIMINARY ANTIMICROBIAL ACTIVITY SCREENING OF THAI PLANTS

2.1 Plant Materials

Nineteen plant species were locally purchased from Pak Khlong Talad market and medicinal shops, Bangkok, Thailand in 2011. All plant samples are displayed in **Table 2.1**.

Table 2.1 Thai plants for antimicrobial activity screening

List	Scientific name	Common name	Plant part
1	Annonaceae *Melodorum fruticosum* L. (ลำดวน)	devil tree, white cheesewood	flower
2	Apocynaceae *Tabernaemontana pandacaqui* Poir. (พุด)	gardenia	flower
3	Asteraceae *Eclipta prostrata* (L.) L. (กะเม็ง)	false daisy	whole plant
4	*Eupotorium odoratum* L. (สาบเสือ)	bitter bush, Siam weed	whole plant
5	Lauraceae *Cinnamomum porrectum* (Roxb.) Kosterm. (เทพธาโร)	citronella laurel	root
6	Myristicaceae *Myristica fragrans* Houtt. (จันทน์เทศ)	nutmeg, mace	seed, flower
7	Myrtaceae *Psidium guajava* Linn. (ฝรั่ง)	guava	leaf
8	Piperaceae *Piper sarmentosum* Roxb. (ชะพลู)	wildbetal leafbush	leaf, stem, fruit
9	Poaceae *Echinochloa crus-galli* (L.) P.Beauv. (หญ้าข้าวนก)	barnyard millet	whole plant
10	*Oryza sativa* L. allelopathy (ข้าว)	rice, paddy rice	whole plant

Table 2.1 (continued)

List	Scientific name	Common name	Plant part
11	Polygonaceae *Polygonum odoratum* Lour. (ผักแพว)	Vietnamese coriander	whole plant
12	*Polygonum tomentosum* Willd. (เอื้องเพ็ดม้า)	west African	whole plant
13	Rubiaceae *Gardenia angusta* (L.) Merr. (พุดซ้อน)	cape gardenia	flower, leaf, twig
14	Rutaceae *Zanthozylum limonella* Alston. (กำจัดต้น หรือมะแขว่น)	ma-khan	fruit
15	Saururaceae *Houttuynia cordata* Thunb. (พลูคาว)	Chinese lizard tail	whole plant
16	Scorphulariaceae *Limnophila aromatica* (Lam.) Merr. (ผักแขยง)	-	whole plant
17	*Otacanthus coeruleus* A. Rose. (บลูฮาวาย)	blue Hawaii	whole plant
18	Solanaceae *Solanum torvum* Sw. (มะเขือพวง)	turkey berry	Fruit
19	Zingiberaceae *Hedychium coronarium* Koenig. (มหาหงส์)	white ginger	Root

2.2 Extraction for antimicrobial activity screening tests

All parts of studied plants were dried, milled to powder, extracted with CH_2Cl_2 and then CH_3OH by Soxhlet apparatus at RT. All extracts were filtered and concentrated using rotary vacuum evaporator. Additionally, some selected plant materials were hydro-distilled for essential oil. The yield of each extract was calculated and collected in **Table 2.2**.

Table 2.2 %Yield of the extraction of studied plants

Species	Plant part	Yield (% w/w)		
		CH_2Cl_2	CH_3OH	Essential oil
Annonaceae *Melodorum fruticosum* L. (ลำดวน)	Flower	-	A	-
Apocynaceae *Tabernaemontana pandacaqui* Poir. (พุดร้อยมาลัย)	Flower	5.17	39.67	-
Asteraceae *Eclipta prostrata* (L.) L. (กะเม็ง)	whole plant	B	B	-
Eupotorium odoratum L. (สาบเสือ)	whole plant	A	A	-
Lauraceae *Cinnamomum porrectum* (Roxb.) Kosterm. (เทพธาโร)	Root	C	C	-
Myristicaceae *Myristica fragrans* Houtt. (จันทน์เทศ)	Seed	-	-	1.15
Myrtaceae *Psidium guajava* Linn. (ฝรั่ง)	Leaf	-	-	Purchase
Piperaceae *Piper sarmentosum* Roxb. (ชะพลู)	leaf	7.30	38.91	0.16
	stem	3.37	6.05	
	fruit	12.00	40.00	
Poaceae *Echinochloa crus-galli* (L.) P.Beauv. (หญ้าข้าวนก)	whole plant	D	D	-
Oryza sativa L.allelopathy (ข้าว)	whole plant	1.80	13.92	-
Polygonaceae *Polygonum odoratum* Lour. (ผักแพว)	whole plant	1.67	10.33	0.03
Polygonum tomentosum Willd. (เอื้องเพ็ดม้า)	whole plant	1.19	7.13	-

Table 2.2 (continued)

Species	Plant part	Yield (% w/w)		
		CH_2Cl_2	CH_3OH	Essential oil
Rubiaceae				
Gardenia angusta (L.) Merr. (พุดซ้อน)	flower	2.07	16.85	Purchase
	leaf	2.20	9.19	
	twig	1.19	10.01	
Rutaceae				
Zanthozylum limonella Alston. (มะแข่น)	Fruit	E	-	8.22
Saururaceae				
Houttuynia cordata Thunb. (พลูคาว)	whole plant	-	-	F
Scorphulariaceae				
Limnophila aromatica (Lam.) Merr. (ผักแขยง)	whole plant	-	-	0.07
Otacanthus coeruleus A. Rose. (บลูฮาวาย)	whole plant	-	-	0.19
Solanaceae				
Solanum torvum Sw. (มะเขือพวง)	Fruit	3.29	16.53	-
Zingiberaceae				
Hedychium coronarium Koenig. (มหาหงส์)	Root	-	-	Purchase

Note: Code A = extracted by Ms. Rachsawan Mongkol
Code B = extracted by Ms. Phijaree Wikitkankosol
Code C = extracted by Ms. Suekanya Jarupinthusophon
Code D = extracted by Ms. Patranit Charoensri
Code E = extracted by Ms. Janpen Tangjitjaroenkun
Code F = extracted by Mr. Veerachai Pongkittiphan

2.3 The screening tests for anti-rice pathogenic microbial activity

The CH_2Cl_2 and CH_3OH extracts, and essential oil from various plant parts were preliminarily screened for antifungal and antibacterial activities using poisoned food technique (Mohana 2007) and agar well diffusion method (Barry 1999), respectively.

2.3.1 Antifungal activity screening

The antifungal activity of the CH_2Cl_2 and CH_3OH extracts, and essential oils was tested against two rice pathogenic fungi, *Rhizoctonia solani* DOAC1406 and *Bipolaris oryzae* DOAC1760. The final concentration of all extracts on poisoned food technique was 1,000 mg/mL and the percentage of mycelia inhibition was calculated as presented in **Table 2.3.**

Table 2.3 Antifungal activity of selected plant extracts and essential oil

Plants	Part	Extract	% Mycelia growth inhibition*,**	
			R. solani	*B. oryzae*
Tabernaemontana pandacaqui	flower	CH_2Cl_2	18.45 ± 2.88^{lm}	43.33 ± 1.67^{g}
		CH_3OH	0.00 ± 0.00^{p}	10.56 ± 3.89^{op}
Gardenia angusta	flower	CH_2Cl_2	35.00 ± 2.22^{fgh}	50.74 ± 1.16^{ef}
		CH_3OH	4.43 ± 0.56^{op}	19.81 ± 5.13^{lm}
	leaf	CH_2Cl_2	14.45 ± 0.96^{mn}	17.04 ± 1.61^{mn}
		CH_3OH	6.48 ± 1.95^{o}	11.29 ± 1.16^{o}
	twig	CH_2Cl_2	21.82 ± 0.86^{l}	4.63 ± 3.69^{qr}
		CH_3OH	0.00 ± 0.00^{p}	24.85 ± 1.32^{jk}
Piper sarmentosum	leaf	CH_2Cl_2	51.77 ± 2.06^{d}	41.45 ± 2.31^{g}
		CH_3OH	0.00 ± 0.00^{p}	11.26 ± 3.09^{o}
Solanum torvum	fruit	CH_2Cl_2	11.22 ± 1.05^{n}	8.36 ± 0.82^{opq}
		CH_3OH	0.00 ± 0.00^{p}	8.18 ± 1.05^{opq}
Limnophila aromatic	whole plant	essential oil	100 ± 0.00^{a}	100 ± 0.00^{a}
Polygonum odoratum	whole plant	essential oil	77.00 ± 0.87^{b}	59.63 ± 0.32^{c}
Otacanthus coeruleus	whole plant	essential oil	100 ± 0.00^{a}	100 ± 0.00^{a}
Piper sarmentosum	leaf	essential oil	100 ± 0.00^{a}	100 ± 0.00^{a}
Oryza sativa (allelopathy)	whole plant	CH_2Cl_2	30.37 ± 1.40^{ijk}	49.26 ± 4.97^{ef}
		CH_3OH	30.37 ± 2.31^{ijk}	47.78 ± 0.56^{f}
Cinnamomum porrectum	root	essential oil	100 ± 0.00^{a}	100 ± 0.00^{a}

Table 2.3 (continued)

Plants	Part	Extract	% Mycelia growth inhibition*,**	
			R. solani	*B. oryzae*
Polygonum odoratum	whole plant	CH_2Cl_2	19.63 ± 1.95^{l}	21.48 ± 1.16^{kl}
		CH_3OH	0.00 ± 0.00^{p}	0.00 ± 0.00^{s}
Polygonum tomentosum	whole plant	CH_2Cl_2	36.11 ± 2.00^{fgh}	18.33 ± 7.71^{lmn}
		CH_3OH	17.96 ± 10.43^{lm}	18.89 ± 6.19^{lmn}
Cinnamomum porrectum	root	CH_2Cl_2	75.89 ± 0.10^{b}	52.30 ± 0.10^{de}
		CH_3OH	19.11 ± 0.11^{l}	15.36 ± 0.03^{n}
Eupotorium odoratum	whole plant	CH_2Cl_2	33.56 ± 0.40^{ghi}	6.67 ± 0.03^{pq}
		CH_3OH	0.00 ± 0.00^{p}	0.00 ± 0.00^{s}
Melodorum fruticosum	flower	CH_3OH	43.56 ± 0.01^{e}	20.56 ± 0.10^{lm}
Echinochloa crus-galli	whole plant	CH_2Cl_2	37.59 ± 4.21^{fg}	0.56 ± 0.96^{s}
		CH_3OH	0.00 ± 0.00^{p}	1.67 ± 0.56^{rs}
Eclipta prostrate	whole plant	CH_2Cl_2	28.70 ± 1.16^{jk}	31.48 ± 3.06^{i}
		CH_3OH	0.00 ± 0.00^{p}	31.67 ± 0.56^{i}
Gardenia angusta	fruit	CH_2Cl_2	26.85 ± 6.99^{k}	27.04 ± 0.85^{j}
		CH_3OH	0.00 ± 0.00^{p}	6.48 ± 1.40^{pq}
Myristica fragrans	fruit	essential oil	76.85 ± 0.32^{b}	42.59 ± 0.64^{g}
Tabernaemontana pandacaqui	flower	essential oil	100 ± 0.00^{a}	76.30 ± 0.32^{b}
Hedycium coronarium	root	essential oil	100 ± 0.00^{a}	77.59 ± 0.85^{b}
Psidium guajava	leaf	essential oil	74.82 ± 2.31^{bc}	55.74 ± 1.79^{d}
Piper sarmentosum	fruit	CH_2Cl_2	71.11 ± 0.56^{c}	60.74 ± 1.28^{c}
		CH_3OH	32.59 ± 4.45^{hij}	35.56 ± 2.23^{h}
	stem	CH_2Cl_2	39.07 ± 2.24^{f}	36.11 ± 0.96^{h}
		CH_3OH	27.96 ± 3.35^{k}	42.78 ± 1.67^{g}
Houttuynia cordata	whole plant	Essential oil	100 ± 0.00^{a}	40.19 ± 2.51^{g}

*Means of three replicate plates for % inhibition for each species

**Values of each species followed by the same letter were not significantly different based on Duncan's multiple – range test ($P \geq 0.05$).

The preliminary screening results for anti-fungal mycelial growth inhibition against *R. solani* revealed that seven essential oils including *L. aromatic*, *O. coeruleus*, *P. sarmentosum*, *C. porrectum*, *T. pandacaqui*, *H. coronarium* and *H. cordata* exhibited complete inhibition. The essential oils from *P. odoratum*, *M. fragrans*, *P. guajava* and the CH_2Cl_2 extracts from *C. porrectum*, *P. sarmentosum* (fruit and leaf) displayed moderate antifungal activity against this fungus.

For *B. oryzae*, four essential oils *viz. L. aromatic*, *O. coeruleus*, *P. sarmentosum* and *C. porrectum* exhibited completely inhibition. Moreover, the essential oils from *H. coronarium*, *T. pandacaqui*, *P. odoratum* and *P. guajava* and the CH_2Cl_2 extracts from *P. sarmentosum* (fruit), *C. porrectum* and *G. angusta* (flowers) showed moderate mycelia inhibition. According to literature reviews, Bhuyan (2010) found that the mycelia growth of these fungi was strongly controlled by the essential oils from *Lippia geminata* (IC_{50} = 420 and 481 ppm) and *Cymbopogon jwarancusa* (IC_{50} = 365 and 337 ppm). The growth of *R. solani* was inhibited by essential oils of *Origanum minutiflorum* (100%), *Satureja cuneifolia* (100%), *Thymbra spicata* (99.6%) (Tugba 2006) and *Brassica rapa* (Dhingra 2004). Udomsilp (2009) have reported that both fungi were completely inhibited by cassia oil (*Acacia farnesiana* Linn.).

In addition, the CH_2Cl_2 extracts of *C. porrectum* and *P. sarmentosum* (fruit and leaf) displayed high inhibition on both fungi. *P. sarmentosum* extracts from fruit and leaf have not been reported about antifungal activity. Therefore, this plant was selected for further investigating for bioactive compounds with antifungal activity.

2.3.2 Antibacterial activity screening

The antibacterial activity screening of CH_2Cl_2 and CH_3OH extracts, and essential oils from various Thai plants was tested against two important pathogenic bacteria of rice: *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and *X. oryzae* pv. *oryzicola* (*Xoc*) at the final concentration 10,000 ppm of 40 µL/agar well using agar well diffusion method. The average of diameter of the inhibition zone was assessed as represented in **Table 2.4**.

Table 2.4 Antibacterial activity of selected plant extracts and essential oil

Plants	Part	Extract	Average of clear zone (mm)[*,**]	
			Xoo	*Xoc*
Tabernaemontana pandacaqui	flower	CH_2Cl_2	0.00[e]	0.00[f]
		CH_3OH	0.00[e]	0.00[f]
Gardenia angusta	flower	CH_2Cl_2	0.00[e]	0.00[f]
		CH_3OH	0.00[e]	0.00[f]
	leaf	CH_2Cl_2	0.00[e]	0.00[f]
		CH_3OH	0.00[e]	0.00[f]
	twig	CH_2Cl_2	11.30±0.12[a]	15.10±0.14[ab]
		CH_3OH	0.00[e]	0.00[f]
Piper sarmentosum	leaf	CH_2Cl_2	6.80±0.15[b]	0.00[f]
		CH_3OH	0.00[e]	0.00[f]
Solanum torvum	fruit	CH_2Cl_2	10.50±0.35[a]	11.50 ± 0.10[bc]
		CH_3OH	4.50±0.24[bcd]	0.00[f]
Limnophila aromatic	whole plant	essential oil	6.83±0.29[b]	3.67 ± 1.53[e]
Polygonum odoratum	whole plant	essential oil	7.17±1.04[b]	11.67 ± 3.51[bc]
Otacanthus coeruleus	whole plant	essential oil	0.00[e]	0.00[f]
Piper sarmentosum	leaf	essential oil	5.50±0.05[bc]	4.00±0.10[e]
Oryza sativa (allelopathy)	whole plant	CH_2Cl_2	0.00[e]	0.00[f]
		CH_3OH	0.00[e]	0.00[f]
Cinnamomum porrectum	root	essential oil	0.00[e]	0.00[f]
Polygonum odoratum	whole plant	CH_2Cl_2	2.30±0.04[d]	3.00±0.10[e]
		CH_3OH	1.50±0.21[d]	1.50±0.25[e]
Polygonum tomentosum	whole plant	CH_2Cl_2	0.00[e]	0.00[f]
		CH_3OH	2.30±0.18[d]	5.00±0.00[de]

Table 2.3 (continued)

Plants	Part	Extract	Average of clear zone (mm)*,**	
			Xoo	*Xoc*
Cinnamomum porrectum	root	CH_2Cl_2	0.00^{e}	0.00^{f}
		CH_3OH	0.00^{e}	0.00^{f}
Eupotorium odoratum	whole plant	CH_2Cl_2	0.00^{e}	0.00^{f}
		CH_3OH	0.00^{e}	0.00^{f}
Melodorum fruticosum	flower	CH_3OH	0.00^{e}	0.00^{f}
Echinochloa crus-galli	whole plant	CH_2Cl_2	0.00^{e}	0.00^{f}
		CH_3OH	0.00^{e}	0.00^{f}
Eclipta prostrate	whole plant	CH_2Cl_2	0.00^{e}	0.00^{f}
		CH_3OH	0.00^{e}	0.00^{f}
Gardenia angusta	fruit	CH_2Cl_2	0.00^{e}	0.00^{f}
		CH_3OH	0.00^{e}	0.00^{f}
Myristica fragrans	seed	essential oil	0.00^{e}	0.00^{f}
Tabernaemontana pandacaqui	flower	essential oil	0.00^{e}	0.00^{f}
Hedycium coronarium	root	essential oil	0.00^{e}	0.00^{f}
Psidium guajava	leaf	essential oil	0.00^{e}	0.00^{f}
Piper sarmentosum	fruit	CH_2Cl_2	13.25±2.47^{a}	16.25±1.06^{a}
		CH_3OH	3.00±1.41^{cd}	8.50±0.71^{cd}
	stem	CH_2Cl_2	2.25±0.35^{d}	13.50±2.12^{ab}
		CH_3OH	3.00±1.41^{cd}	2.50±0.71^{e}
Houttuynia cordata	whole plant	essential oil	5.75±1.06^{bc}	4.50±0.71^{de}

* Means of two replicate plates for % inhibition for each species

**Values of each species followed by the same letter were not significantly different based on Duncan's multiple – range test ($P \geq 0.05$).

The CH_2Cl_2 extracts of *P. sarmentosum* (fruit) and *G. angusta* (twig) showed the highest antibacterial activity with a maximum average of clear zone against *Xoo*

(13.25 and 11.30 mm) and *Xoc* (16.25 and 15.1 mm) at 40 µL/well. *Xoo* was strongly controlled by the CH_2Cl_2 extract of *S. torvum* (10.50 mm), the essential oils of *P. odoratum* (7.17 mm), *L. aromatic* (6.83 mm), the CH_2Cl_2 extract of *P. sarmentosum* (leaf) (6.80 mm), the essential oils of *H. cordata* (5.75 mm) and *P. sarmentosum* (5.50 mm); moderately controlled by the CH_3OH extract of *S. torvum* (4.50 mm). In the case of *Xoc*, the antibacterial activity was crucially affected by the CH_2Cl_2 extract from the stems of *P. sarmentosum* (13.50 mm), the essential oil from *P. odoratum* (11.67 mm), the CH_2Cl_2 extract from *S. torvum* (11.50 mm), the CH_3OH extract from *P. sarmentosum* (8.50 mm) and *P. odoratum* (5.00 mm); moderately affected by the essential oils from *H. cordata, P. sarmentosum* and *L. aromatic* with 4.50, 4.00 and 3.67 mm, respectively.

Many previous reports were addressed for antibacterial activity from natural sources. For example, Kagale (2004) found that anti-*X. oryzae* pv. *oyzae* activity was affected by the CH_3OH extract of Datura metal leaves. Atiax et al., (2011) reported that the aerial part extract of *P. sarmentosum* could inhibit on Gram-negative bacteria including *Pseudomonas aeruginosa* and *E. coli*. Moreover, Pukdeekumjorn (2012) reported that 50 and 95% EtOH extracts of woods displayed antibacterial activity against *S. aureus* ATCC 25923, *Salmonella typhi* ATCC 14028, *B. subtilis* ATCC 6633 and *E. coli* ATCC 25922 with inhibition zone range of 8-10 mm and related the range of total phenolic content values of 118.18-189.88 mg GAE/g. Nutmeg and mace of 85% CH_3OH extracts displayed anti-food-borne bacterial activities against *S. aureus* and *B. cereus* with MIC of 50 µg/mL, and some phenolic compounds were found (Sulaiman 2012). In addition, water, CH_3OH and $CHCl_3$ extracts of dry guava leaves inhibited *S. aureus* and β-*streptococcus* group A ($P<0.01$) (Jaiarj et al., 1999).

2.4 Experimental section

2.4.1 Extraction of plant materials

All parts of studied plants were oven dried, milled and extracted with CH_2Cl_2 and then CH_3OH for three times. The extracts were filtered, evaporated and kept for antimicrobial activities. The fresh plant (200 g) was hydro-distilled using a modified Dean-Stark apparatus. After extraction with Et_2O, the extract was evaporated under reduced pressure in rotatory evaporator. The oil yield and all test data are the average of triplicate analyses (Ho 2012).

2.4.2 Preliminary bio-screening tests for antibacterial activity

Antibacterial activity of CH_2Cl_2 and CH_3OH extracts, and essential oil was tested against two gram negative bacteria of rice pathogenic bacteria: *Xoo* and *Xoc*. The agar diffusion method (Barry 1999) was used to screen the antibacterial activity of plant essential oil and all compounds. Initially, two bacterial strains (*Xoo* and *Xoc*) were cultivated in nutrient agar (NA) and incubated at 37 °C for 18–24 h. Selected 4–5 single colonies of each tested bacteria were cultivated in nutrient broth (NB) and incubated at 37 °C for 2-5 h. Freshly cultured bacterial suspensions in NB were standardized to cell density of 1.5×10^8 CFU/mL (McFarland No. 0.5) and adjusted by 0.85% sterile NaCl. Each tested NA media (19 mL) was mixed with adjusted bacterial suspension (1 mL), poured into petri plates, and then allowed to set. The tested culture plates created the wells using 6 mm cork border, added 40 µL of dissolved extract and DMSO (control) into these wells, incubated the plates at 37 °C overnight, and also recorded the zones of inhibition in duplicate.

2.4.3 Preliminary bio-screening tests for antifungal activity

Antifungal activity of all extracts was tested against two rice pathogenic fungi including *R. solani* and *B. oryzae*. The extract and major constituents were dissolved in DMSO and 100 µL was added to PDA to execute a final concentration of 1,000 mg/L. A 5 mm agar disc containing mycelia was transferred to the center of the PDA plate containing the extract or compounds. Plates were incubated at 25 °C for 3 days (*R. solani*) and 8 days (*B. oryzae*). 1% DMSO was used as negative control. When the mycelium of fungi reached the edges of the control petri dishes (those without extracts), the antifungal indices were calculated. The formula of antifungal indices is shown as **Eq 2.1**.

$$\text{Percentage inhibition} = \frac{(C - T) \times 100}{C} \quad \% \qquad \text{(Eq 2.1)}$$

Where, C = colony diameter (cm) of the control.

T = colony diameter (cm) of the test plate.

2.4.4 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.

CHAPTER III

ANTI-MICROBIAL CONSTITUENTS FROM *Piper sarmentosum* Roxb.

3.1 Introduction

3.1.1 Botanical description

P. sarmentosum distributes around the tropical areas in southern China, India, Cambodia, Indonesia, Laos, Philippines, Malaysia, Vietnam and Thailand (Jensen 1999, Mathew 2004). It is a member of the family Piperaceae used in many Southeast Asia cuisines as far as the Andaman Islands. It, locally known as "Cha–plu", is a glabrous, creeping terrestrial herb about 20 cm tall (**Figure 3.1**). The plant and fruits are used in Thailand as an expectorant (Rukachaisirikul et al., 2004), additionally; leaves were traditionally used in many South Asian cuisines (Raman 2012).

(a) (b) (c)

Figure 3.1 *P. sarmentosum*, leaves (a), mature plant (b) and fruits (c)

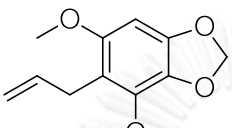
(Sources: (a) http://watprodesktop.blogspot.com/2011/03/blog-post_17.html,
(b) http://en.academic.ru/dic.nsf/enwiki/11173015,
(c) http://toptropicals.com/catalog/uid/piper_sarmentosum.htm)

Various biological activities including pharmacological study (Hussain 2009), neuromuscular blocking study (Ridtitid et al., 1998), antioxidant study (Hafizah et al., 2010), antitumor study (Atiax et al., 2011), antitermite study (Chieng 2008), antifeedant study (Qin 2010), antimalarial study (Rahman 1999), anti-inflammatory study (Zakaria 2010) antiplasmodial and antituberculosis study (Rukachaisirikul et al., 2004) have been addressed.

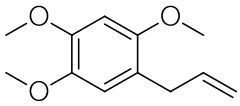
3.1.2 Chemical investigation and biological activities

Masuda (1991) investigated the chemical constituents of the leaves and identified as 1-allyl-2,6-dimethylenedioxybenzene (**1**), 1-allyl-2,4,5-trimethoxy benzene (**2**), 1-(1-*E*-propenyl)-2,4,5-trimethoxybenzene (**3**) and 1-allyl-2-methoxy-4,5-methylene-dioxybenzene (**4**). These compounds revealed antimicrobial activity against *E. coli* and *B. subtilis*.

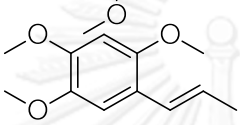
(1)



(2)



(3)



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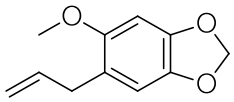
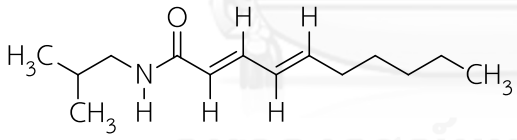


Rukachaisirikul et al., (2004) reported that the chemical compositions of the hexane and MeOH extracts of fruits were identified as pellitorine (**5**), guineensine (**6**), brachystamide B (**7**), sarmentine (**8**), 1-piperettyl pyrrolidine (**9**), brachyamide B (**10**), 3′,4′,5′-trimethoxycinnamoyl pyrrolidine (**11**), sarmentosine (**12**), (+)-asarinin (**13**), sesamin (**14**), 1-(3,4-methyleneoxyphenyl)-1*E*-tetradecene (**15**), methyl piperate (**16**) and a mixture of β-sitosterol (**17**) and stigmasterol (**18**). Sarmentine (**8**) and 1-piperettyl pyrrolidine (**10**) showed antituberculosis and antiplasmodial activities.

(5)



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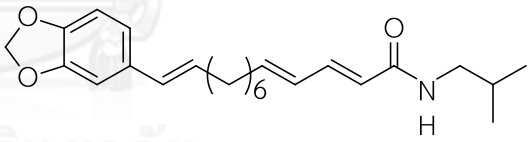

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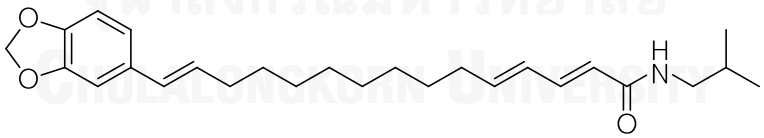

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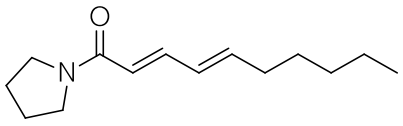

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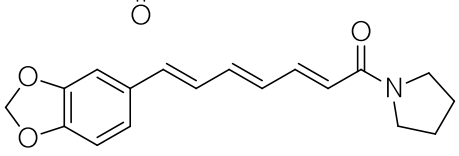

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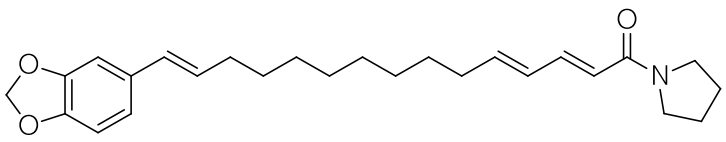
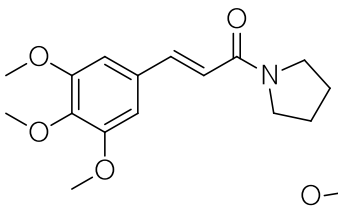
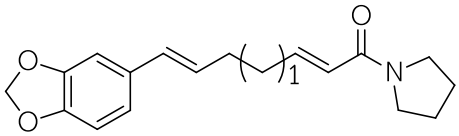
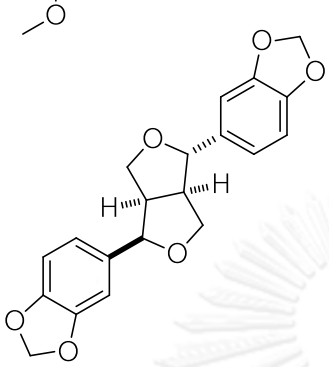
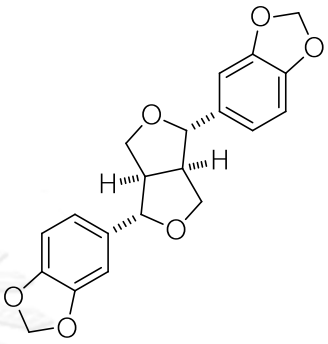
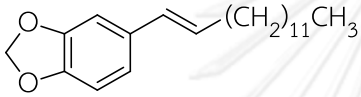


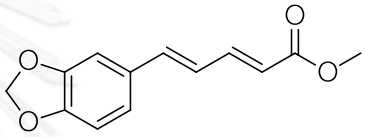
(**11**) 

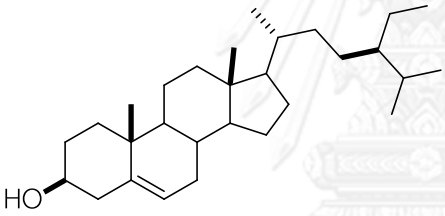
(**12**) 

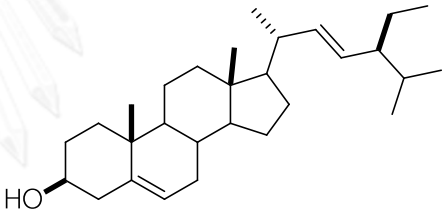
(**13**) 

(**14**) 

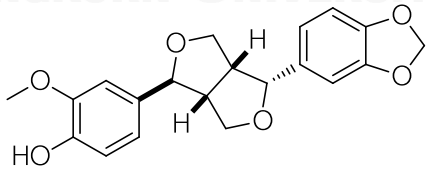
(**15**) 

(**16**) 

(**17**) 

(**18**) 

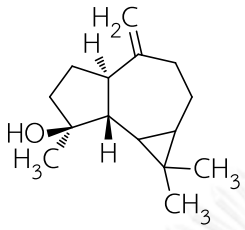
Tuntiwachwuttikul (2006) identified sixteen chemical constituents from the roots as 1-allyl-2-methoxy-4,5-methylene-dioxybenzene (**4**), β-sitosterol (**17**), sarmentine (**8**), sarmentosine (**12**), pellitorine (**5**), (+)-sesamin (**14**), horsfieldin (**19**), guineensine (**6**) and brachystamide B (**7**). All compounds showed antiplasmodial, antimycobacterial and antifungal activities.

(19) 

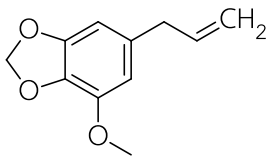
Chieng (2008) studied the toxicity and antitermite activities of the leave essential oil. GC-MS analyzes of this oil revealed the presence of 31 components; spathulenol (**20**) (21.0%), myristicin (**21**) (18.8%), *trans*-caryophyllene (**22**) (18.20%) and (*E*,*E*)-farnesol (**23**) (10.5%) as the major compounds. The crude oil displayed anti-larvae activity against *Artemia salina* with LC_{50} value of 35.2 µg/mL and 100%

mortality for two days at 1% concentration against the subterranean termite (*Coptotermes* sp.). In addition, *trans*-caryophyllene (**22**) and myristicin (**21**) were indentified from crude oil extract and myristicin (**21**) revealed the potent larvae inhibition activity against *A. salina* with LC_{50} of 7.5 µg/mL.

(**20**)



(**21**)



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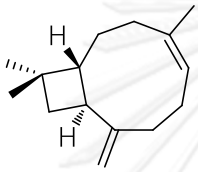
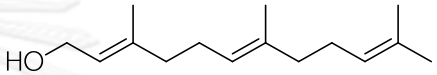


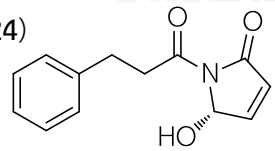
(**23**)



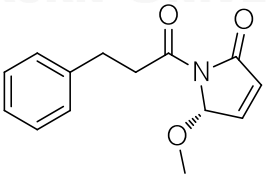
Qin (2010) showed the results of antifeedant activity from the essential oil on *Brontispa longissima*. The eggs and pupae stage of lavae could be controlled by essential oil within 43.34 d (2,000 mg/L). The major compositions of this oil were analyzed as myristicin (**21**) (65.2%) and *trans*-caryophyllene (**22**) (13.9%) using GC-MS. Additionally, myristicin (**21**) showed the strong antifeedant and contact toxicity effects on the 3rd stage of *B. longissima* larvae.

Damsud (2013) reported that three new phenylpropanoyl amides from the CH_2Cl_2 extract were identified as chaplupyrrolidones A (**24**) and B (**25**) and deacetylsarmentamide B (**26**). Chaplupyrrolidones B (**25**) displayed the most potent inhibition against α-glucosidase.

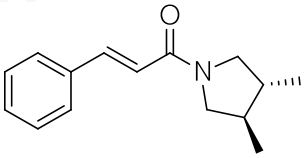
(**24**)



(**25**)



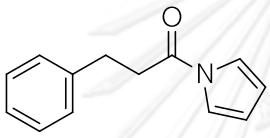
(**26**)

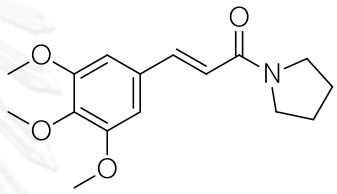


The CH_3OH extract of the leaves also exhibited potential antibacterial activity against both gram positive *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA) and gram negative *Pseudomonas aeruginosa* (Zaidan 2005).

Therefore, it can be seen that a number of phytochemical investigations of the genus *Piper* revealed the presence of various physiologically active chemical constituents including unsaturated amides, flavonoids, lignans, aristolactams, long and short chain esters, terpenes, steroids, prophenylphenols, and alkaloids (Navickiene et al., 2000, Parmar 1997). The chemical constituents of various parts of *P. sarmentosum* have also been reported.

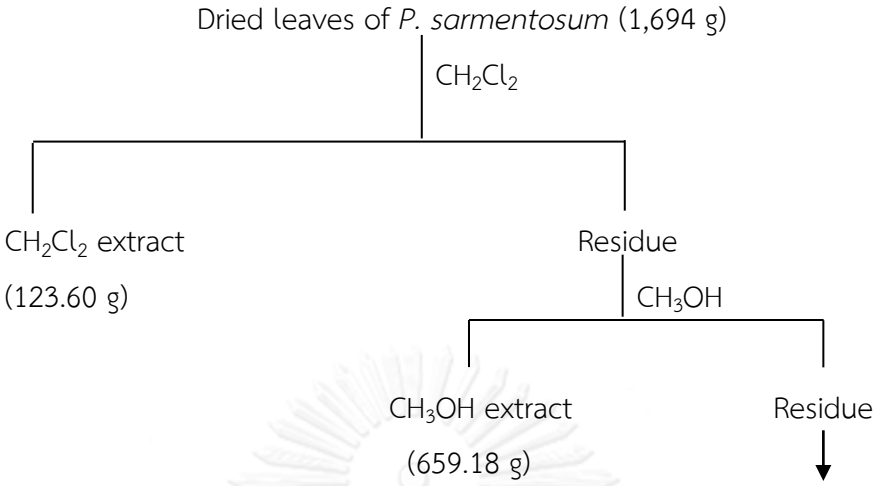
Atiax et al., (2011) investigated the phytochemical and antibacterial activities of *P. sarmentosum*. *N*-(3-phenylpropanoyl) pyrrole (**27**), 3-(3′,4′,5′-trimethoxyphenylpropanoyl) pyrrolidine (**28**) and β-sitosterol (**17**) showed inhibition against *E. coli*; additionally, the hexane and EtOAc extracts were also active on this bacterium.

(**27**) 

(**28**) 

3.2 Extraction of *P. sarmentosum*

The fresh leaves of *P. sarmentosum* were purchased from Pak Klong Talad Market, Bangkok in 2012. The fresh leaves (10 kg) were dried in the open air for two weeks and powdered using electronic blender. The dried leave powder (1,694 g) was firstly extracted by maceration with CH_2Cl_2 at RT for seven days and then filtered. The process was repeated for three times. The leave residue was continually extracted with CH_3OH. Both extracts were concentrated by rotary evaporator under reduced pressure and gave dark brown crude extract 123.6 g (7.3% wt by wt) of CH_2Cl_2 extract and bottle green crude extract 659.2 g (38.9% wt by wt) of CH_3OH extract (**Scheme 3.1**).



Scheme 3.1 The extraction of the leaves of *P. sarmentosum*

For the fruit, the dried and powdered fruits (5 kg) were extracted with CH_2Cl_2 using the procedure as above-mentioned to yield the CH_2Cl_2 extract as viscous brown extract 600.0 g (12.0% wt by wt). For CH_3OH extract, it was not used for further separation because it revealed lower antimicrobial activities than CH_2Cl_2 extract (**CHAPTER II**).

3.3 Fractionation and anti-rice pathogenic fungal activity of the CH_2Cl_2 leave extract

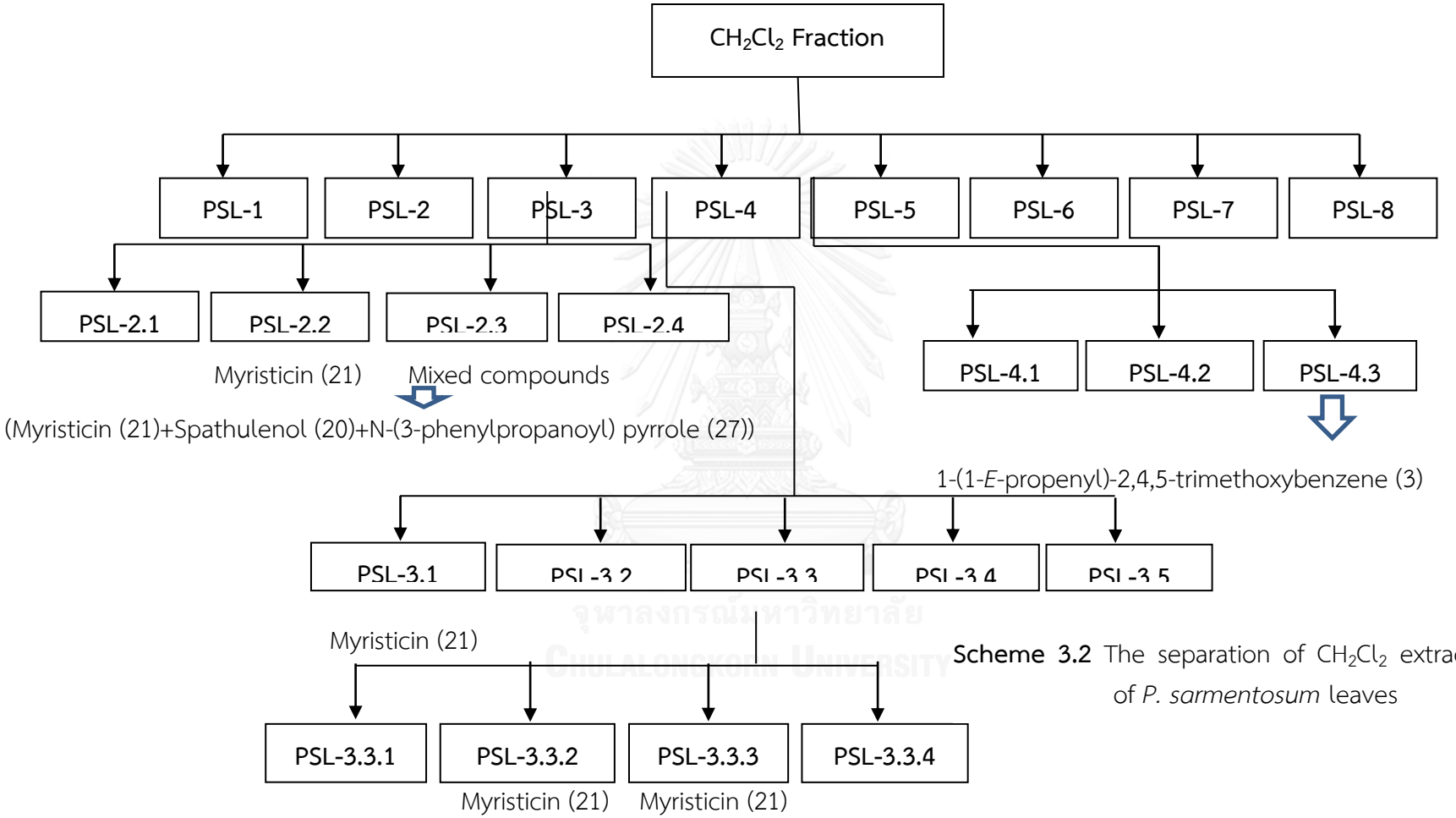
3.3.1 Fractionation

The CH_2Cl_2 leave extract exhibited better antifungal activity than the CH_3OH extract (**CHAPTER II**). Thus, this effective extract was subjected to silica gel quick column eluting with increasing solvent polarity of hexane, hexane-EtOAc, EtOAc and EtOAc-MeOH. Each fraction was examined and combined according to TLC to yield eight fractions (**Table 3.1**, **Scheme 3.2**).

Table 3.1 Fractionation of the CH_2Cl_2 extract of the leaves of *P. sarmentosum*

Fraction	Solvent system	Remarks	Weight (g)
PSL-1	100% hexane	canary yellow oil	1.66
PSL-2	100% hexane - 5%EtOAc	orange liquid	6.46
PSL-3	5-10% EtOAc-hexane	dark brown sludge	19.88
PSL-4	10-20% EtOAc-hexane	dark yellow liquid	2.56
PSL-5	20-60% EtOAc-hexane	ocher solid	2.56
PSL-6	60-100% EtOAc-hexane	brownish black liquid	19.36
PSL-7	60-100% EtOAc-hexane	amber liquid	9.32
PSL-8	100% EtOAc – 10% MeOH-EtOAc	cinnamon liquid	3.71

Among eight fractions, fractions **PSL-3** and **-6** gave the highest yield as 19.88 and 19.36 g, respectively.



Scheme 3.2 The separation of CH_2Cl_2 extracts of *P. sarmentosum* leaves

3.3.2 Antifungal activity assay

1,000 ppm of eight fractions separated from the CH_2Cl_2 extract and the original CH_2Cl_2 extract were tested against both rice pathogenic fungi *R. solani* and *B. oryzae*. The percentage of mycelia growth inhibition is presented in **Figures 3.2-3.3**.

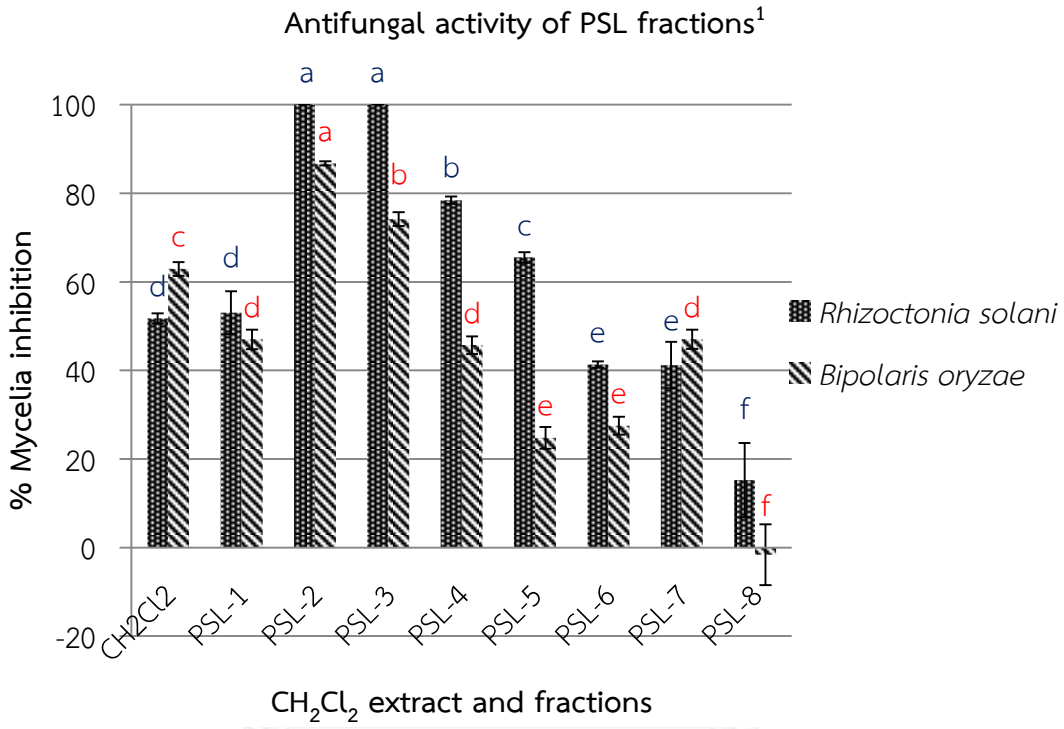


Figure 3.2 Mycelia growth inhibition of rice pathogenic fungi of CH_2Cl_2 extract and its fractions

[1]Bar charts of each fungus followed by the same letter were not significantly ($P \geq 0.05$) difference according to Duncan's multiple comparison tests.

According to **Figures 3.2**, **PSLs -2** and **-3** significantly displayed complete antifungal activities against *R. solani*; on the other hand, **PSL-8** showed the least mycelia growth inhibition. Additionally, **PSLs -1**, **-4** and **-5** revealed higher activity than the CH_2Cl_2 extract. For *B. oryzae*, **PSLs -2** and **-3** represented higher mycelia inhibition than other fractions and the CH_2Cl_2 crude extract, while **PSL-8** showed the lowest mycelia inhibition. **PSLs -2**, **-3** and **-4** were thus selected for further separation and antifungal activity test.

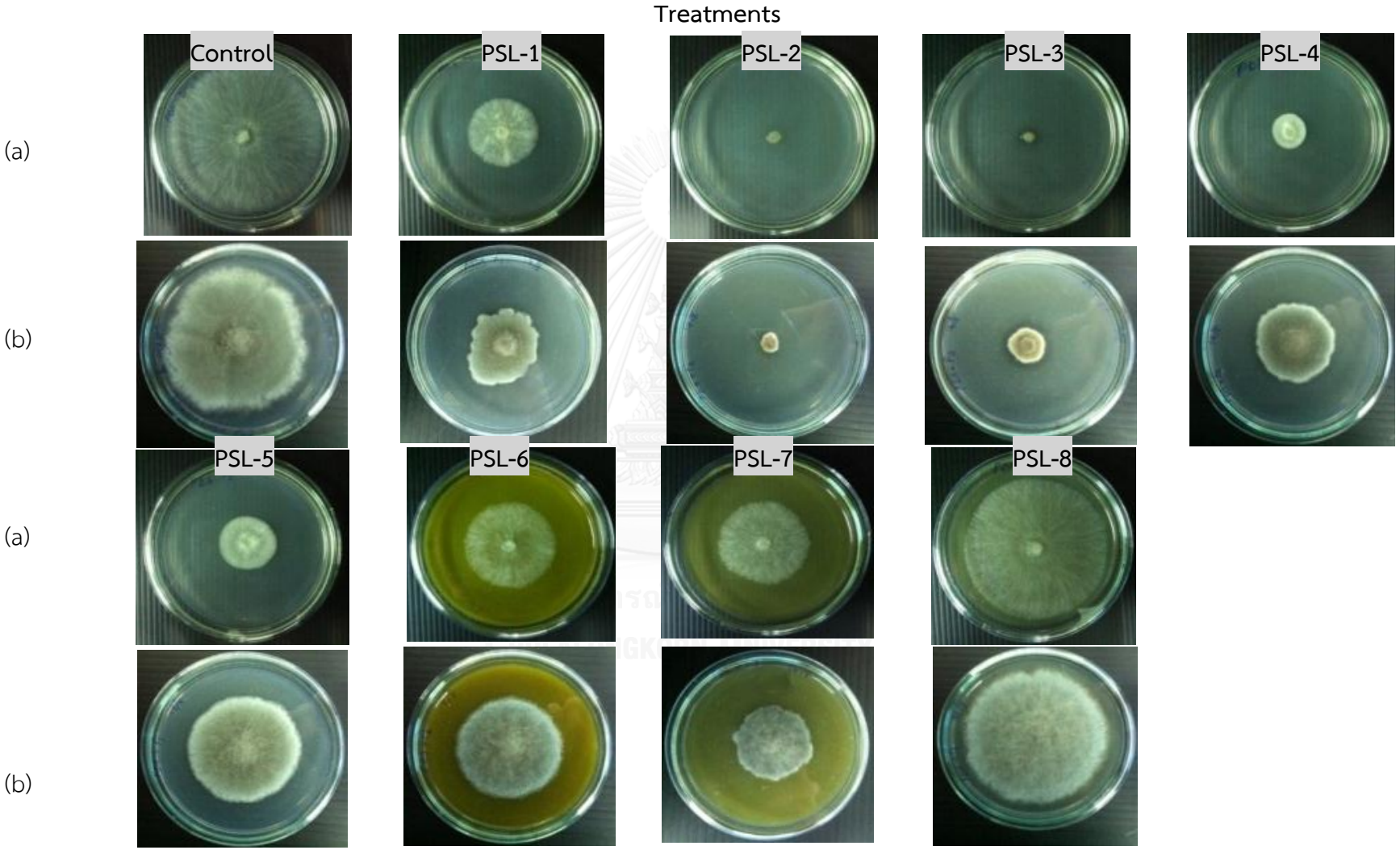


Figure 3.3 The mycelia growth inhibition of control, the CH_2Cl_2 extract and its fractionations against *R. solani* (a) and *B. oryzae* (b)

3.3.3 Separation, antifungal activity and structural elucidation of bioactive compounds

3.3.3.1 Separation of PSL-2

The selected fraction (**PSL-2**) was separated using silica gel column. Each portion was examined by TLC using 10% CH_2Cl_2 in hexane as a developing solvent. The results of the separation of **PSL-2** are presented in **Table 3.2** and **Scheme 3.2**.

Table 3. 2 The separation of **PSL-2** by siliga gel column

Fraction	Solvent system	Remarks	Weight (g)
PSL-2.1	Hexane	Lutescent liquid	0.66
PSL-2.2	hexane - 5% EtOAc in hexane	Yellow liquid	2.13
PSL-2.3	5% EtOAc in hexane	Darkened yellow viscous liquid	0.64
PSL-2.4	5 – 10% EtOAc in hexane	Canary yellow viscous liquid	0.13

The separation of **PSL-2** by column chromatograph gave 4 fractions. **PSL-2.2** gave the highest yield of 42.60%.

Table 3.3 Mycelia growth inhibition of rice pathogenic fungi of **PSL-2**

Fractions	%inhibition ± SD[a,1]	
	R. solani	*B. oryzae*
PSL-2.1	28.67 ± 6.94[b]	15.74 ± 3.70[d]
PSL-2.2	100.00 ± 0.00[a]	100.00 ± 0.00[a]
PSL-2.3	100.00 ± 0.00[a]	74.44 ± 0.56[b]
PSL-2.4	100.00 ± 0.00[a]	70.19 ± 1.15[c]

[a]Value, an average ± standard deviation of 3 replicates, of mean mycelia growth inhibition of both fungi species
[1]Row values followed by the same letter were not significantly (P ≥ 0.05) difference according to Duncan's multiple comparison tests.

According to antifungal activity results, **PSL-2.2** displayed completely mycelia growth inhibition on both fungi at 1,000 ppm. **PSLs-2.2**, **2.3** and **2.4** did not reveal

significant inhibition on mycelia growth of *R. solani* as well as these fractions was highly combated on *B. oryzae* mycelia growth. The result is presented in **Table 3.3** and **Figure 3.4**.

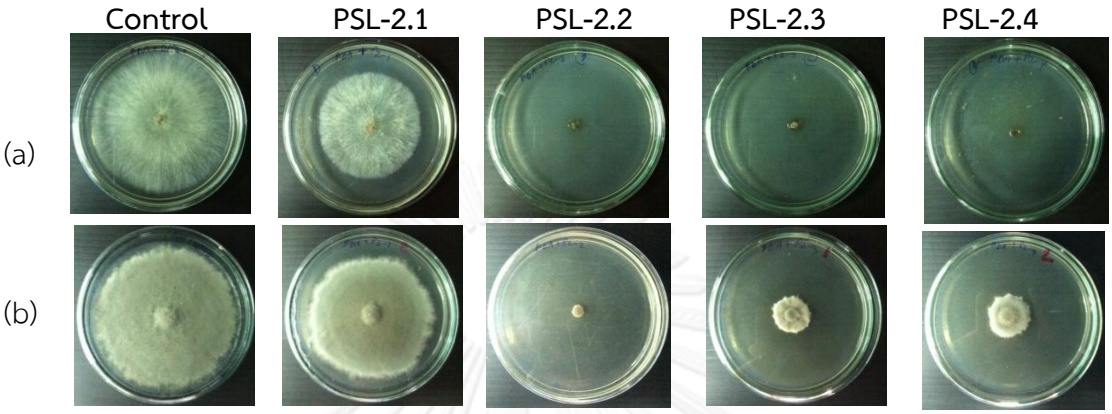


Figure 3. 4 The mycelia growth inhibition of control, **PSL-2.1 -2.4** at 1,000 ppm against *R. solani* (a) and *B. oryzae* (b)

GC analysis of **PSLs-2.2** and **2.3** was performed (**Figure 3.5**). **PSL-2.2** displayed only one peak at R_t 13.67 min **(Figure 3.5 (a))**. **PSL-2.3** showed three peaks at R_t 13.73, 15.08 and 17.40 min **(Figure 3.5 (b))**. It could be observed that the peak at R_t ≈ 13.6 min was common in all three subfractions. **PSL-2.3** was subjected to GC/MS analysis (**Figure 3.6**), whereas **PSL-2.2** was analyzed by ^{1}H NMR (**Figure 3.7**).

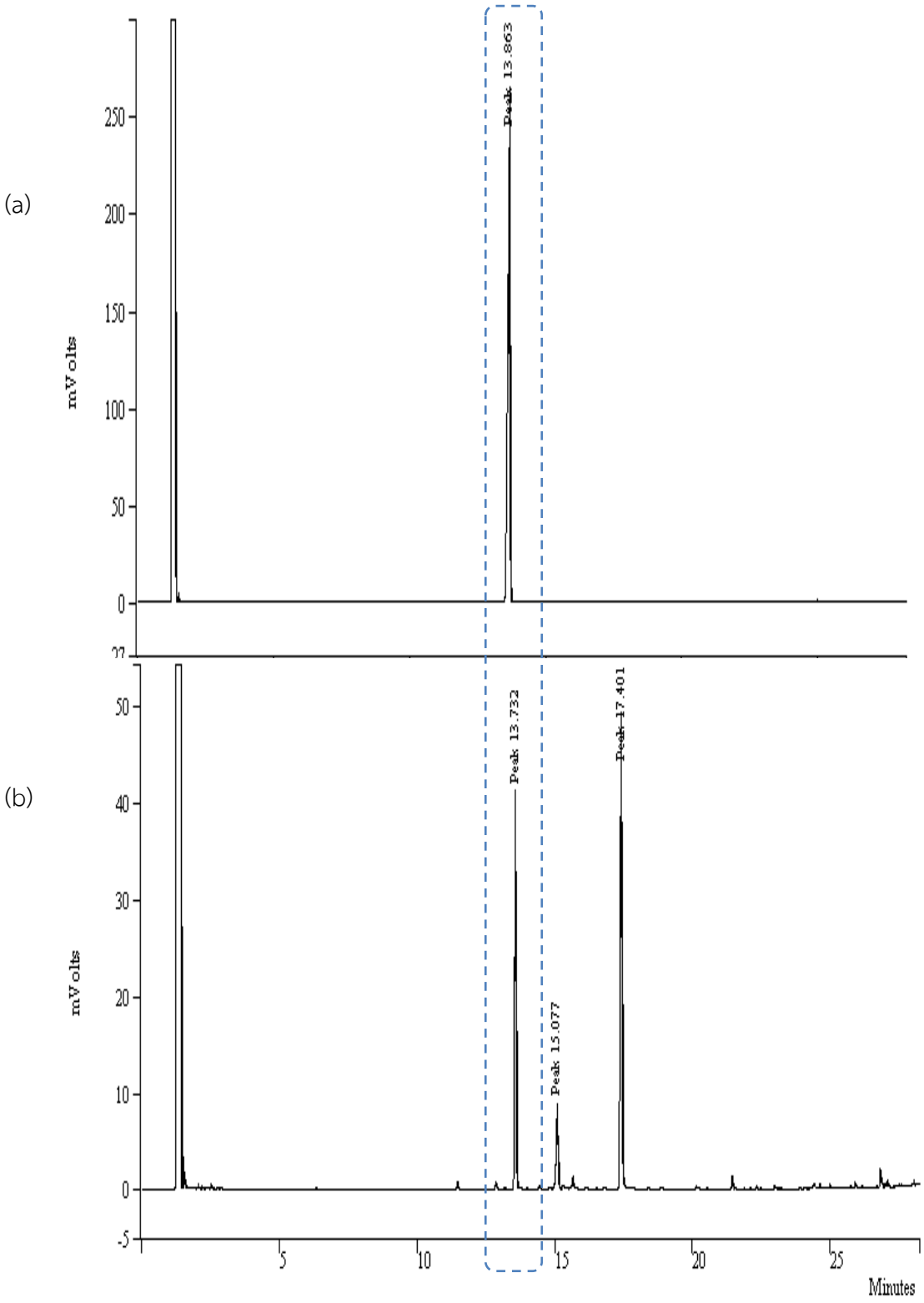


Figure 3. 5 GC chromatograms of effective subfractions **PSLs- 2.2** (a) and **-2.3** (b)

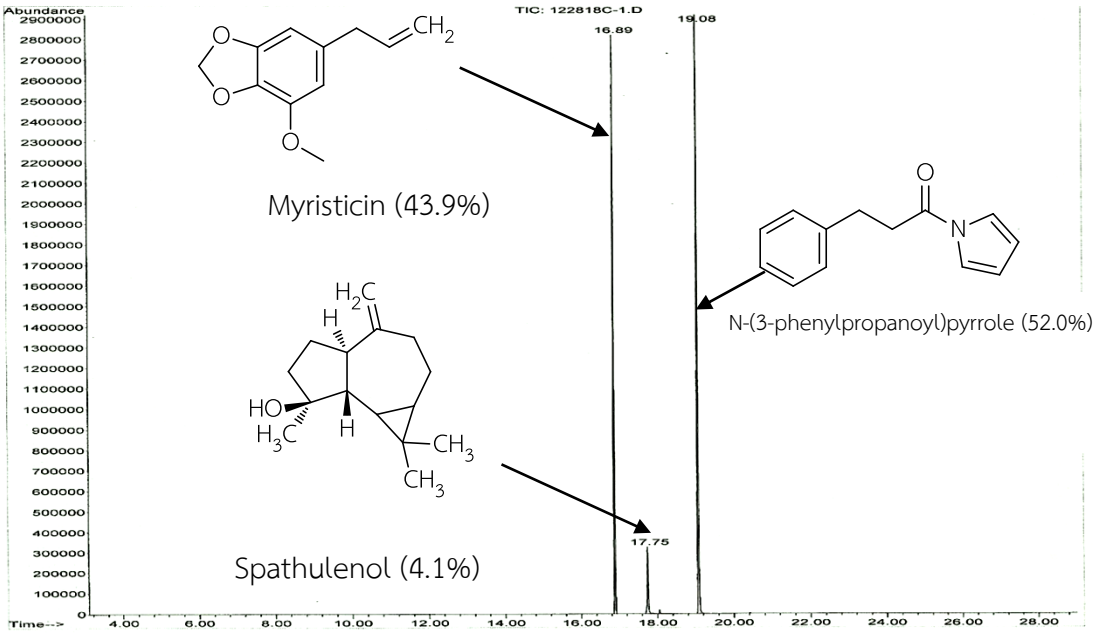


Figure 3. 6 GC-MS analysis of **PSL-2.3**

The GC/MS chromatogram of **PSL-2.3** revealed three peaks which were identified as myristicin (**21**) (R_t: 16.9 min, 43.9%), spathulenol (**20**) (R_t: 17.8 min, 4.1%) and N-(3-phenylpropanoyl)pyrrole (**27**) (R_t: 19.1 min, 52.0%). All compounds were compared with the Wiley7n library. The first peak of two fractions (**PSLs -2.2** and **2.3**) could be confirmed to be myristicin (**21**) supporting by MS.

The structure of compound **A (myristicin, 21)** was further confirmed by ^{1}H-NMR **(Figure 3.7**). The comparative ^{1}H-NMR data between compound **A** and myristicin (**21**) is shown in **Table 3.4** and **Figure 3.7**.

Table 3. 4 ^{1}H-NMR data of myristicin (**21**) and compound **A**

Positions	Myristicin (21)	Compound A
	δ_H (multiplicity *J* in 300 Hz)*	δ_H (multiplicity *J* in 400 Hz)
2	6.36 (d, 1H), *J* = 1.4 Hz, H-6	6.35 (s, 1H)
6	6.39 (d, 1H), *J* = 1.4 Hz, H-2	6.38 (s, 1H)
7	3.25 (d, 2H), *J* = 6.7 Hz	3.29 (d, *J* = 6.7 Hz, 2H)
8	5.90 (m, 1H, H-8)	5.98 – 5.86 (m, 1H)
9	5.09 (dd, 1H), *J*=1.7; 17.1 Hz, H-9a 5.01 (dd, 1H), *J*=1.7; 8.0 Hz, H-9b	5.19 – 4.91 (m, 2H)
OCH_2O	5.92 (s, 2H)	5.93 (s, 2H)
OCH_3	3.81 (s, 3H)	3.88 (s, 3H)

* (Stefano 2011)

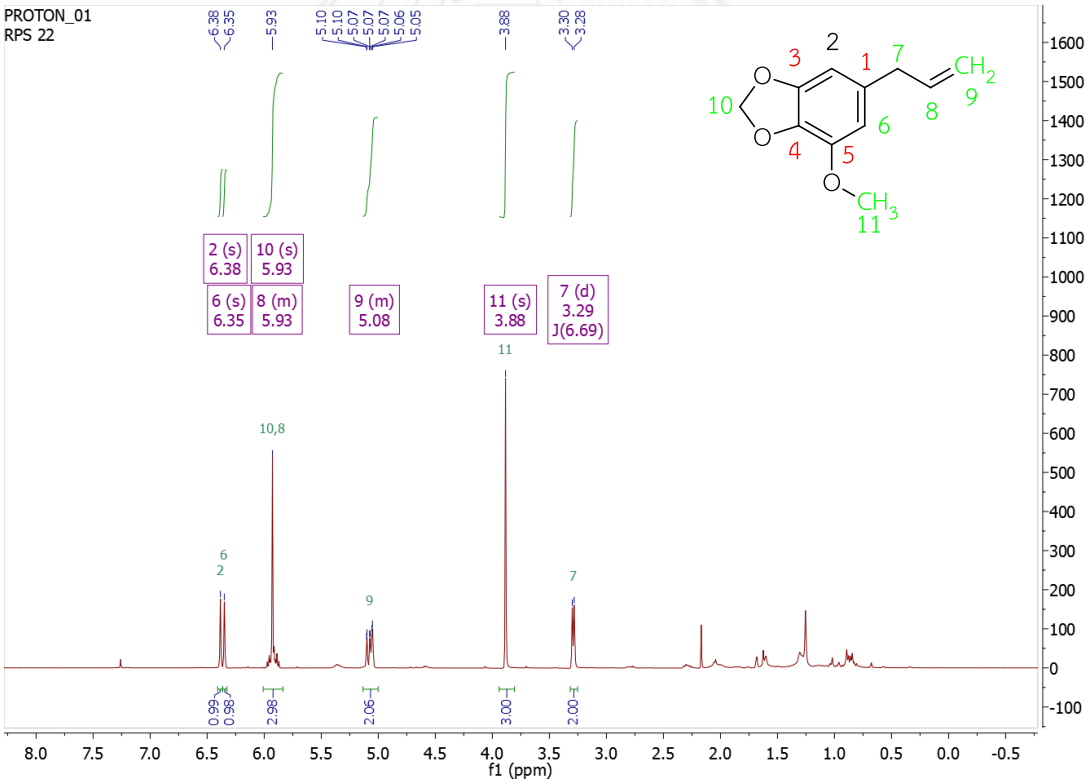


Figure 3. 7 ^{1}H-NMR and structure of myristicin **(21)**

For **PSL-2.4**, two major peaks were the same as those observed in **PSL-2.3**. Thus, the major compound of **PSL-2** was myristicin (**21**). This compound was further tested for IC_{50} on both fungi. For *R. solani*, the average percentage at 50, 100, 200,

400 and 800 ppm of anti-mycelia inhibition was 0, 52.78, 85.56, 100 and 100%, respectively (**Figure 3.8**). These results were plotted and calculated by polynomial equation (**Figure 3.9, Table 3.5**) and R^2 was 0.8707. The IC_{50} was 133 mg/L. With the same concentration range, the average percentage of anti-mycelia inhibition on *B. oryzae* was 48.70, 55.93, 82.78 and 100%, respectively (**Figure 3.9**). The IC_{50} 81 mg/L at R^2 = 0.9354 was obtained (**Figure 3.9**).

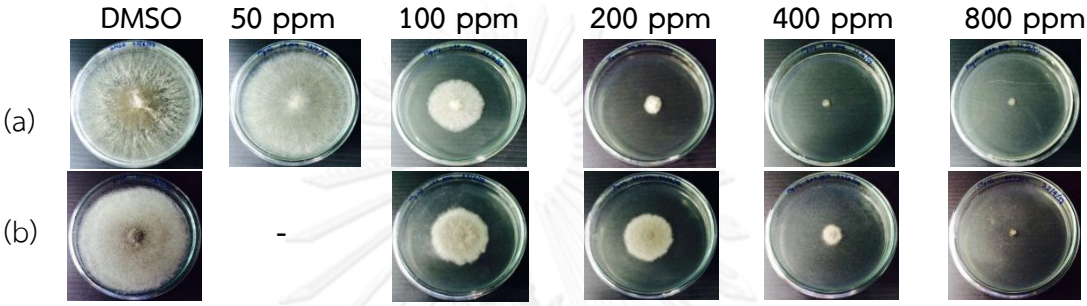


Figure 3. 8 Anti-mycelia inhibition of *R. solani* (a) and *B. oryzae* (b)

Table 3. 5 IC_{50} of compound **A**

	Strains	**mg/mL**	**mM**
IC_{50}	*R. solani*	0.133	0.69
	B. oryzae	0.081	0.42

From **Table 3.5**, the IC_{50} results were converted to mM with 0.69 and 0.42 mM against *R. solani* and *B. oryzae*, respectively. The molecular weight of myristicin is 192.21 g/mol. The results would be further compared with the other compounds.

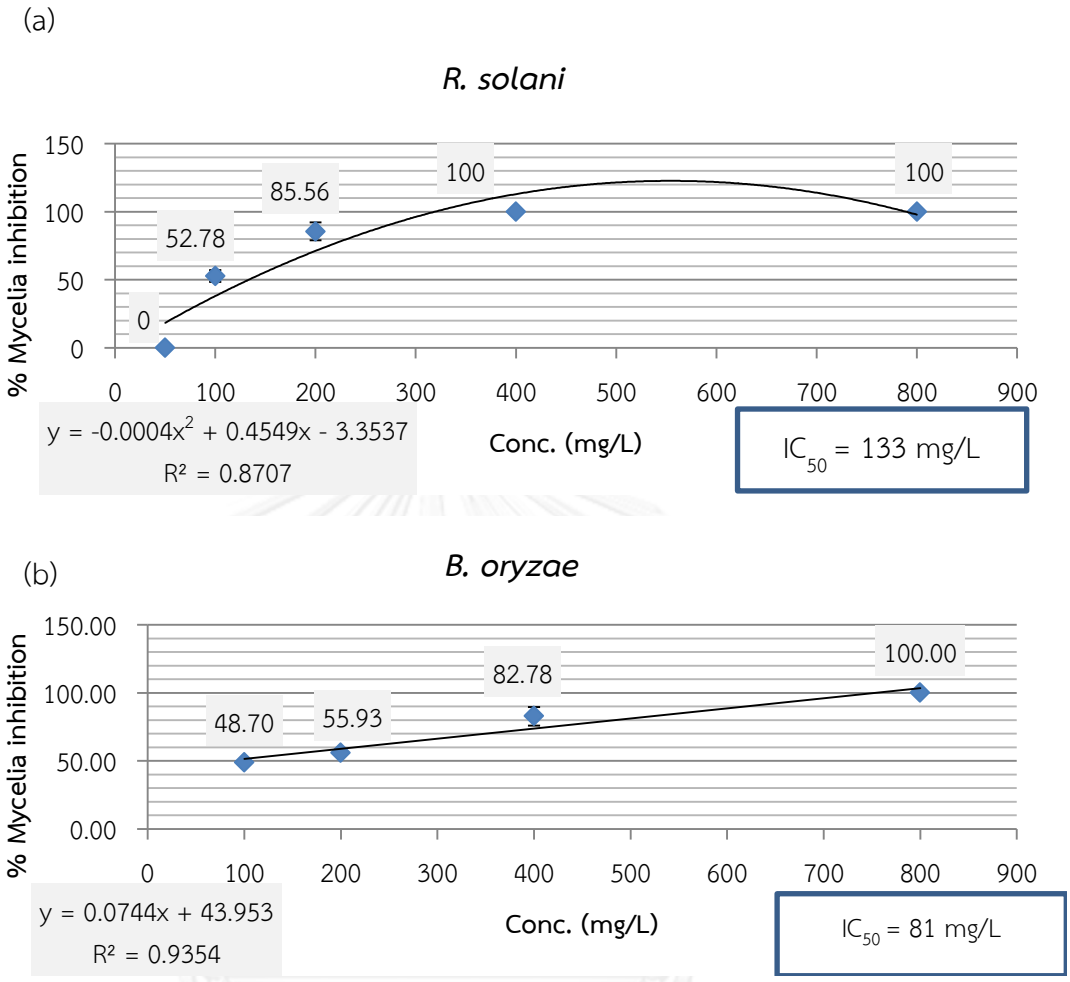


Figure 3. 9 Relationship between the concentration of myristicin (**21**) and %inhibition on *R. solani* and *B. oryzae*

Stefano (2011) reported that myristicin (**21**) was inactive against Gram-positive and Gram-negative bacteria (*S. aureus*, *S. epidermidis*, *E. coli* and *P. aeruginosa*), and antifungal activity (*C. albicans* and *C. tropicalis*). This may be because myristicin (**21**) might not be attached with bacterial cell wall and cell membrane of unicellular fungi. Nevertheless, this compound has not been reported to possess anti-mycelia fungal activity against *R. solani* and *B. oryzae*. Therefore, this study was the first report for anti-mycelia activity on rice pathogenic fungi. The mode of action of myristicin (**21**) was believed to combine and damage chitins which were mycelia fungal cell wall components. In addition, the antimicrobial activity of this compound which belonged to phenylpropanoids would depend on the aromatic ring, selected

microorganisms and the experimental parameters (growth medium, temperature and conditions) (Pauli 2010).

3.3.3.2 Separation of PSL-3

PSL-3 (15 g) as yellow liquid was chromatographed on silica gel. The column was initially eluted with hexane and increasing polarity by mixing with EtOAc. Each portion was examined by TLC using 10% CH_2Cl_2 in hexane as a developing solvent. Fractions with similar chromatographic patterns were combined as presented in **Table 3.6**.

Table 3.6 The separation of **PSL-3**

Fraction code	Solvent system	Remarks	Weight (g)
PSL-3.1	Hexane	yellow solid	0.38
PSL-3.2	5% EtOAc in hexane	orange solid	0.08
PSL-3.3	5% EtOAc in hexane	brown liquid	13.22
PSL-3.4	5–10% EtOAc in hexane	brown solid	0.88
PSL-3.5	10% EtOAc in hexane	pale solid	0.59

The separation of **PSL-3** by silica gel column gave 5 fractions; **PSL-3.3** gave the highest yield (77%). All portions were further searched for active compounds against *R. solani* and *B. oryzae* at a final concentration of 1,000 ppm. The results are shown in **Table 3.7**.

Table 3.7 The mycelia growth inhibition of **PSL-3**

Fractions	% Mycelia growth inhibition±SD[a]	
	R. solani[1]	*B. oryzae*[1]
PSL-3.1	100.00±0.00[a]	100.00±0.00[a]
PSL-3.2	100.00±0.00[a]	88.15±0.64[b]
PSL-3.3	100.00±0.00[a]	81.48±2.10[c]
PSL-3.4	100.00±0.00[a]	70.19±1.79[d]
PSL-3.5	100.00±0.00[a]	50.93± 2.10[e]

[a]Value, an average ± standard deviation of 3 replicates, of mean mycelia growth inhibition of both fungi species, [1]Row values followed by the same letter were not significantly (P > 0.05) difference according to Duncan's multiple comparison tests.

All fractionated **PSL-3** showed complete mycelia growth inhibition of *R. solani* at 1,000 ppm (100%). For *B. oryzae*, only **PSL-3.1** inhibited complete mycelia growth (100%), followed by **PSL-3.2** (88%), **PSL-3.3** (81%), **PSL-3.4** (70%) and **PSL-3.5** (51%), respectively (**Figure 3.10**).

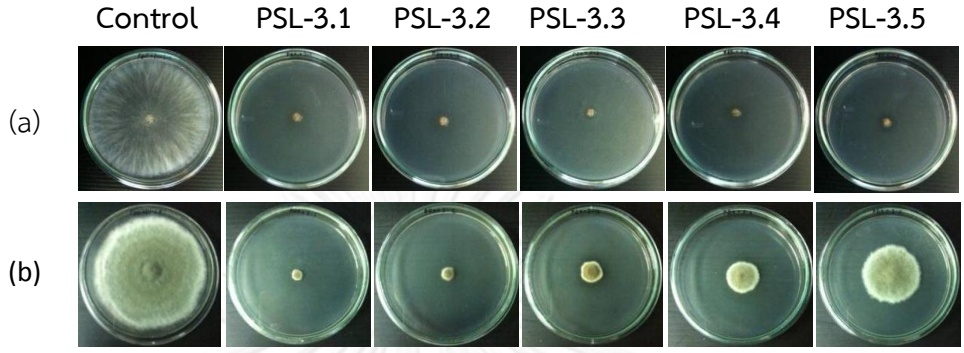


Figure 3. 10 The mycelia growth inhibition of control, **PSLs-3.1-3.5** at 1,000 ppm against (a) *R. solani* and (b) *B. oryzae.*

Even though **PSLs -3.1** and **-3.2** revealed high anti-fungal activity on both fungi, both fractions were not sufficient to lead to the separation. **PSL-3.1** was further analyzed by GC as shown in **Figure 3.11.**

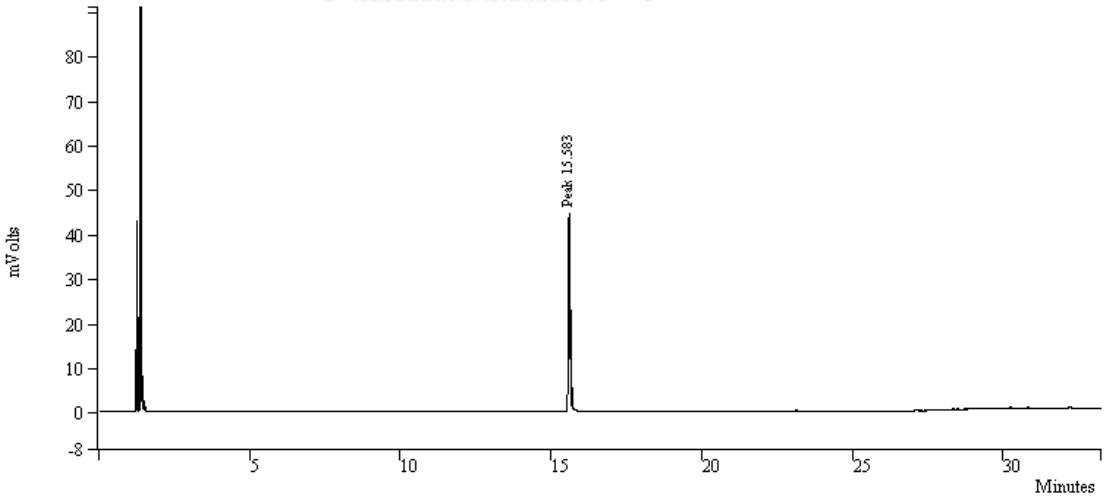


Figure 3. 11 GC chromatogram of **PSL-3.1**

The GC chromatogram of **PSL-3.1** gave only one peak at R_t 15.58 min which was the same as that of standard myristicin (**21**)**.**

PSL-3.3 was separated by silica gel column to give four fractions. Both P**SLs -3.3.2** and **-3.3.3** revealed completely anti-fungal activity against *R. solani*, while only **PSLs –3.3.2** displayed anti-fungal activity against *B. oryzae.* These fractions might

contain active compound that controlled the fungi. So, these two fractions were further analyzed by GC. The GC chromatograms of **PSLs -3.3.2** and **-3.3.3** are shown in **Figure 3.12**. The GC chromatogram of **PSL–3.3.2** revealed only one peak which was the same as standard myristicin (**21**). **PSL-3.3.3** showed two peaks; based on the information obtained previously as discussed in **Figure 3.5**, the former was identified as myristicin (**21**), whereas the latter was *N*-(3-phenylpropanoyl)pyrrole (**27**).

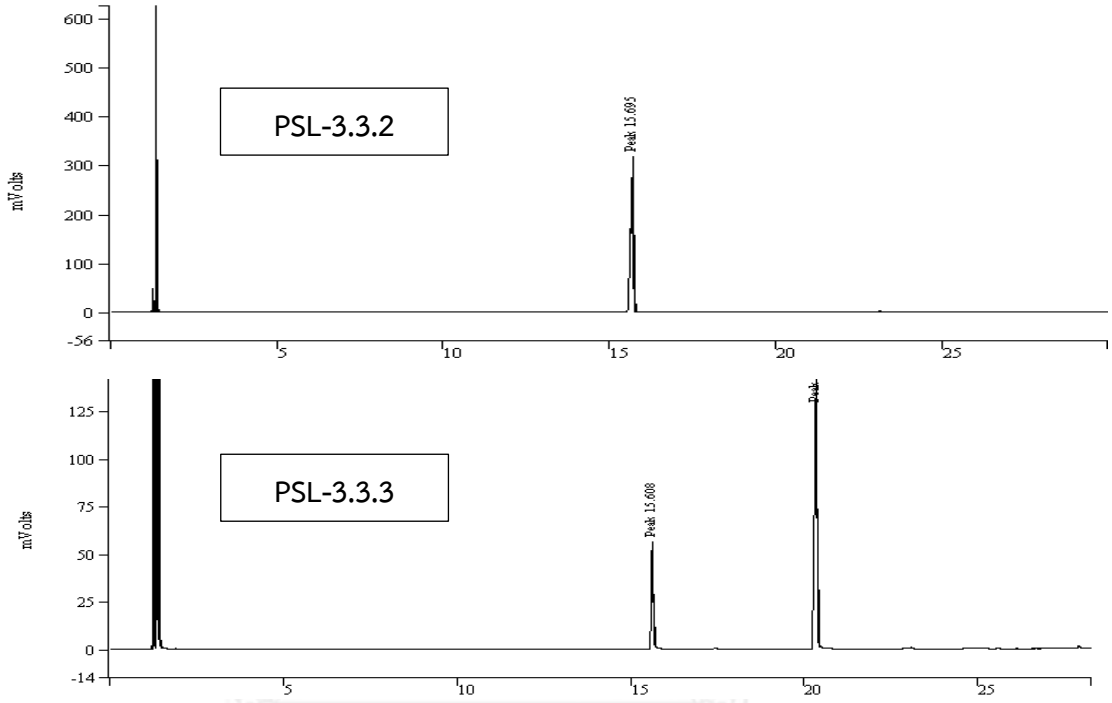


Figure 3. 12 GC chromatograms of **PSLs -3.3.2** and **-3.3.3**

3.3.3.3 Separation of PSL-4

PSL-4 (2.0 g) was separated by silica gel column and examined by TLC. Fractions with similar chromatographic pattern are presented in **Table 3.8**.

Table 3.8 The separation of **PSL-4**

Fractions	Solvent system	Remarks	Weight (g)
PSL-4.1	5% EtOAc in hexane	pale yellow solid	0.18
PSL-4.2	5%-10% EtOAc in hexane	yellow solid	0.01
PSL-4.3	5%-10% EtOAc in hexane	yellow solid	0.54

The separation of **PSL-4** by silica gel column gave 3 fractions; **PSL-4.3** gave the highest yield (27%). Two portions (**PSLs -4.1** and **-4.3**) were tested against *R. solani* and *B. oryzae* and the results are displayed in **Table 3.9** and **Figure 3.13**.

Table 3.9 The mycelia growth inhibition of **PSLs -4.1** and **-4.3**

Fractions	%mycelia inhibition ± SD[a,1]	
	R. solani	*B. oryzae*
PSL-4.1	52.78 ± 2.55[b]	4.07 ± 2.10[b]
PSL-4.3	100.00 ± 0.00[a]	72.96 ± 1.16[a]

[a]Value, an average ± standard deviation of 3 replicates, of mean mycelia growth inhibition of both fungi species
[1]Row values followed by the same letter were not significantly (P > 0.05) difference according to Duncan's multiple comparison tests.

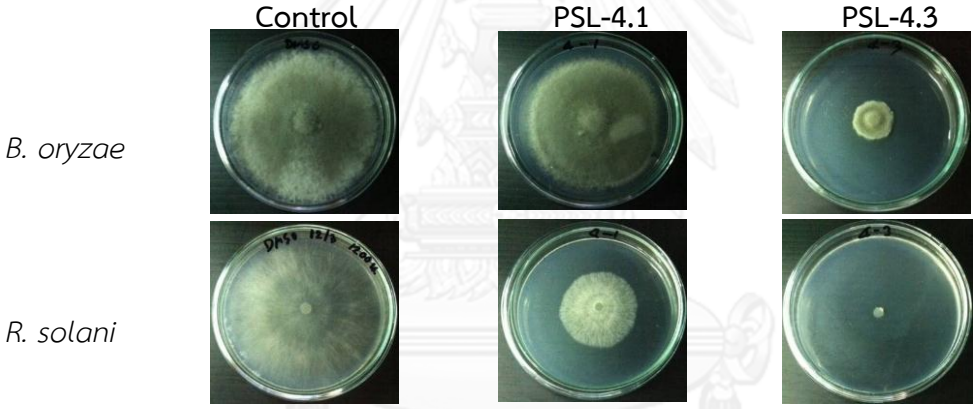


Figure 3. 13 The mycelia growth inhibition of control, **PSLs -4.1** and **-4.3** against *R. solani* and *B. oryzae*

The data from **Table 3.8** showed that **PSL-4.3** was completely inhibited against *R. solani* and highly controlled of *B. oryzae*. The mass spectrum (**Figure 3.14**) of **PSL-4.3** which was the same as that of 1-(1-*E*-propenyl)-2,4,5-trimethoxybenzene (**3**) or **α**-asarone (Masuda 1991). This compound was subordinate compound for enhancing the main compound.

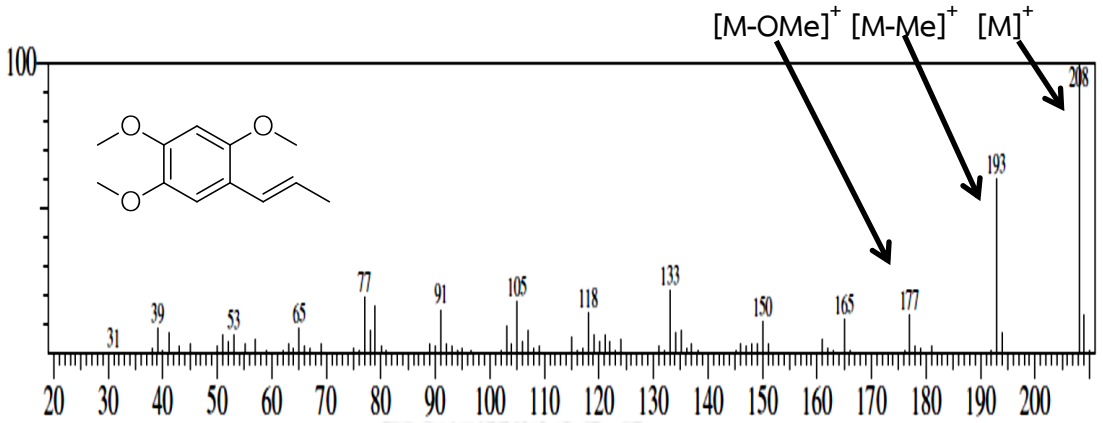


Figure 3. 14 Mass spectrum of **PSL-4.3**

In conclusion, the results indicated that the most antifungal activity of myristicin (**21**) of the CH_2Cl_2 extract which might be further used for a new anti-rice pathogenic fungal agent. Additionally, this compound was the major constituent of *P. sarmentosum* leaves because it was found in many fractions.

3.4 Fractionation and anti-rice pathogenic microbial activity of the CH_2Cl_2 extract from the fruits of *P. sarmentosum*

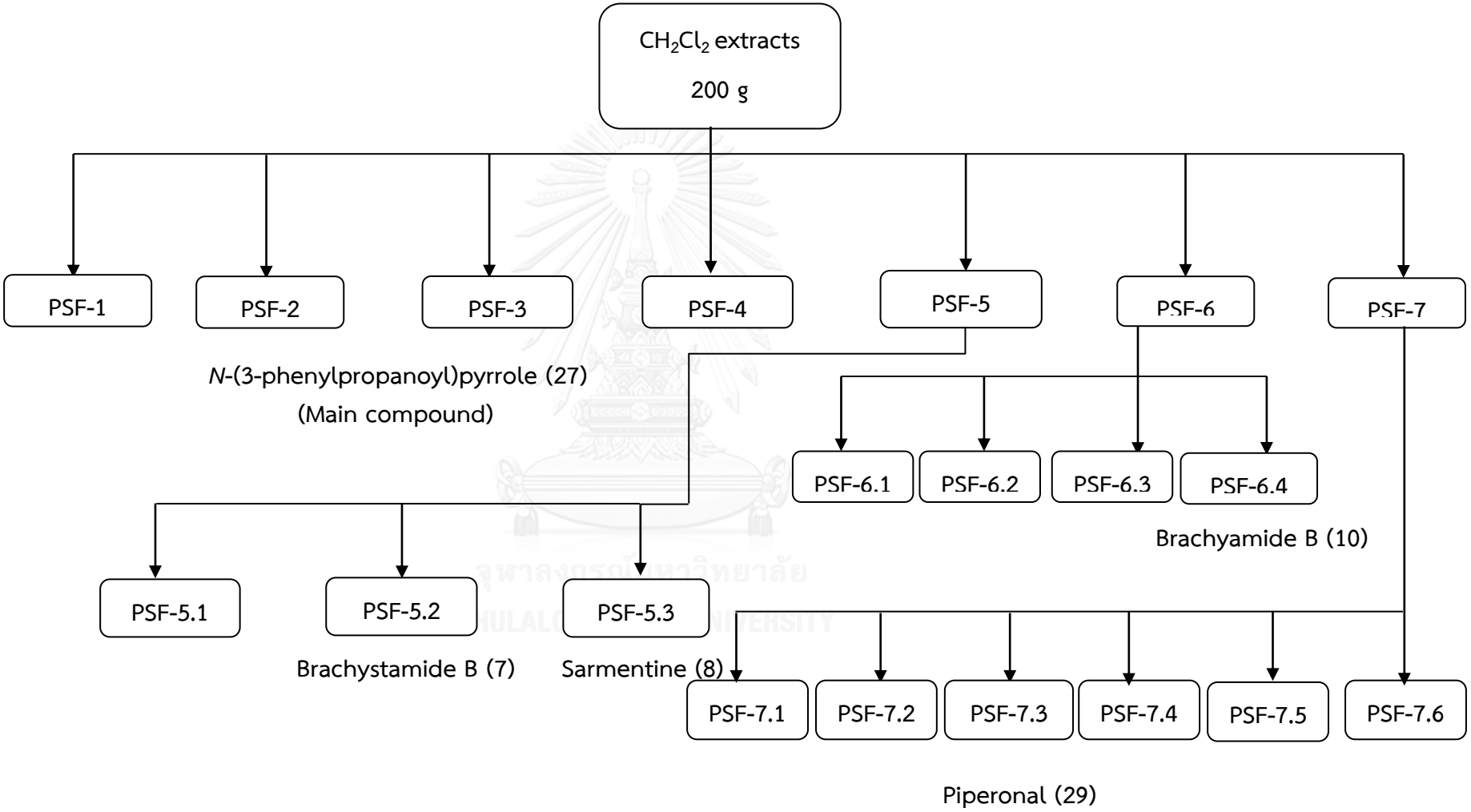
3.4.1 Fractionation

5.2 Kg of the dried fruits were grinded and extracted with CH_2Cl_2 using sohxlet's apparatus to give 600 g of dark brown liquid. 200 grams were separated by quick column chromatography. The separated fractions were eluded by increasing polar solvents and collected to 7 fractions according to TLC results (**Table 3.10**, **Scheme 3.3**).

Table 3.10 Fractionation of the CH_2Cl_2 extract of *P. sarmentosum* fruits using silica gel column

Fractions	Solvent system	Remarks	Weights (g)
PSF-1	100% hexane	fallow liquid	29.28
PSF-2	5% EtOAc in hexane	dark-yellow liquid	13.32
PSF-3	5%-10% EtOAc in hexane	darkbrown semi-liquid	17.85
PSF-4	10% EtOAc in hexane	brownish solid	2.20
PSF-5	20% – 40% EtOAc in hexane	bottle-green liquid	35.42
PSF-6	40% - 60% EtOAc in hexane	dark-brown sticky liquid	17.36
PSF-7	60% EtOAc in hexane -10% MeOH in EtOAc	bottle-green solid	32.50

Among seven fractions, fractions **PSF-5** gave the highest yield as 35.42 g.



Scheme 3.3 Separation of the CH_2Cl_2 extract of *P. sarmentosum* fruits

3.4.2 Antifungal activity assay

Seven fractions of the CH_2Cl_2 extract (1,000 mg/L) were subjected against both rice pathogenic fungi. The percentage of mycelia growth inhibition is presented in **Figures 3.15** and **3.16**.

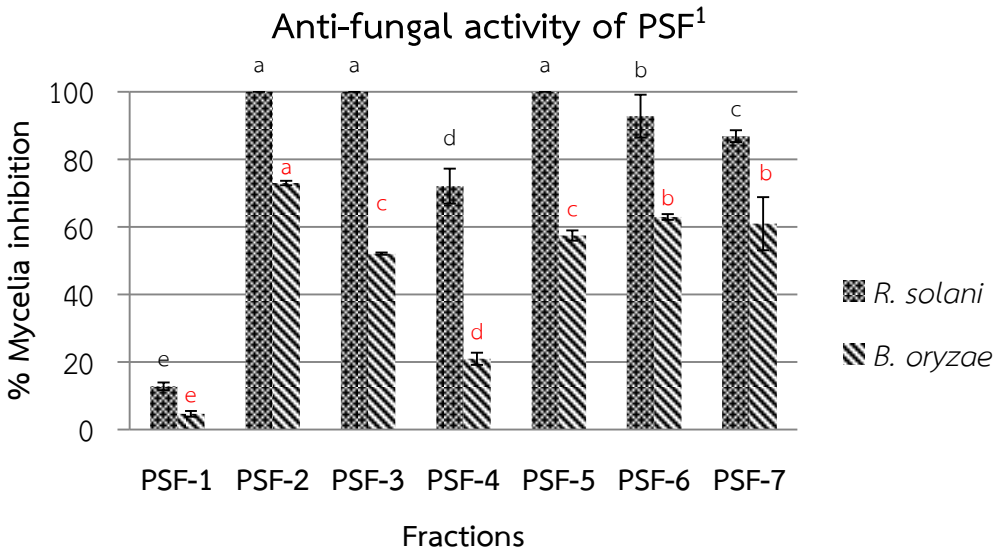


Figure 3. 15 Mycelia growth inhibition of rice pathogenic fungi in agar poisoned food technique of fractions at 1,000 mg/L

[1]Bar charts of each fungus followed by the same letter were not significantly (P ≥ 0.05) difference according to Duncan's multiple comparison tests.

According to **Figure 3.15, PSFs -2**, **-3** and -5 significantly displayed complete antifungal activities against *R. solani*. **PSF-1** showed the least mycelia growth inhibition. For *B. oryzae*, **PSF-2** only revealed more high mycelia inhibition than other fractions, while **PSF-1** was the lowest mycelia inhibition. Thus these three fractions (**PSFs -2**, **-3** and **-5**) were further separated and tested for antifungal activity. All tested fractions are represented in **Figure 3.16**.

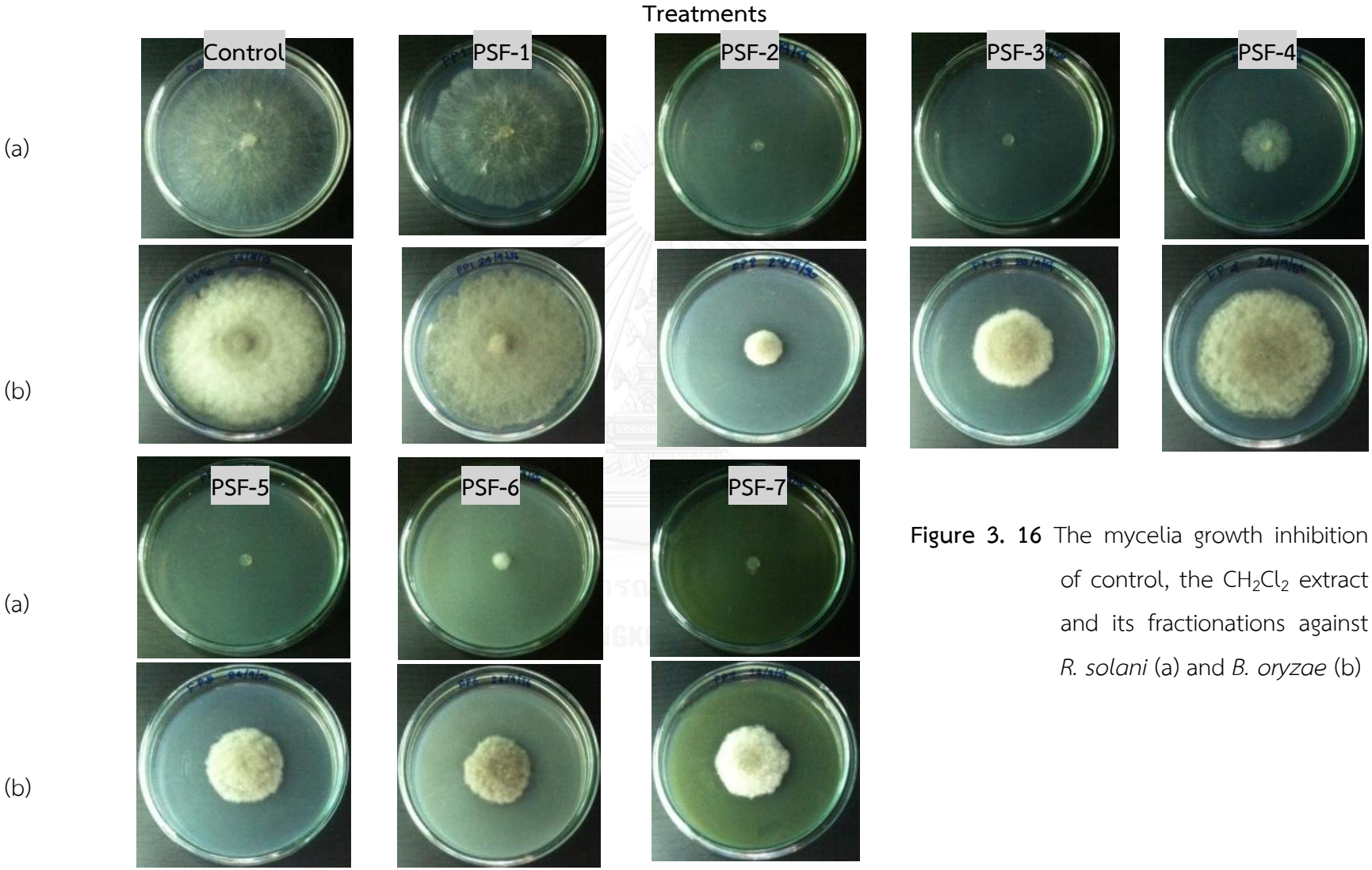


Figure 3. 16 The mycelia growth inhibition of control, the CH_2Cl_2 extract and its fractionations against *R. solani* (a) and *B. oryzae* (b)

3.4.3 Antibacterial activity Assay

Fractionation of the CH_2Cl_2 of *P. sarmentosum* fruits displayed antibacterial activity. **PSFs -5**, **-6** and **-7** were highly inhibited on both bacteria. **PSF-3** was not inhibition on all bacteria. **PSFs -5** and **-6** showed high inhibition on *Xoc* with 9.75 and 10 mm of average clear zone. **PSFs -5** and **-7** displayed high inhibition on *Xoo* with 3.5 and 2.75 mm of average clear zone (**Table 3.11**).

Table 3.11 Inhibition zones (mm ± SD[*,1]) of rice pathogenic bacteria in agar diffusion method of all fractions

Fractions	*X. oryzae* pv. *oryzae*	*X. oryzae* pv. *oryzicola*
PSF-1	1.75 ± 0.35^{abc}	0.00 ± 0.00^{c}
PSF-2	1.00 ± 0.00^{bc}	0.00 ± 0.00^{c}
PSF-3	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
PSF-4	1.50 ± 0.71^{abc}	0.00 ± 0.00^{c}
PSF-5	3.50 ± 2.12^{a}	9.75 ± 1.06^{a}
PSF-6	1.75 ± 0.35^{abc}	10.00 ± 2.12^{a}
PSF-7	2.75 ± 1.06^{ab}	4.75 ± 1.06^{b}

[*] Value, an average ± standard deviation of 3 replicates, of mean mycelia growth inhibition of both fungi species

[1] Row values followed by the same letter were not significantly (P > 0.05) difference according to Duncan's multiple comparison tests.

PSFs -5, -6 and **-7** were selected for further separation because these fractions displayed anti-bacterial and anti-fungal activities.

3.4.4 Fractionation of PSF-5

PSF-5 was separated using silica gel column. According to TLC pattern, three fractions were combined as shown in **Table 3.12**.

Table 3.12 The separation of **PSF-5**

Fractions	Solvent system	Remarks	Weight (g)
PSF-5.1	20% EtOAc in hexane	yellow liquid	0.03
PSF-5.2	20% - 40% EtOAc in hexane	green-yellow solid	27.42
PSF-5.3	40% - 100% EtOAc in hexane	dark brown liquid	7.97

PSF-5.2 and **PSF-5.3** were subjected to antibacterial activity against *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and *X. oryzae* pv. *oryzicola* (*Xoc*) as shown in **Figure 3.17**.

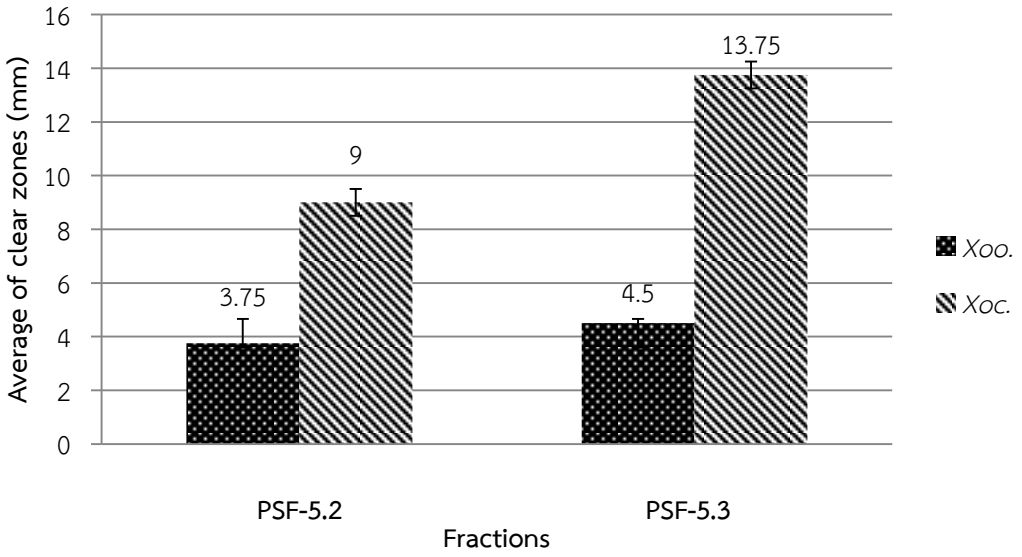
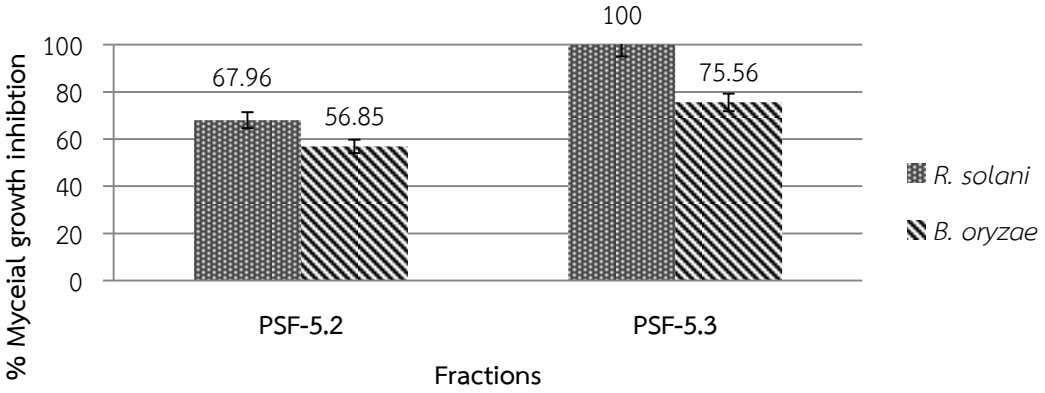


Figure 3. 17 Anti-bacteria activity of **PSF-5.2** and **PSF-5.3**

PSF-5.3 showed more inhibition than **PSF-5.2**. **PSF-5.3** might contain the effective compound for attaching cell wall components of Gram-negative bacteria better than the compound of **PSF-5.2**. The two mentioned fractions were tested for antifungal activity. The results are presented in **Figure 3.18**.

(a)



(b)

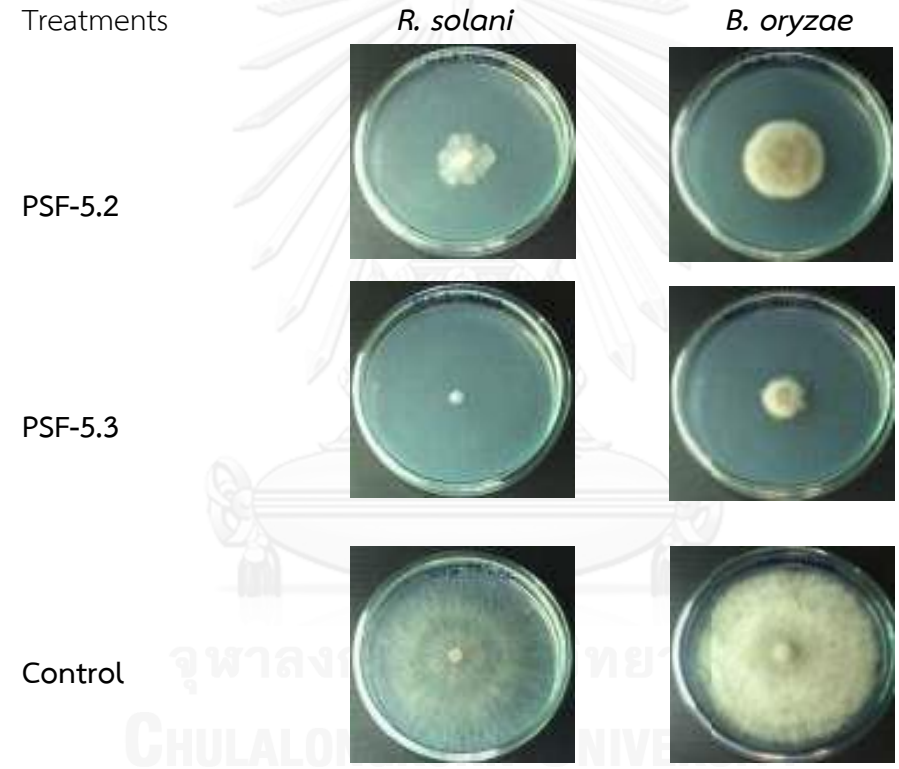


Figure 3. 18 Anti-fungal activities of **PSF-5.2** and **-5.3**; % mycelia growth inhibition (a) and mycelia growth plates (b)

3.4.4.1 Identification of PSF-5.3

Both fractions (**PSFs-5.2-5.3**) displayed mycelia inhibition on fungi. **PSF-5.3** showed more significant antifungal activity than **PSF-5.2**. Thus, **PSF-5.3** was further analyzed. The GC/MS analysis of **PSF-5.3** is shown in **Figure 3.19**.

GC/MS chromatogram

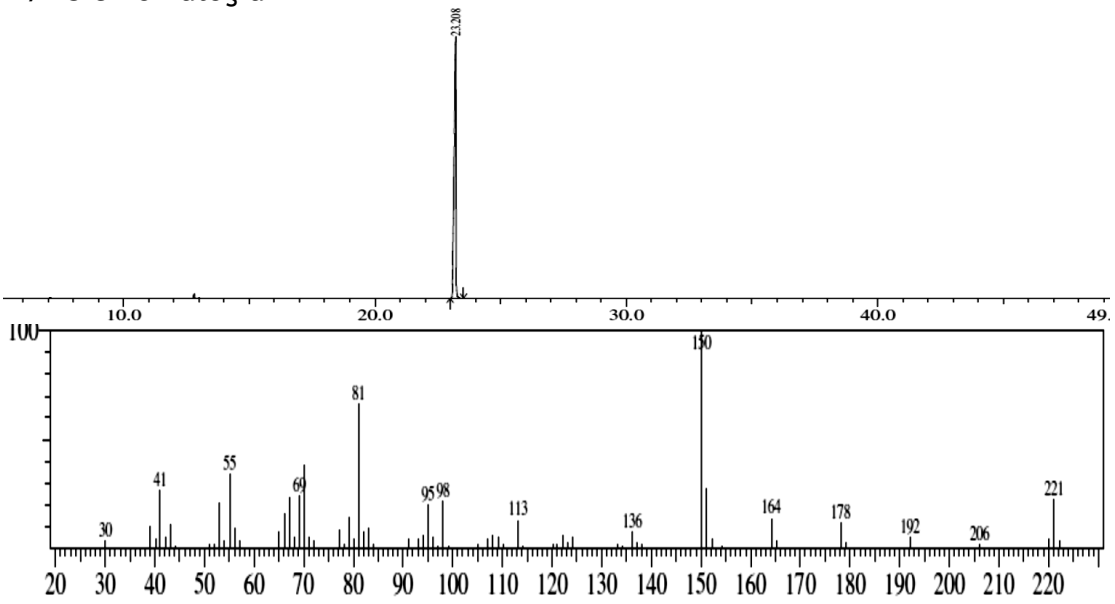


Figure 3. 19 GC/MS of **PSF-5.3**

The ^{1}H-NMR spectrum of **PSF-5.3** is shown in **Figure 3.21**. The comparative assignments of **PSF-5.3** and sarmentine (**8**) are presented in **Table 3.13** and **Figure 3.20**.

Table 3.13 ^{1}H-NMR data of sarmentine (**8**) and compound **B**

Position	Sarmentine (8)* δ_H (multiplicity *J* in 600 Hz)	Compound B δ_H (multiplicity *J* in 400 Hz)
2, 5	6.08 (d, *J* = 14.4 Hz, 1H) 6.07 (dt, *J* = 14.4, 7.2 Hz, 1H)	6.04 (dd, *J* = 14.3, 7.7 Hz, 2H)
3	7.26 (dd, *J* = 14.4, 10.8 Hz, 1H)	7.21 (dd, *J* = 14.7, 10.7 Hz, 1H)
4	6.16 (dd, *J* = 10.8, 9.6 Hz, 1H)	6.13 (dd, *J* = 15.1, 10.7 Hz, 1H)
6	2.13 (quart., *J* = 7.2 Hz, 2H)	2.09 (quart., *J* = 7.1 Hz, 2H)
7	1.39 (quint., *J* = 7.2 Hz, 2H)	1.37 (quint., *J* = 7.2 Hz, 2H)
8, 9	1.27 (m, 4H)	1.30 – 1.18 (m, 4H)
10	0.87 (t, *J* = 7.2 Hz, 3H)	0.83 (t, *J* = 6.9 Hz, 3H)
1´, 4´	3.50 (t, *J* = 7.2 Hz, 2H) 3.52 (t, *J* = 7.2 Hz, 2H)	3.52 – 3.42 (m, 4H),
2´	1.95 (quint., *J* = 7.2 Hz, 2H)	1.91 (quint., *J* = 6.7 Hz, 2H)
3´	1.85 (quint., *J* = 7.2 Hz, 2H)	1.80 (quint., *J* = 6.8 Hz, 2H)

*(Huang 2011)

Based on spectroscopic method and comparison with previous reports, compound **B** (or **PSF-5.3**) was identified as sarmentine (**8**). Sarmentine (**8**) is generally found in *Piper* species; especially in *P. sarmentosum* (Likhitwitayawuid 1987).

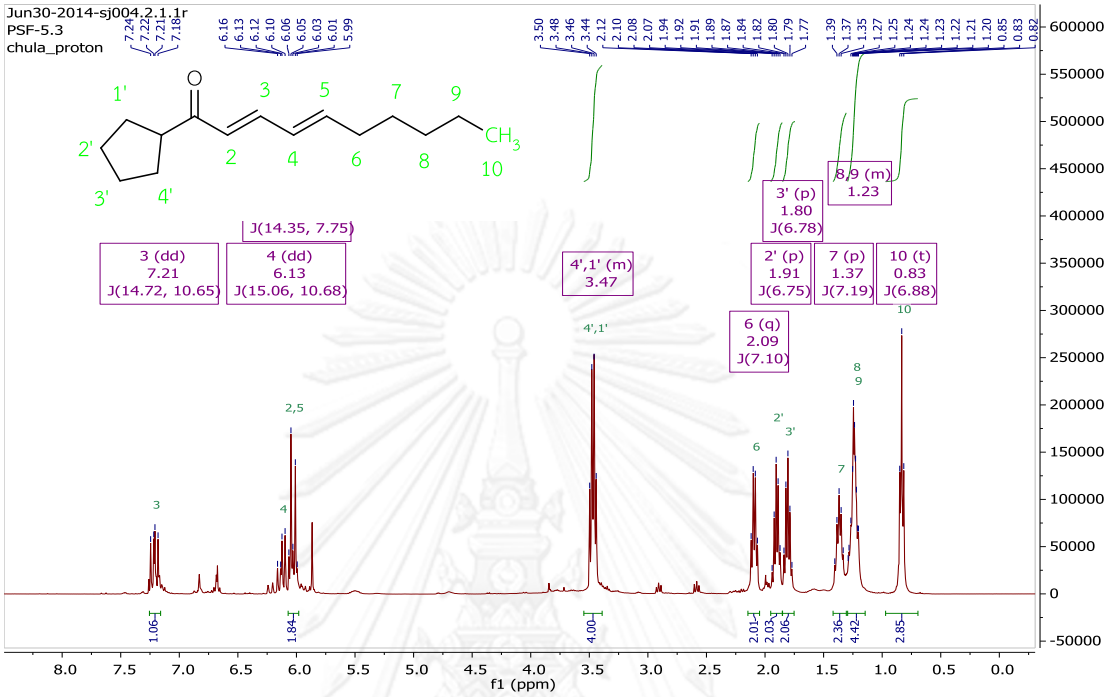


Figure 3. 20 ^{1}H-NMR spectrum of sarmentine (**8**)

This compound revealed the most antimicrobial activity on tested rice pathogenic microorganisms. So, this compound was supposed to further test for MIC/MBC and IC_{50}. MIC/MBC and IC_{50} of compound **B** are shown in **Table 3.14**.

Table 3.14 MIC/MBC and IC50 of compound **B**

	Strains	Concentration	
		mg/mL	mM
MIC/MBC	*Xoo*	3.12/12.50	14.10/56.50
	Xoc	1.56/6.25	7.05/28.24
IC_{50}	*R. solani*	0.226	1.02
	B. oryzae	0.203	0.92

The molecular weight of sarmentine (**8**) is 221.34 g/mol. The results were converted to mM of 14.10/56.50, 7.05/28.24, 1.02 and 0.92 mM against *Xoo*, *Xoc*, *R. solani* and *B. oryzae*, respectively. The results would be further compared with the other compounds.

3.4.4.2 Separation of PSF-5.2

PSF-5.2 (24.35 g) was separated using flash column eluting by increasing polarity of solvents. All fractions were combined based on TLC pattern (**Table 3.15**).

Table 3.15 The separation of **PSF-5.2**

Fractions	Solvent system	Remarks	Weight (g)
PSF-5.2.1	100% hexane - 15% EtOAc in hexane	yellow-orange solid	0.14
PSF-5.2.2	15% EtOAc in hexane	green solid	0.12
PSF-5.2.3	15% EtOAc in hexane	dark green solid	7.13
PSF-5.2.4	15% EtOAc in hexane	dark green liquid	11.38
PSF-5.2.5	15-40% EtOAc in hexane	brown liquid	8.65

PSF-5.2.3 (7.13 g) was further separated using flash column with increasing polarity of solvents. All fractions were combined based on TLC pattern. The results of the separation of **PSF-5.2.3** are collected in **Table 3.16**.

Table 3.16 The separation of **PSF-5.2.3**

Fractions	**Solvent system**	**Remarks**	**Weight (g)**
PSF-5.2.3.1	100% Hexane and 5-10% EtOAc	canary yellow solid	0.10
PSF-5.2.3.2	10% EtOAc in hexane	yellow solid	0.03
PSF-5.2.3.3	10-12% EtOAc in hexane	olive-green liquid	0.61
PSF-5.2.3.4	12% EtOAc in hexane	white powder	1.35
PSF-5.2.3.5	12-15% EtOAc in hexane	dark-green liquid	0.85

The ^{1}H-NMR spectrum of **PSF-5.2.3.4** is displayed in **Figure 3.22** and **Figure 3.21.** The comparative assignments of **PSF-5.2.3.4** or compound **C** (brachystamide B, **7**) are presented in **Table 3.17**.

Table 3.17 ^{1}H-NMR data of brachystamide B (**7**) and compound C

Position	Brachystamide B (7)* δ_H (multiplicity *J* in 400 Hz)	Compound C δ_H (multiplicity *J* in 400 Hz)
2, OCH_2O	5.76 (d, *J* = 15.0 Hz, 1H) 5.95 (s, 2H)	6.01 – 5.87 (m, 3H)
3	7.21 (dd, *J* = 15.0, 9.9 Hz, 1H),	7.10 (dd, *J* = 15.2, 10.8 Hz, 1H),
4, 5, 14	6.09 (overlapped, 1H) 6.09 (overlapped, 1H) 6.05 (dt, *J* = 13.8, 6.9 Hz, 1H),	6.23 – 6.01 (m, 3H)
6, 13	2.18 (dt, *J* = 13.8, 6.9 Hz, 2H) 2.18 (dt, *J* = 13.8, 6.9 Hz, 2H)	2.23 – 2.09 (m, 4H)
7, 8, 9, 10, 11, 12	1.44 (quint., *J* = 7.2 Hz, 2H) 1.31 (m, 8H) 1.44 (quint., *J* = 7.2 Hz, 2H)	1.54 – 1.22 (m, 12H)
15	6.30 (d, *J* = 15.6 Hz, 1H)	6.33 (d, *J* = 15.8 Hz, 1H)
2′	6.91 (d, *J* = 1.2 Hz, 1H)	6.97 (d, *J* = 1.6 Hz, 1H)
5′, 6′	6.74 (d, *J* = 7.8 Hz, 1H) 6.78 (dd, *J* = 7.8, 1.2 Hz, 1H)	6.76 (d, *J* = 7.9 Hz, 1H) 6.81 (dd, *J* = 8.1, 1.6 Hz, 1H)
7′	3.18 (t, *J* = 6.6 Hz, 2H)	3.07 (t, *J* = 6.4 Hz, 2H)
8′	1.82 (9 lines, *J* = 6.6 Hz, 1H)	1.85 – 1.68 (m, 1H)
9′,9″	0.94 (d, *J* = 6.6 Hz, 6H)	0.88 (d, *J* = 6.7 Hz, 6H)
NH	5.49 (br s, 1H)	-

*(Phansa 2005)

Brachystamide B (**7**) has been firstly found by Phansa (2005). It usually contains in *Piper brachystachyum* leaves (Parmar *et al.*, 1997). This compound was

used for anti-inflammatory, inhibitory activity on prostaglandin and leukotriene biosynthesis (Thomas 2006).

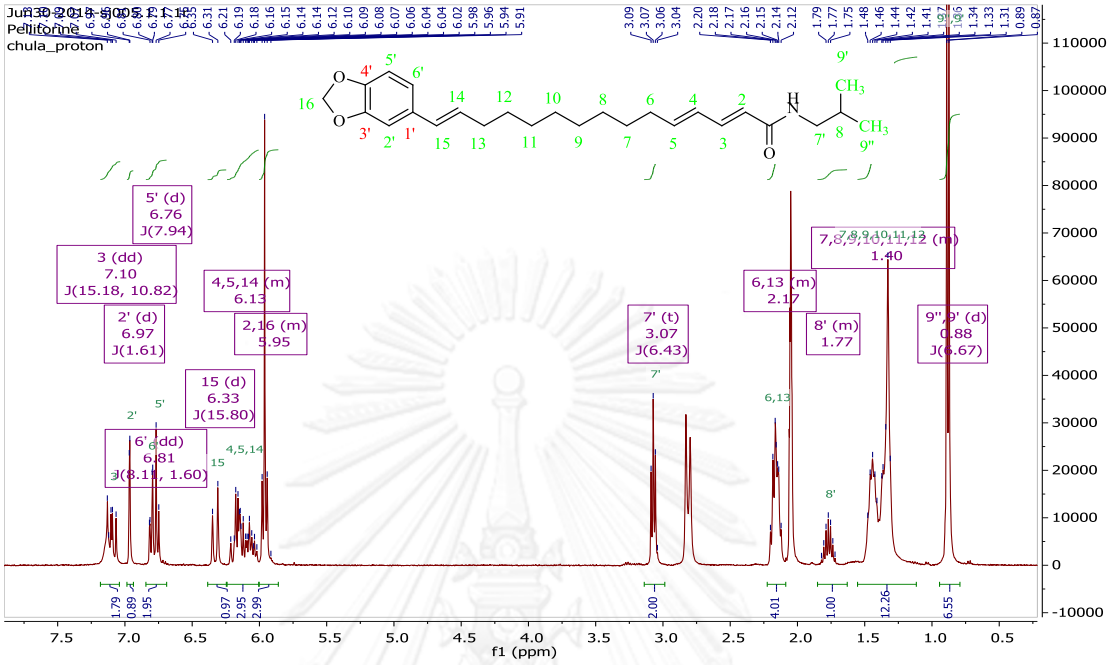


Figure 3. 21 ^{1}H-NMR and structure of brachystamide B (**7**)

This compound revealed the potential antimicrobial activity on tested rice pathogenic microorganisms. So, this compound was supposed to further test for MIC/MBC and IC_{50}. MIC/MBC and IC_{50} of compound **C** are shown in **Table 3.18**.

Table 3. 18 MIC/MBC and IC50 of compound **C**

	Strains	Concentration	
		mg/mL	mM
MIC/MBC	*Xoo*	6.25/6.25	15.19/15.19
	Xoc	6.25/12.50	15.19/30.37
IC_{50}	*R. solani*	1.179	2.87
	B. oryzae	0.745	1.81

Sarmentine (**8**) is a long unsaturated fatty acid chain and pyrrolidine. Brachystamide B (**7**) is an amide compound. Sarmentine (**8**) was more antimicrobial activity than brachystamide B (**7**) because the mode of action of sarmentine (**8**) was harmful to microbial cell and its mechanisms more than brachystamide B (**7**).

3.4.5 Separation of PSF-6

PSF-6 (15.0 g) was separated using flash column eluting with increasing polarity of solvents. All fractions were combined based on TLC pattern (**Table 3.19**).

Table 3. 19 The separation of **PSF-6**

Fractions	Solvent system	Remarks	Weight (g)
PSF-6.1	100% Hexane and 30% EtOAc in hexane	yellow-orange solid	0.02
PSF-6.2	40% EtOAc in hexane	dark-brown liquid	2.43
PSF-6.3	40-60% EtOAc in hexane	brown liquid	11.71
PSF-6.4	60% EtOAc in hexane-100% EtOAc	canary brown solid	1.56

PSF-6.3 was high yield. **PSFs -6.2-6.4** were further tested for antimicrobial activity at 1,000 mg/L (**Table 3.20** and **Figure 3.22**).

Table 3. 20 Antimicrobial activity of **PSF-6.2-6.4** at 1,000 mg/L

Fractions	Mycelia inhibion (% ± SD)[a]		Bacterial clear zones (mm ± SD)[b]	
	R. solani[1]	*B. oryzae*[1]	*Xoo*[1]	*Xoc*[1]
PSF-6.2	48.33 ± 5.30[b]	47.41 ± 1.40[c]	13.50 ± 0.71[ab]	7.25 ± 0.35[a]
PSF-6.3	35.00 ± 1.47[c]	57.59 ± 1.70[b]	13.00 ± 0.00[b]	8.25 ± 0.35[a]
PSF-6.4	63.33 ± 4.94[a]	60.74 ± 1.28[a]	14.50 ± 0.00[a]	8.50 ± 0.71[a]

[a]Value, an average ± standard deviation of 3 replicates, of mean mycelia growth inhibition of both fungi species, [b]Value an average± standard deviation of duplicates, [1]Row values followed by the same letter were not significantly (P > 0.05) difference according to Duncan's multiple comparison tests.

PSF-6.4 displayed high mycelia inhibition (63%) against *R. solani*, followed by **PSFs -6.3** and **-6.2** (**Figure 3.22a**), whereas *B. oryzae* was highly inhibited by **PSFs -6.4**

and **-6.3** with 61 and 58% mycelia inhibition, respectively. For antibacterial activity, **PSF-6.4** revealed bacterial clear zones of 14.5 and 8.5 mm against *Xoo* and *Xoc*, respectively (**Figure 3.22b**). **PSF-6.4** revealed broad spectrum on anti-rice pathogenic microoraganisms.

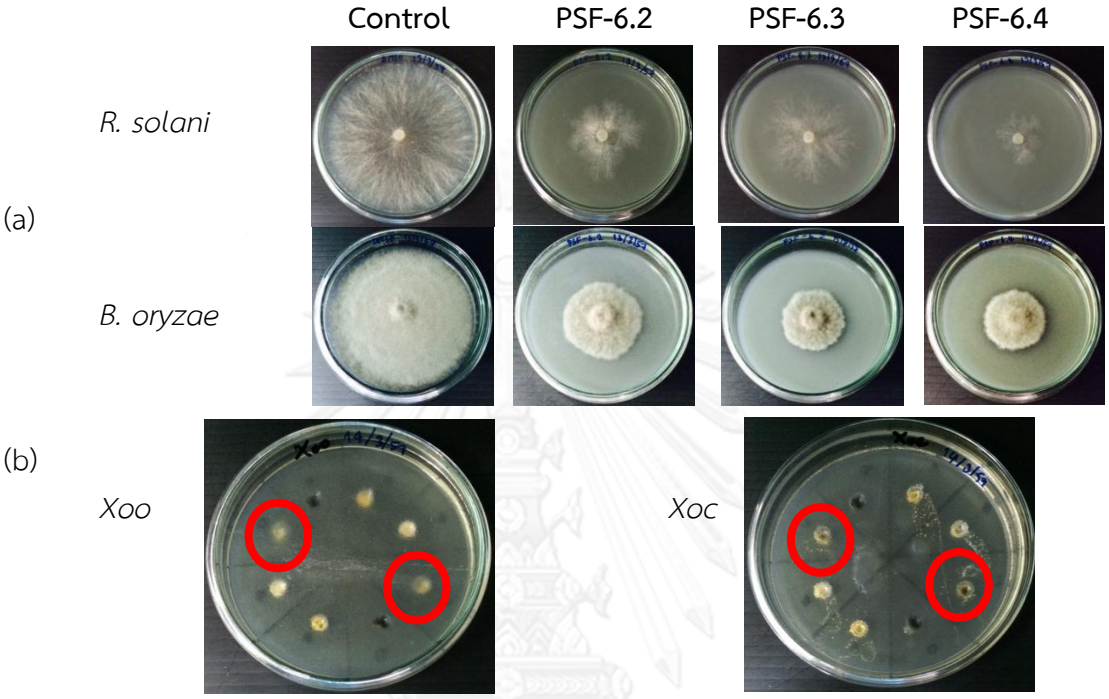


Figure 3. 22 Antifungal (a) and antibacterial (b) activities of **PSF-6** fractions

(Note: Red circles are clear zones of **PSF-6.4**.)

PSF-6.4 showed significant antimicrobial activity better than other fractions. Thus, **PSF-6.4** was further investigated.

The ^{1}H-NMR spectrum of **PSF-6.4** is shown in **Figure 3.23**. The comparative assignments of **PSF-6.4** (compound **D**) and brachyamide B (**10**) are presented in **Table 3.21**.

Table 3. 21 ^{1}H-NMR data of brachyamide B **(10)** and compound **D**

Position	Brachyamide B (10)	Compound D
	δ_H (multiplicity *J* in 60 and 90 Hz)*	**δ_H (multiplicity *J* in 400 Hz)**
1	6.06 (d, *J* = 15.0 Hz, 1H)	6.32 (d, *J* = 15.8 Hz, 1H)
2,	7.10 – 7.30 (m, 1H)	6.79 – 6.70 (m, 2H)
5′	6.60 – 7.00 (m, 4H)	
2′		6.87 (s, 1H)
6′		6.99 – 6.89 (m, 1H)
8		6.13 (d, *J* = 15.1 Hz, 1H)
3, 6	2.10 – 2.40 (m, 4H)	2.41 – 2.25 (m, 4H)
4, 5	1.50 (m, 4H)	2.20 – 1.79 (m, 4H)
7	6.15 – 6.23 (m, 1H)	6.07 – 5.96 (m, 1H)
9	5.88 (s, 2H)	5.93 (s, 2H)
1″ , 4″	3.48 (t, , *J* = 6.0 Hz, 4H)	3.51 (dt, *J* = 10.2, 6.9 Hz, 4H)
2″	1.80 – 2.00 (m, 4H)	1.95 (quint., *J* = 6.7 Hz, 2H)
3″		1.85 (quint., *J* = 6.8 Hz, 2H)

*(Koul 1988)

Based on spectroscopic method and comparison with previous reports, compound **D** (or **PSF-6.4**) was identified as brachyamide B (**Figure 3.24**)

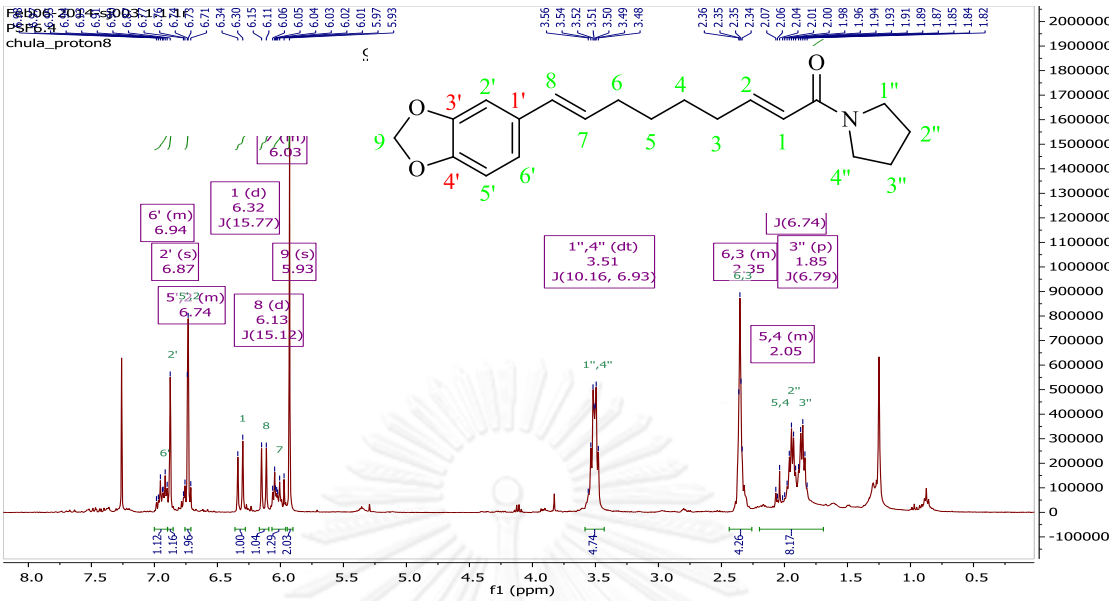


Figure 3. 23 ^{1}H-NMR and structure of brachyamide B (**10**)

This compound revealed the potential antimicrobial activity on tested rice pathogenic microorganisms. So, this compound was supposed to further test for MIC/MBC and IC_{50}. MIC/MBC and IC_{50} of compound **D** are shown in **Table 3.22**. Brachyamide B displayed the most antifungal activity of IC_{50} with 0.12 mM against *B. oryzae*.

Table 3. 22 MIC/MBC and IC_{50} of compound **D**

	Strains	Concentration	
		mg/mL	mM
MIC/MBC	*Xoo*	3.12/0.78	7.62/1.90
	Xoc	12.50/0.78	30.52/1.90
IC_{50}	*R. solani*	0.396	0.97
	B. oryzae	0.048	0.12

Brachyamide B (**10**) displayed marginal anti-unicellular fungi with IC_{50} of 41.82-32.82 μg/mL against *Candida albicans* (Tuntiwachwuttikul *et al.*, 2006). The mode of action of brachystamide B (**7**) and brachyamide B (**10**) was the inhibition of ribonucleic acid (RNA) synthesis affecting towards mycelial growth, spore formation and structural cell wall. Moreover, these amides could inhibit fungal respiration and destroyed with microtubules, mitosis and cell division (Mueller 2008).

3.4.6 The separation of SPF-7

PSF-7 (10.0 g) was separated using flash column eluting with increasing polarity of solvents. All fractions were combined based on TLC pattern (**Table 3.23**).

Table 3. 23 The separation of **PSF-7**

Fractions	Solvent system	Remarks	Weight (g)
PSF-7.1	100% Hexane -40% EtOAc in hexane	yellow solid	0.12
PSF-7.2	40% EtOAc in hexane	dark-orange solid	0.06
PSF-7.3	40-60% EtOAc in hexane	brown liquid	3.18
PSF-7.4	60% EtOAc in hexane-100% EtOAc	dark-green solid	0.62
PSF-7.5	5% MeOH in EtOAc	dark-green solid	2.43
PSF-7.6	10% MeOH in EtOAc	dark-green solid	31.93

All fractions except **PSF-7.2** were further tested against rice pathogenic microorganisms. The results of antifungal activities of **PSFs -7.1, -7.3-7.6** are shown in **Figures 3.24-3.25**.

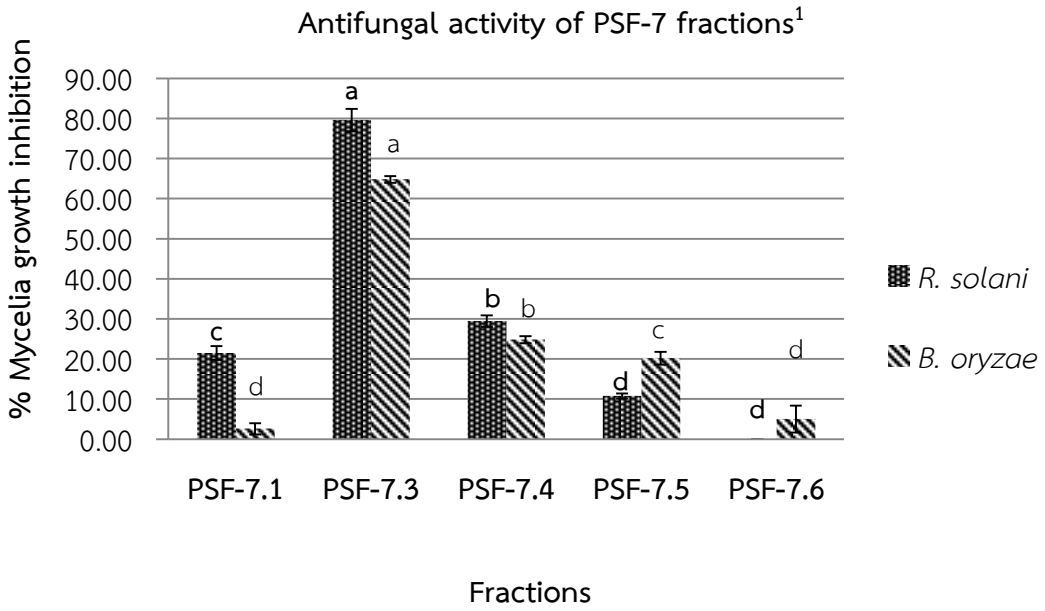


Figure 3. 24 Mycelia growth inhibition of rice pathogenic fungi of **PSFs -7.1,** and **- 7.3-7.6**

[1]Bar charts of each fungus followed by the same letter were not significantly (P ≥ 0.05) difference according to Duncan's multiple comparison tests.

For anti-fungal activity, **PSF-7.3** showed significant anti-fungal activity (P<0.05) on both fungi following by **PSFs -7.4**, **-7.5**, **-7.1** and **-7.6**.

PSF-7.3 showed significantly the highest anti-bacterial activity against *Xoo*, whereas that against *Xoc* was comparable with other fractions (**Figure 3.25**).

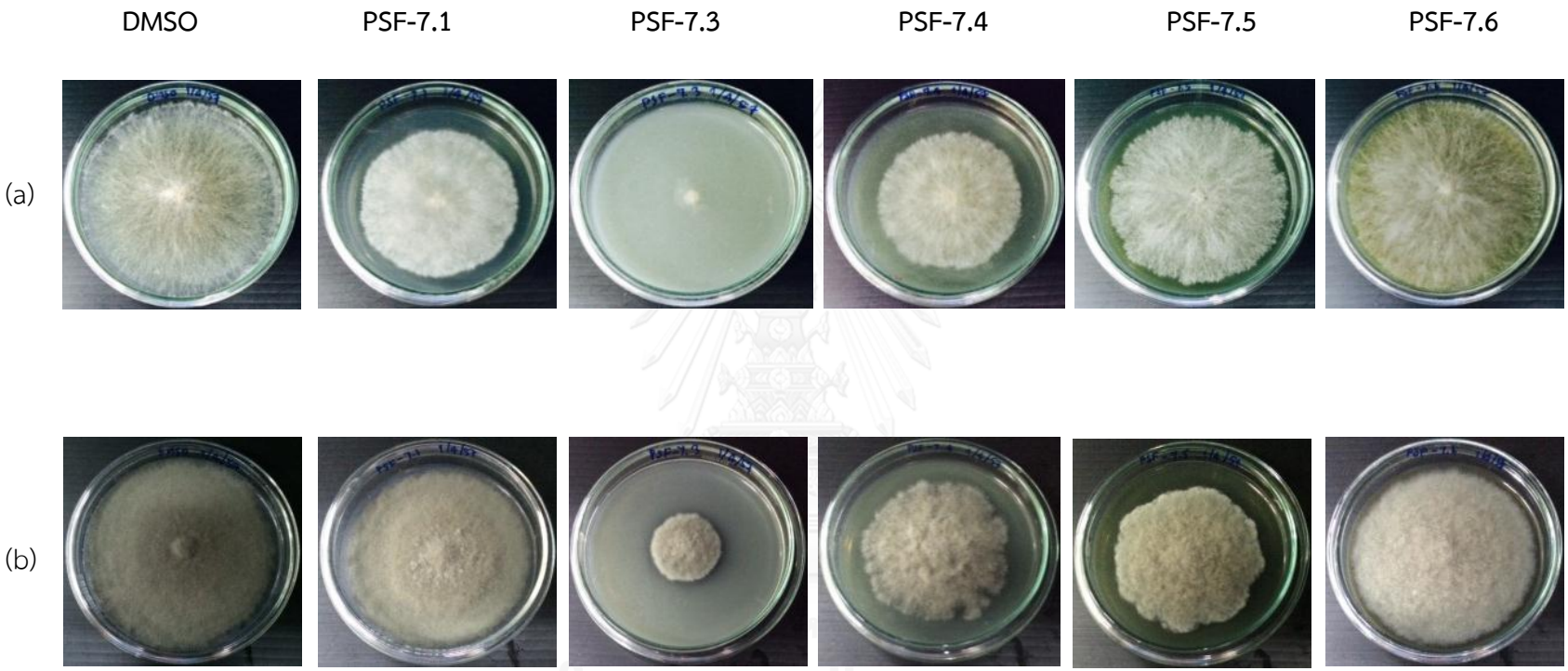
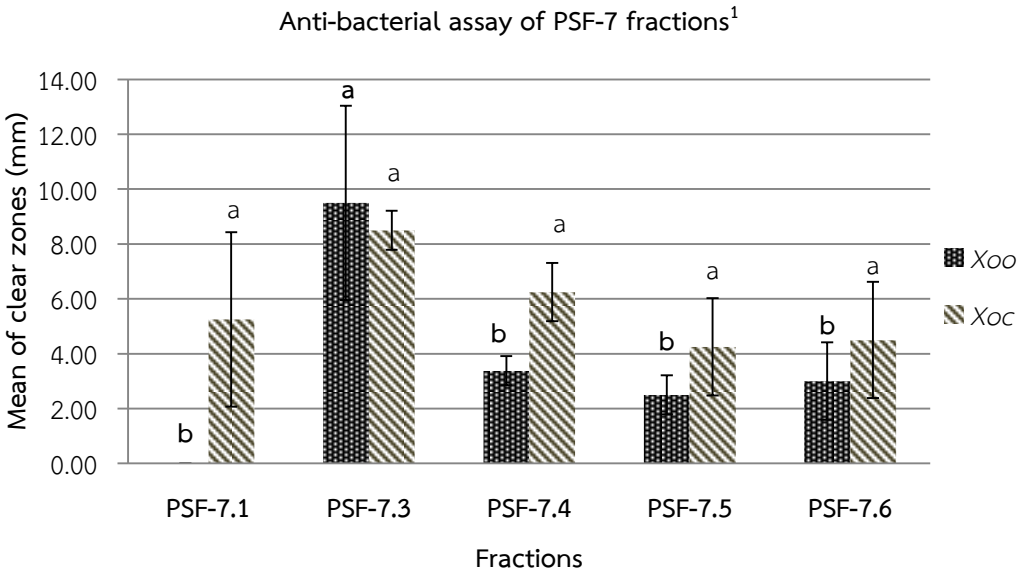


Figure 3. 25 Mycelia inhibition of **PSFs -7.1,** and **-7.3-7.6** against *R. solani* (a) and *B. oryzae* (b)

(a)



(b)

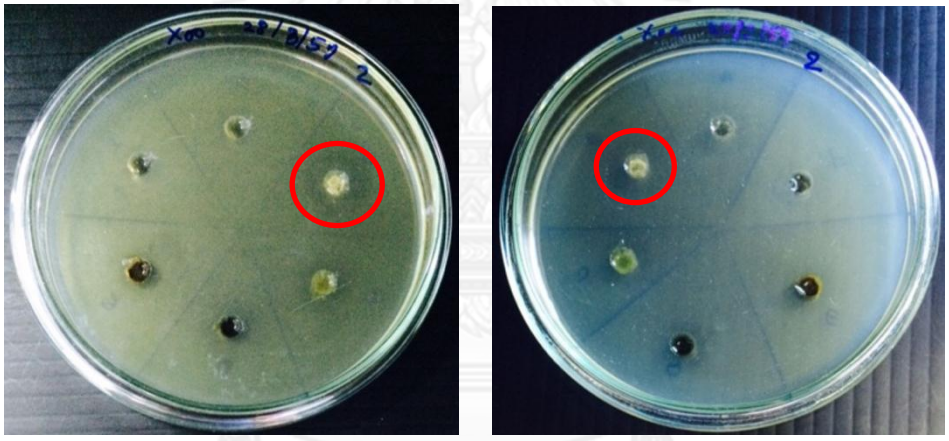


Figure 3. 26 Anti-bacterial activity of **PSFs -7.1,** and **-7.3-7.6**; average of clear zones (a) and agar diffusion assay

[1]Bar charts of each fungus followed by the same letter were not significantly (P ≥ 0.05) difference according to Duncan's multiple comparison tests.

PSF-7.3 may compose of antimicrobial agents which disturbed the cell mechanisms (Rafii 2008). Therefore, **PSF-7.3** was selected for further study. GC/MS revealed a single peak whose the mass spectrum is shown in **Figure 3.27**. According to the natural products' library, this compound was suggested to be piperonal (**29**). This compound was further confirmed by ^{1}H-NMR spectrum **(Figure 3.28**). The comparative assignments of **PSF-7.3** (compound **E**) and piperonal (**29**) are presented in **Table 3.24**.

Table 3. 24 ^{1}H-NMR data of piperonal **(29)** and compound **E**

Position	Piperonal (29)*	Compound E
	δ_H (multiplicity *J* in 300 Hz)	δ_H (multiplicity *J* in 400 Hz)
1	9.82 (s, 1H)	9.73 (s, 1H)
2	6.08 (s, 2H)	6.01 (s, 2H)
2´	7.34 (d, *J* = 1.5 Hz, 1H)	7.24 (d, *J* =1.6 Hz, 1H)
5´	6.94 (d, *J* = 7.9 Hz, 1H)	6.86 (d, *J* = 8.0 Hz, 1H)
6´	7.42 (dd, *J* = 7.9 Hz, 1H)	7.34 (dd, *J* = 7.9, 1.6 Hz, 1H)

*(Wan 2007)

Based on spectroscopic method and comparison with previous reports, compound **E** (**PSF-7.3**) was identified as piperonal (**29**). This compound has been usually found in other *Piper* species including *P. maginatum* and *P. nigrum* (Parmar *et al*., 1997).

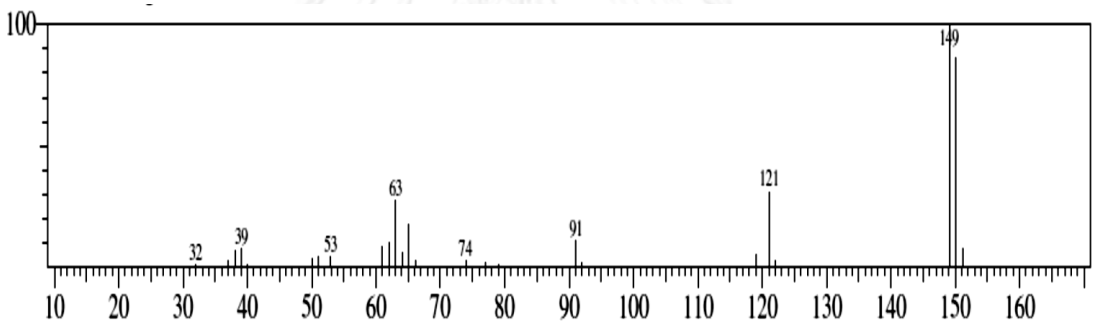


Figure 3. 27 Mass spectrum (GC/MS) of compound **E**

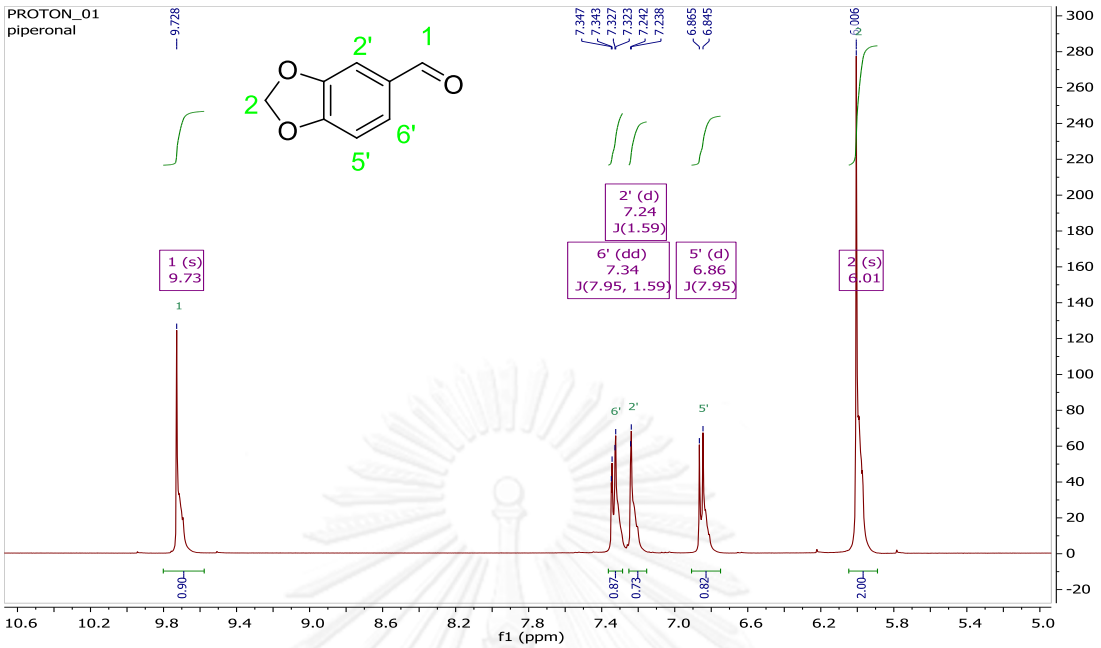


Figure 3. 28 The ^{1}H NMR spectrum of **PSF-7.3**

This compound revealed the potential antimicrobial activity on tested rice pathogenic microorganisms. So, this compound was supposed to further test for MIC/MBC and IC_{50}. MIC/MBC and IC_{50} of compound **E** are shown in **Table 3.25**. Brachyamide B displayed the most anti-fungal activity of IC_{50} with 0.26 mM against *B. oryzae*.

Table 3. 25 MIC/MBC and IC_{50} of compound **E**

	Strains	Concentration	
		mg/mL	mM
MIC/MBC	*Xoo*	6.25/6.25	41.57/41.57
	Xoc	0.39/3.12	2.59/20.75
IC_{50}	*R. solani*	0.166	1.11
	B. oryzae	0.038	0.26

3.4.7 Investigation on PSF-3

3.4.7.1 GC/MS analysis of PSF-3

PSF-3 was the most active against both fungi (**Figure 3.16**). The GC/MS analysis of this fraction is shown in **Figure 3.29**.

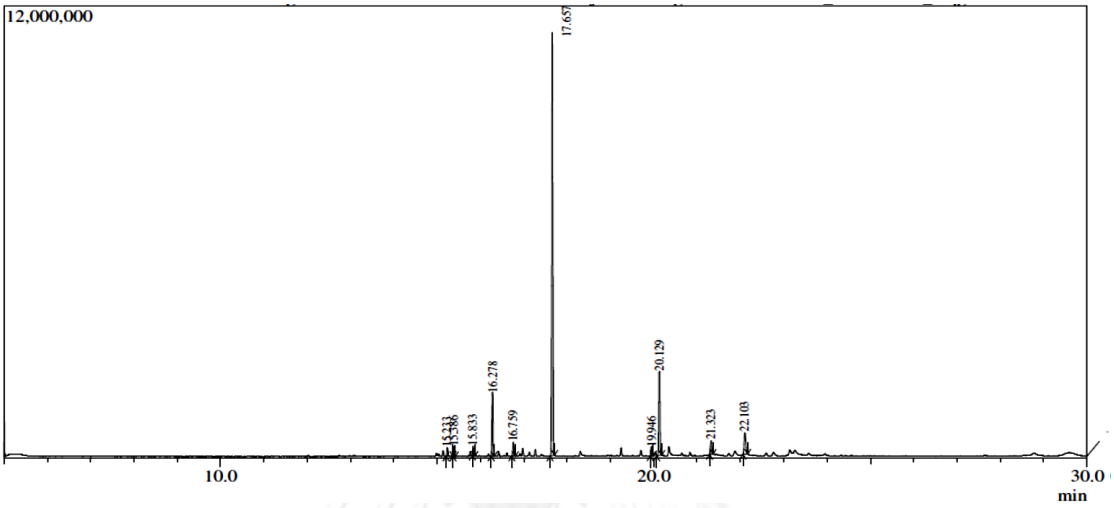


Figure 3. 29 The GC/MS chromatogram of **PSF-3**

Ten compounds were detected and represented 99.9% of the fraction. The chemical compounds and %content are summarized in **Table 3.26**.

Table 3. 26 The analysis of **PSF-3**

No.	R_t (min)	Compounds	% Content[a]
1	15.23	Phenylpropanamide	1.3
2	15.39	Isoledene	1.3
3	15.83	1,6,10-Dodecatrien-3-ol	1.1
4	**16.28**	***trans*-Caryophyllene (22)**	**9.7**
5	16.76	Spathulenol (20)	2.1
6	**17.66**	***N*-(3-phenylpropanoyl)pyrrole (27)**	**60.9**
7	19.95	Methyl hexadecanoate	1.4
8	**20.13**	**Pellitorine (5)**	**14.5**
9	21.32	Methyl heptadecanoate	2.6
10	22.06	Pyrimidin-4-ol	5.0
		Total identified	99.9

[a]%content from the peak area relative to the total peak area

N-(3-phenylpropanoyl)pyrrole (**27**) (60.9%), pellitorine (**5**) (14.5%), *trans*-caryophyllene (**22**) (9.7%), pyrimidin-4-ol (5.0%) and methyl-heptadecanoate (2.6%) were five major compounds in this fraction. The three major compounds (**27**, **5** and **22**) were reported from the prior reports (Atiax et al., 2011, Qin 2010, Rukachaisirikul et al., 2004). *N*-(3-phenylpropanoyl)pyrrole (**27**) has been firstly found by Likhitwitayawuid (1987). Three main compounds (**27**, **5** and **22**) might active alone or synergistic for anti-fungal activity against both fungi.

3.4.8 Investigation on PSF-2

3.4.8.1 GC/MS analysis of PSF-2

PSF-2 was the most active against both fungi (**Figure 3.17**). The GC/MS analysis of this fraction is shown in **Figure 3.30**.

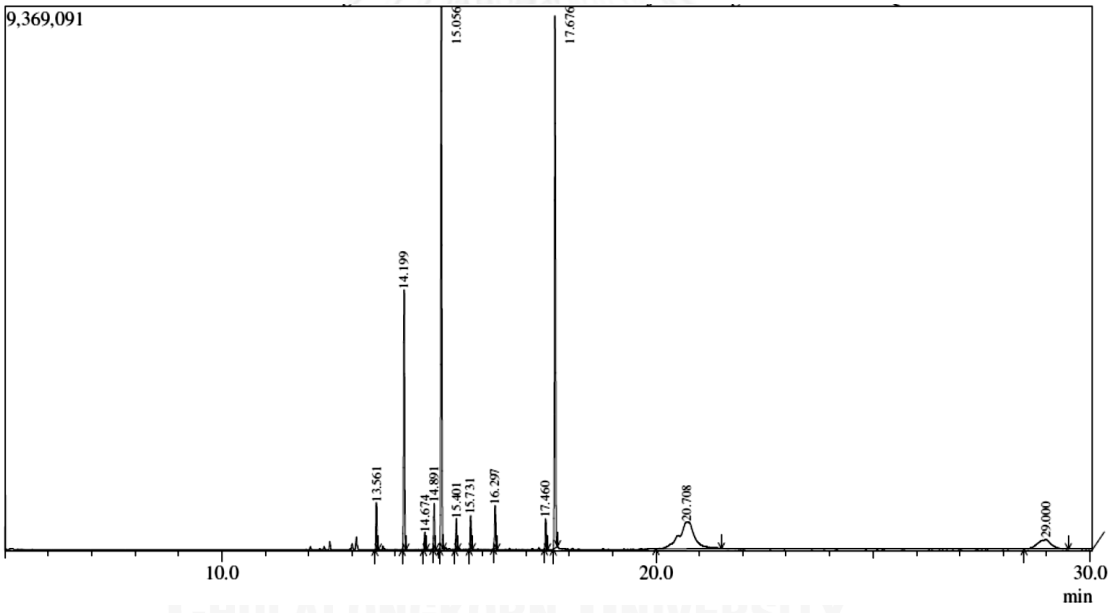


Figure 3. 30 The GC/MS chromatogram of **PSF-2**

Twelve compounds were detected and represented 100% of the fraction. The chemical compounds and %content are summarized in **Table 3.27**.

Table 3. 27 The analysis of **PSF-2**

No.	R_t (min)	Compounds	% Content[a]
1	13.56	**α**-Cubebene	2.2
2	**14.20**	**Isocaryophyllene**	**12.3**
3	14.67	**α**-Caryophyllene	0.8
4	14.89	9-Octadecene	1.9
5	**15.06**	**Pentadecane**	**24.3**
6	15.40	Napthalene	1.4
7	15.73	1-Allyl-2,4,5-trimethoxy benzene (2)	1.5
8	16.30	Cycloheptane	2.4
9	17.46	Heptadecane	1.3
10	**17.68**	***N*-(3-phenylpropanoyl)pyrrole (27)**	**25.7**
11	**20.71**	**Sarmentosine (12)**	**19.9**
12	29.00	Cyclohexasiloxane	6.3
		Total identified	100

[a]%content from the peak area relative to the total peak area

N-(3-phenylpropanoyl)pyrrole (**27**) (25.7%), pentadecane (24.3%), sarmentosine (**12**) (19.9%), isocaryophyllene (12.3%) and cyclohexasiloxane (6.3%) were the major large amount compounds in this fraction. The compounds **27** and **12** have been reported (Likhitwitayawuid 1987, Rukachaisirikul et al., 2004). Additionally, sarmentosine (**12**) was firstly found in this fraction.

In conclusion, the results revealed that brachystamide B (**7**), sarmentine (**8**), brachyamide B (**10**) and piperonal (**29**) had the broad spectrum of antimicrobial activities against rice pathogenic fungi and bacteria. The antifungal agent of this extract was *N*-(3-phenylpropanoyl)pyrrole (**27**) which the major compound of two effective fractions (**PSF-2** and **PSF-3**).

3.5 Hydrodistillation of *P. sarmentosum*

The fresh leaves of *P. sarmentosum* (10 kg) were cut into small pieces and hydrodistillated. The crude oil was further extracted with Et_2O, filtrated, dried over anhydrous Na_2SO_4 and evaporated to furnish the pale yellow oil 3.36 g (0.03% yield).

3.5.1 Chemical compositions of the essential oil

The obtained essential oil was subjected to GC/MS analysis. Fourteen compounds were detected (**Figure 3.31**), representing 99.99% of the total oil. The chemical constituents of the oil and %content are summarized in **Table 3.28**.

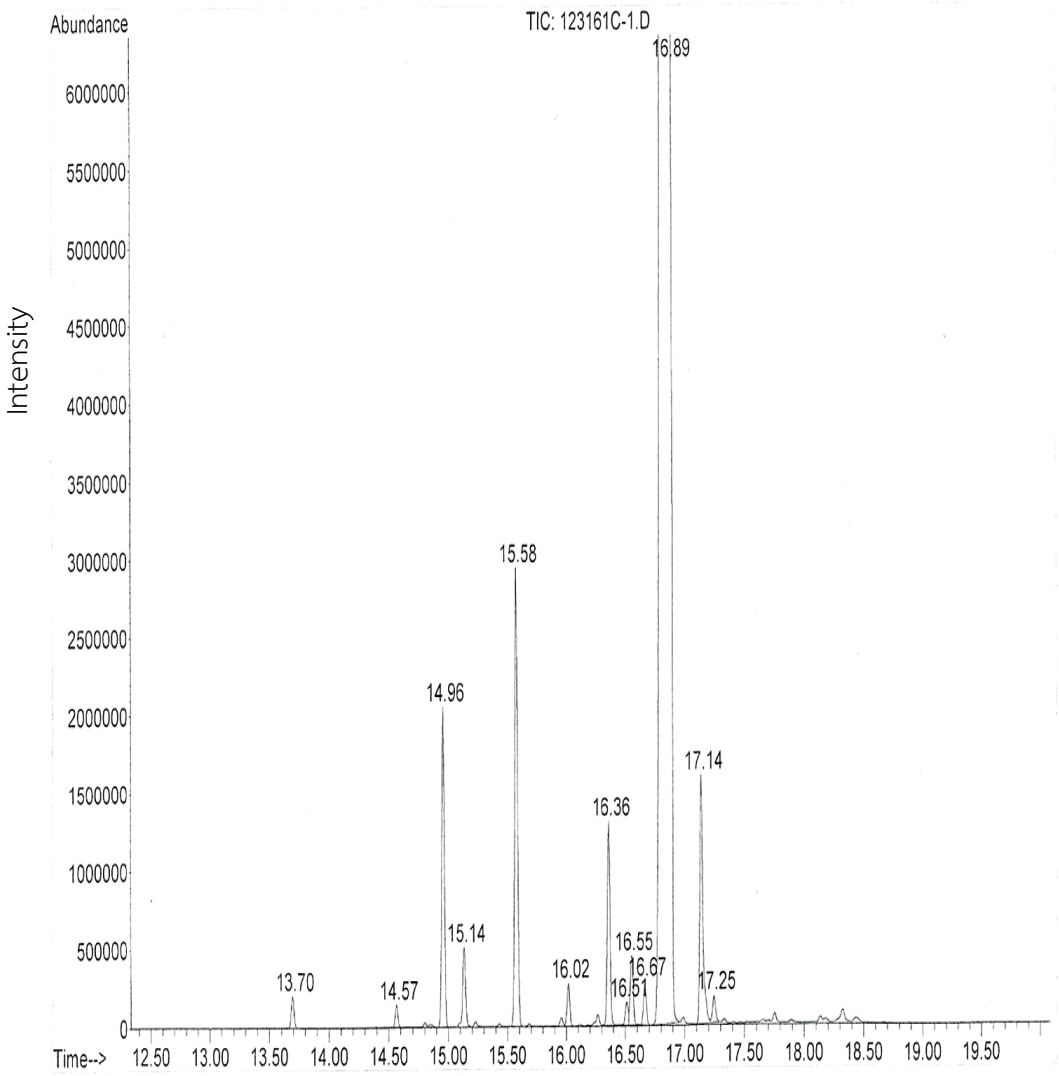


Figure 3. 31 GC/MS chromatogram of the essential oil of *P. sarmentosum*

Table 3. 28 The analysis of the essential oil of *P. sarmentosum*

No.	R_t (min)	Compounds	% Content[a]
1	3.42	Pyrrole	0.2
2	13.70	Safrole	0.3
3	14.57	**α**-Cubebene	0.2
4	14.96	**α**-Copaene	3.1
5	15.14	*Epi*-Bicyclosesquiphellandrene	0.8
6	15.58	*trans*-Caryophyllene (22)	4.5
7	16.02	**α**-Humulene	0.4
8	16.36	Germacrene-D	1.9
9	16.51	(+)-delta-Cadinene	0.2
10	16.55	Germacrene B	0.7
11	16.67	Ionole	0.4
12	**16.89**	**Myristicin (21)**	**84.3**
13	17.14	Elemicin	2.6
14	17.25	(E)-Farnesene	0.3
		Total identified	99.9

[a] %content from the peak area relative to the total peak area

Myristicin (**21**) (84.3%), *trans*-caryophyllene (**22**) (4.5%), **α**-copaene (3.1%), elemicin (2.6%) and germacrene D (1.9%) were five major components in this essential oil. Phenylpropanoid was the group of natural products within the oil. The two major constituents (**21** and **22**) were reported from a previous study (Qin 2010). Myristicin (**21**) was also found in nutmeg and tripe spice (Dawidowicz 2012).

MS spectrum of myristicin (**21**) displayed as *m/z* (EI, 70 eV.) = 192.20. The molecular formula was $C_{11}H_{12}O_3$ and the compound was matched as 98% quality comparing with the wiley 7n library and the percentage area was 84.31%. *trans*-Caryophyllene (**22**) displayed as *m/z* (EI, 70 eV.) = 204.35. The molecular formula was $C_{15}H_{24}$ and the compound was matched as 99% quality comparing with the library and the percentage area was 4.51%. **α**-Copaene displayed as *m/z* (EI, 70 eV.) = 204.35. The molecular formula was $C_{15}H_{24}$. Moreover, this compound was matched as 99% quality comparing with the library and the percentage area was 3.06%. Elemicin displayed as *m/z* (EI, 70 eV.) = 208.25. The molecular formula was $C_{12}H_{16}O_3$

and the compound was matched as 95% quality comparing with the library and the percentage area was 2.62%. Germacrene D displayed as *m/z* (EI, 70 eV.) = 204.35. The molecular formula was $C_{15}H_{24}$ and the compound was matched as 97% quality comparing with the library and the percentage area was 1.93%. Five major constituents could be found in the previous studied of Qin (2010); especially, myristicin (**21**) was the most abundant compounds as same as my study. It was differed from Chieng (2008) studied that the spathulenol (**20**) revealed more abundant than myristicin (**21**).

3.5.2 Antimicrobial activities of the essential oil

This essential oil completely inhibited on both mycelia and spore germination of fungi (*R. solani* and *B. oryzae*) at 1,000 mg/L (**Figure 3.32**).

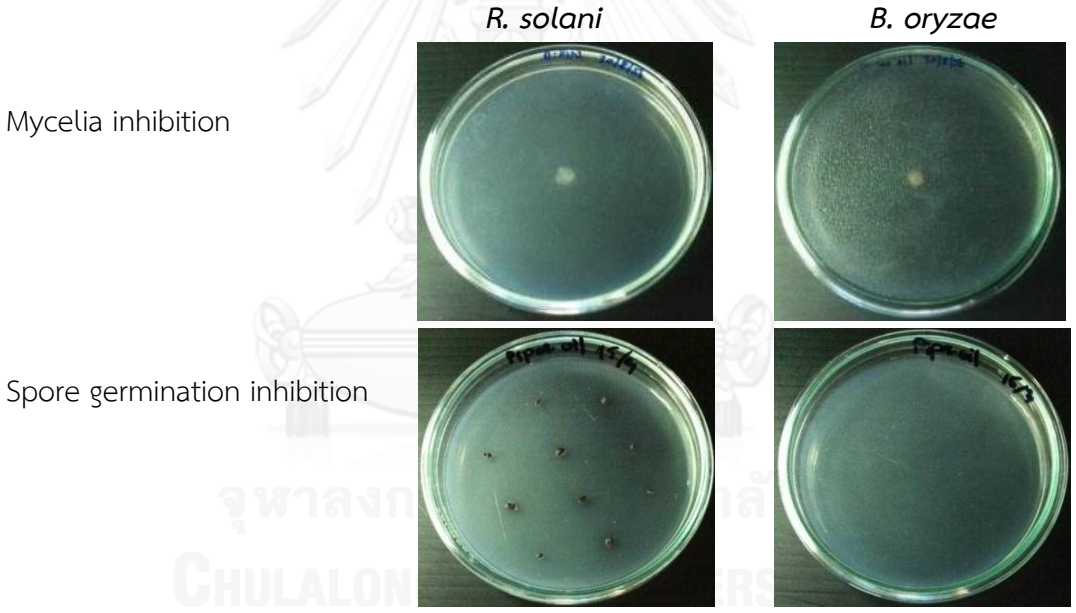


Figure 3. 32 Anti-fungal activity and spore germination inhibition of the essential oil at 1,000 mg/L

IC_{50} of the essential oil against mycelia growth of rice pathogenic fungi are presented in **Table 3.29**.

Table 3. 29 IC_{50} of the essential oil

Fungal strains	IC_{50} (mg/mL)
R. solani	0.048
B. oryzae	0.015

R. solani and *B. oryzae* were relatively mycelia fungi sensitivity with their IC_{50} as 0.048 and 0.015 mg/mL, respectively. According to IC_{50} of *R. solani*, the fruit oil of *Illicium verum* and the oil of *Eucalyptus tereticornis* exhibited lower anti-fungal activity than *P. sarmentosum* oil (Huang 2010, Kaur 2011) and the leaf oil of *Calocedrus macrolepis* var. *formosana* was close to the *Piper* oil (Yen 2008). On the behalf of anti-*B. oryzae*, it has been suppressed by basil oil (*Ocimum basilicum* L.) and sweet fennel oil (*O. gratissimum*) (Piyo 2009). Shin (2007) reported that anti-fungal activities of plumbagin, purified from *Nepenthes ventricosa* x *maxima* leave oil, could inhibit on both fungi (*R. solani* and *B. oryzae*) with MIC of 4.8 and 9.7 μg/mL. The antimicrobial activity of the essential oil of *P. sarmentosum* was investigated on oral microorganisms, *Aspergilus niger*, *A. oryzae*, *Penicillium* sp., *Microsporum canis*, *A. flavus*, *C. albicans*, *Trichophyton rubrum* and *T. mentagrophyte* (Cheeptham 2002, Nazmul 2011, Taweechaisupapong 2010).

3.6 Experimental section

3.6.1 Extraction of *P. sarmentosum*

Fresh Leaves (10 kg) and seeds (5 kg) of *P. sarmentosum* were oven dried, milled and extracted with CH_2Cl_2 for three times. The extracts were filtered, evaporated and kept for antimicrobial activities. The fresh plant (10 kg) was hydro-distilled using a modified Dean-Stark apparatus. After extraction with Et_2O, the extract was evaporated under reduced pressure in rotatory evaporator. The oil yield and all tested data are the average of triplicate analyses (Ho 2012).

3.6.2 Antibacterial assay

Antibacterial activity of CH_2Cl_2 and essential oil was tested against two gram negative bacteria of rice pathogenic bacteria: *Xoo* and *Xoc*. The agar diffusion method (Barry 1999) was used to screen the antibacterial activity of plant essential

oil and all compounds. Initially, two bacterial strains (*Xoo* and *Xoc*) were cultivated in nutrient agar (NA) and incubated at 37°C for 18–24 h. Selected 4–5 single colonies of each tested bacteria were cultivated in nutrient broth (NB) and incubated at 37°C for 2-5 h. Freshly cultured bacterial suspensions in NB were standardized to cell density of 1.5×10^8 CFU/mL (McFarland No. 0.5) and adjusted by 0.85% sterile NaCl. Each tested NA media (19 mL) was mixed with adjusted bacterial suspension (1 mL), poured into petri plates, and then allowed to set. The tested culture plates created the wells using 6 mm cork border, added 40 µL of dissolved extract and DMSO (control) into these wells, incubated the plates at 37°C overnight, and also recorded the zones of inhibition in duplicate.

3.6.3 Antifungal activity

Antifungal activity of all extracts was tested against two rice pathogenic fungi including *R. solani* and *B. oryzae*. The extract and major constituents were dissolved in DMSO and 100 µL was added to PDA to execute a final concentration at 1,000 mg/L. A 5 mm agar disc containing mycelia was transferred to the center of the PDA plate containing the extract or compounds. Plates were incubated at 25°C for 3 days (*R. solani*) and 8 days (*B. oryzae*). 1% DMSO was used as negative control. When the mycelium of fungi reached the edges of the control petri dishes (those without extracts), the antifungal indices were calculated. The formula of antifungal indices is shown as **Eq 3.1**.

$$\text{Percentage inhibition} = \frac{(C - T) \times 100}{C} \% \qquad \text{(Eq 3.1)}$$

Where, C = colony diameter (cm) of the control.

T = colony diameter (cm) of the test plate.

3.6.4 Minimum inhibitory concentration (MIC)

MIC's were measured by the macro-dilution broth susceptibility assay recommended by NCCL (Lalitha 2010). Stock solutions of the oil were prepared in DMSO. Ten-fold serial dilutions of oil or major constituent were prepared from 100 mg/mL to 0.08 mg/mL in 10 test tubes with sterile NB (1 mL) in as diluents. Each

dilution was pipetted with 1 mL of test bacteria to the standard concentration (1.5 x 10^8 CFU/mL). Oil or major constituent were used as experimental positive control and only medium as a negative control. The tubes were incubated at 37°C for 24 h. The least concentration of the oil extract or major constituent showing no visible growth was taken as the MIC. After 24 h of incubation period, mean MIC values were calculated. The test was performed in triplicates for each bacterium used and the final results were expressed as the arithmetic average of triplicate experiments. 100 µL test media from each MIC broth tube was spread over the NA plates. Plates were incubated at 37°C for 24 h. The test MIC concentration showing no bacterial growth on agar plates was considered as MBC of the extract (Singh 2011).

3.6.5 GC-MS analysis

The GC-MS analysis was performed by Agilent 6890 gas chromatograph in electron impact (EI, 70eV) mode coupled to an HP 5973 mass selective detector and fitted with a fused silica capillary column (HP-5MS) (30 m x 0.25 mm x 0.25 µm film thickness). Helium (1.0 mL/min) was used as a carrier gas. Samples were injected in the split less mode at ratio of 1:10-1:100. The injector was kept at 250°C and the transfer line at 280°C. The MS was EM mode at 1,576.5 EM Voltage, in the *m/z* range 50-550. The identification of the compounds was performed by comparing their retention indices and mass spectra with those found in the literature and supplemented by the Wiley 7n and Natural Products GC/MS libraries.

3.6.6 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.

CHAPTER IV
ANTIMICROBIAL ACTIVITY OF THE ESSENTIAL OIL FROM *POLYGONUM ODORATUM* LOUR.

4.1 Introduction

4.1.1 Botanical description

P. odoratum (Lour.), (family Polygonaceae) - Vietnamese coriander (*syn*. *Persicaria odorata* Lour.) (Wilson 1988) - is locally known in Thai as Pak - Paeow (Pak-Phai) distributing in tropical areas such as Malaysia, Southern Australia, Singapore, Vietnam and Thailand. This plant is a constant herb 30 - 35 cm height with spiked leaves 6-15 cm, the center of leaves is dark purple and has individual smell (Starkenmann et al., 2006) (**Figure 4.1 a**, **b** and **c**). The plant has usually been used for Southeast Asian cuisine such as in salads, meat dishes, some hot soup and spicy soup (Quynh 2009).

(a) (b) (c)

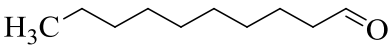
Figure 4. 1 *P. odoratum*, mature plant (a), leaves and twigs (b) and sketch (c)

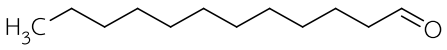
(Sources: (a) http://gb.eurohydro.com/aromatics.html
(b) http://www.digthedirt.com/plants/12519-herbs-persicaria-odorata
(c) http://runeberg.org/nordflor/391.html)

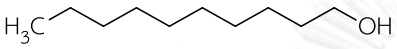
Various biological activities such as antioxidant (Somparn 2007, Zheng 2001), anti-breast cancer (Rafi 2007), antimicrobial (Nanasombat 2009) and antibacterial activities (Sasongko 2011) have been reported.

4.1.2 Chemical investigation and biological activities

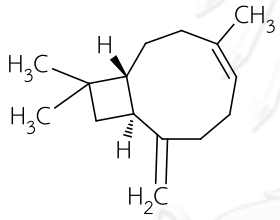
Hunter (1997) analyzed the oil of *P. odoratum* from Southern Australia by GC and GC/MS. Forty-nine compounds were detected and among those, the major constituents were decanal (28%) (**1**), dodecanal (44%) (**2**) and decanol (11%) (**3**).

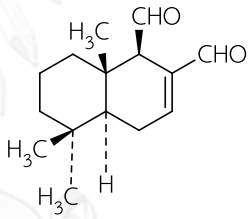
(**1**) 

(**2**) 

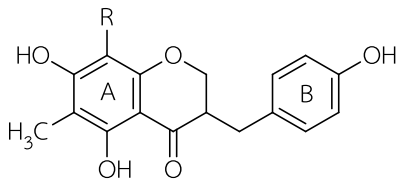
(**3**) 

Starkenmann et al., (2006) reported the volatile constituents of the aerial part of *Persicaria odorata* (Lour.) from Singapore. The major compounds were identified as decanal (1), dodecanal (2), β-caryophyllene (4) and polygodial (5) by GC/MS analysis.

(**4**) 

(**5**) 

Rafi (2007) examined the active compositions and anti-cancer activity. Two active compounds: 2,3-dihydro-3-[(15-hydroxyphenyl)methyl]-5,7-dihydroxy-6-methyl-8-methoxy-4H-1-benzopyran-4-one (**6**) and 2,3-dihydro-3-[15-hydroxyphenyl)methyl]-5,7-hydroxy-6,8-dimethyl-4H-1-benzopyran-4-one (**7**) revealed anti-breast tumor cells which were induced by G2/M cell cycle arrest.

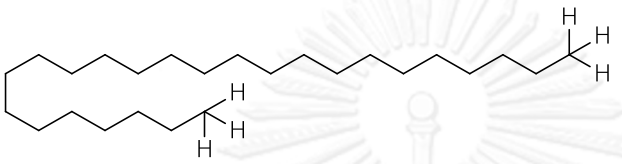


(**6**) R = -OCH_3

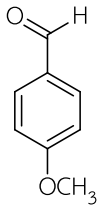
(**7**) R = -CH_3

According to anti-microbial activities, the EtOH extract of *P. odoratum* showed high activity on *Fusarium* sp. with 51% at 10,000 ppm (Sawatdikarn 2011). Wongratsameetham (2010) studied the anti-bacterial activity of the essential oil. The major constituents were dodecanal (**2**), decanal (**1**), pentacosane (**8**), *p*-anisaldehyde (**9**), *n*-dodecanol (**10**), 2*E*-dodecenal (**11**) and *n*-decanol (**3**). 2*E*-dodecanal (**11**) displayed the most anti-bacterial activity (*Salmonella choleraesuis*) with IC_{50} value of 7.8 µg/mL followed by dodecanal (**2**, IC_{50} 16.9 µg/mL).

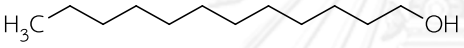
(**8**)



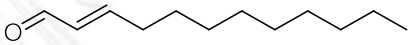
(**9**)



(**10**)



(**11**)



The objective of this study was to explore anti-rice pathogenic microbial activity of the essential oil of *P. odoratum*.

4.2 Extraction of *P. odoratum*

The fresh whole plants of *P. odoratum* were purchased from Pak Klong Talad market, Bangkok in November 2012. The fresh plants (7.82 kg) were cut and hydrodistilled. The hydrodistillate was extracted by Et_2O, dried over anhydrous Na_2SO_4 and evaporated to yield white-muddy colored oil 0.0021 g (0.027 % based on the fresh weight of whole plants).

4.3 Chemical constituents of the essential oil

With the aids of GC/MS, thirteen constituents (**Figure 4.2**) were identified. All retention indices and the percentage contents are summarized in **Table 4.1**.

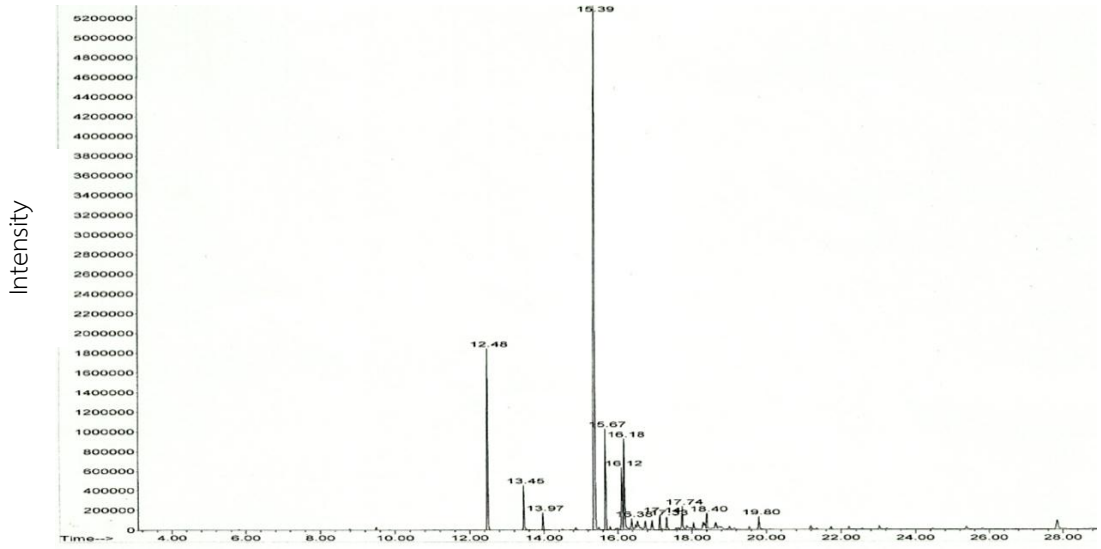
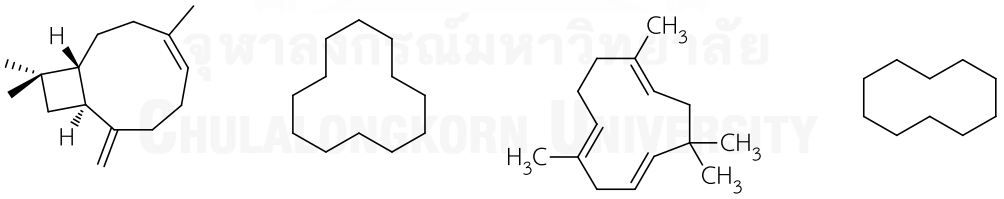
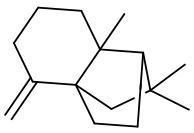


Figure 4. 2 GC/MS chromatogram of the essential oil of *P. odoratum*

Seven major constituents were analyzed as dodecanal (**2**, 54%), decanal (**1**, 15%), *trans*-caryophyllene (**12**, 8%), cyclododecane (**13**, 7%), α-humulene (**14,** 5%), cyclodecane (**15**, 3%), and β-neoclovene (**16**, 2%) (**Table 4.1**). The class of main compositions of this oil was aliphatic aldehydes. These chemical compositions were similar to those previously observed (Hunter 1997). Dodecanal (**2**), the dominant compound was widely found in many essential oils such as *Coriandrum sativum* (Matasyoh 2009), *Machilus zuihoensis* (Ho 2012), *Hypericum richeri* subsp. *Grisebachii* (Dordevic 2011), *Paeonia daurica* (Tosun 2011), and *Etlingera* species (Chiang 2010).



(**12**) (**13**) (**14**) (**15**)



(**16**)

Table 4. 1 The composition of *P. odoratum* essential oil

No.	R_t (min)	Compound	%Content
1	12.48	Decanal	15
2	13.45	Cyclodecane	3
3	13.97	Undecanal	1
4	15.39	Dodecanal	54
5	15.67	*trans*-caryophyllene	8
6	16.12	α-humulene	5
7	16.18	cyclododecane	7
8	16.38	α-curcumene	1
9	17.14	α-cedrene	1
10	17.33	(*E*)-farnesene	1
11	17.74	β-neoclovene	2
12	18.40	6-acetyl-7-hydroxy-2,2-dimethylbenzopyran	1
13	19.80	1-napthalenemethanol	1
		Total identified	100

[a] %content from the peak area relative to the total peak area

Dodecanal (**2**), a major constituent was selected to explore anti-rice pathogenic microbial activity in more details compared with the obtained essential oil.

4.4 Anti-rice pathogenic microbial activities of the essential oil and dodecanal **(2)**

4.4.1 Antimicrobial activity of the essential oil

The antimicrobial activity of the essential oil against rice pathogenic microorganisms was investigated. The antifungal activities against *R. solani* and *B. oryaze* of the original essential oil were 77.00±0.87% and 59.63±0.32% at 1,000 mg/L (**Chapter II**). The germination inhibition of sclerotia of *R. solani* was 75.00±7.07% at 2,000 mg/L and the highest inhibition spore germination of *B. oryzae* was 91.50±6.30% at 1,000 mg/L. Similar to the present findings, Seema (2010) reported the inhibitory effect of the concentrations of the essential oils from *Cinnamomum zeylanicum* Breyne., *Syzygium aromaticum* L., and *Foeniculum vulgare* ssp *piperitum*

on the mycelia growth of *R. solani* was anti-*R. solani* activity. Moreover, more effective essential oils could be seen from other plants such as *Spiraea alpine* Pall. (Teng 2010), *Origanum minutiflorum*, *Satureja cuneifolia*, *Thymbra spicata*, *O. nites* (Tugba 2006) and sweet fennel oil (*Ocimum gratissimum*) (Piyo 2009). Harish (2008) reported that the neem cake oil, mahua and castor oil revealed the mycelia growth inhibition and spore germination against *B. oryzae*.

For anti-rice pathogenic bacteria, the diameters of zones of inhibition were 7.17±1.04 mm and 11.67±3.51 mm on *X. oryzae* pv. *oryzae* (*Xoo*) and *X. oryzae* pv. *oryzicola* (*Xoc*) at 10,000 mg/L, respectively. This study was consistent with the findings of numerous studies (Bajpai 2010, Jabeen 2011, Teng 2010) which represented that anti-*Xoc* and *Xoo* activities were from the essential oils of *Terminalia chebula* Retz., *Metasequoia glyptostroboides* Miki ex Hu. and *Spiraea alpine* Pall.

4.4.2 Determination of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and the half maximal inhibitory concentration (IC_{50}) of the essential oil

4.4.2.1 MIC and MBC

The MIC and MBC values of the oil against both rice-gram negative bacteria (*Xoo* and *Xoc*) were evaluated as shown in **Table 4.2** and **Figure 4.3**.

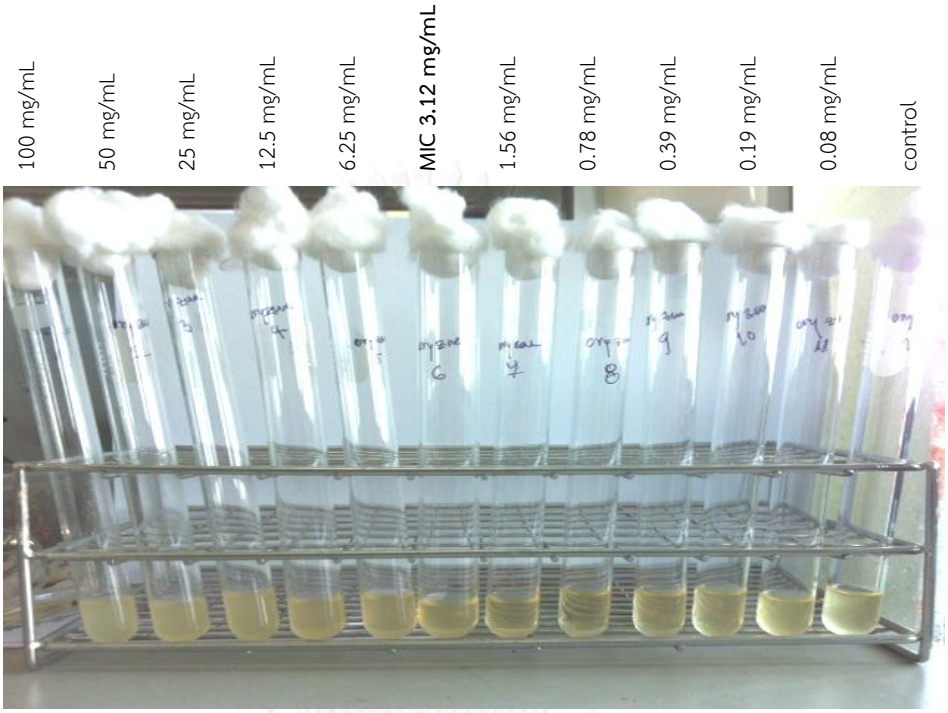
Table 4. 2 The MIC, MBC and MIC index of *P. odoratum* oil.

Bacterial strain	MIC (mg/mL)	MBC (mg/mL)	MIC index (MBC/MIC)	Indication
Xoo	3.12	12.50	4.00	Bactericidal
Xoc	0.78	12.50	16.02	Bacteriostatic

MIC was determined as the minimum concentration that is capable to inhibit visible bacterial growth (Levison 2004, Nuryastuti 2009). *Xoo* and *Xoc* were suppressed by this oil with MIC of 3.12 and 0.78 mg/mL, respectively. The MBC of the essential oil against both bacteria was 12.50 mg/mL (**Figure 4.4**). The MIC is the lowest concentration that can inhibit bacterial growth while the MBC is the lowest concentration for killing bacterial cells. The MIC is mostly measured for bacterial

susceptibility to drugs. There are only certain circumstances that need to be treated with the MBC, such as meningitis, endocarditis, sepsis in granulocytopenic host *etc*. (Lalitha 1997).

(a)



(b)

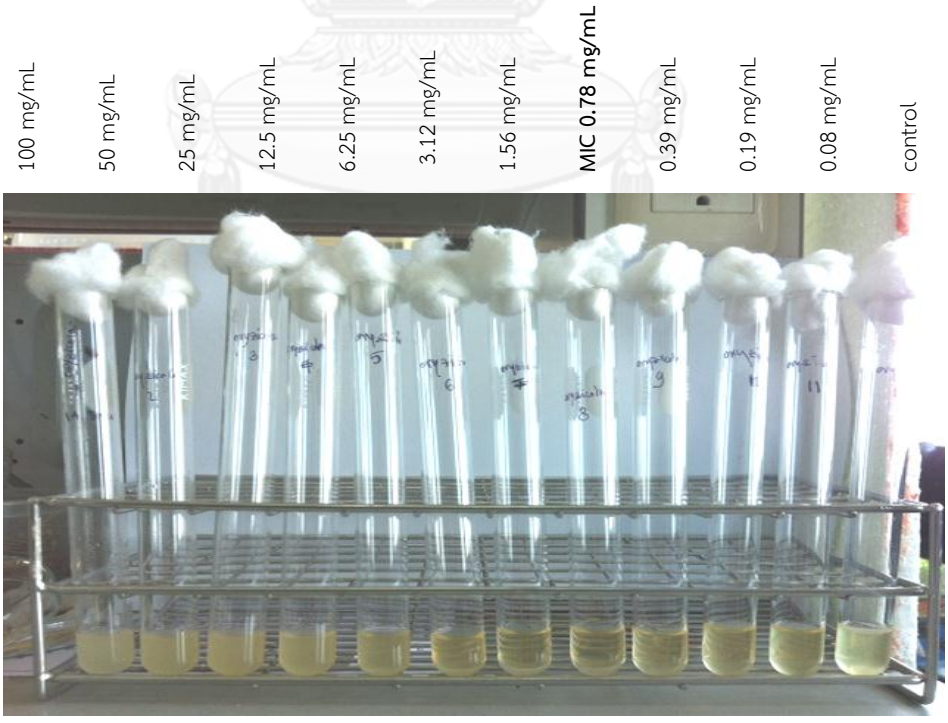
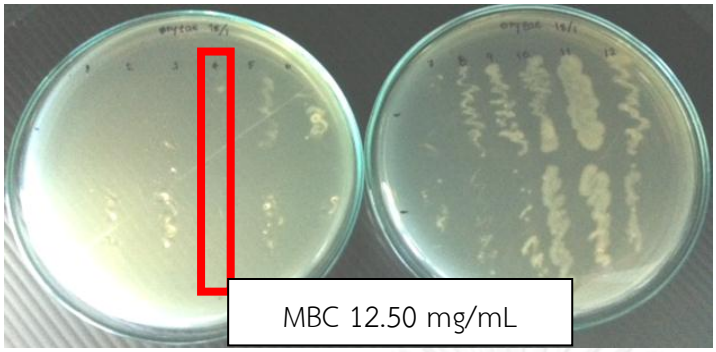
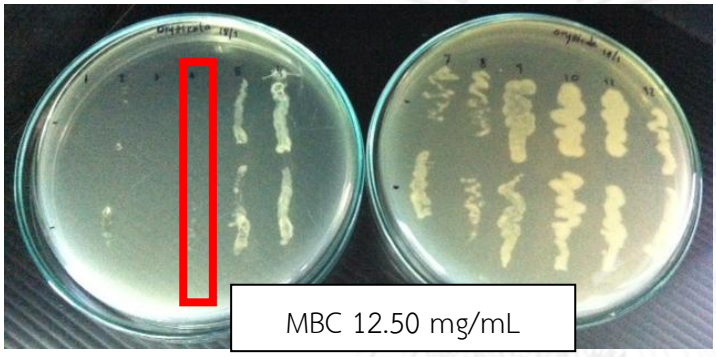


Figure 4. 3 The MIC of the essential oil against Xoo (a) and Xoc (b).



(a) MBC = 12.50 mg/mL



(b) MBC = 12.50 mg/mL

Figure 4. 4 Representative petri plates showing MBC of Xoo (a) and Xoc (b).

The MIC index (MBC/MIC) was calculated to determine if the plant extract possessed bactericidal or bacteriostatic properties (Fernandez 2012). When the MBC/MIC is ≤ 4, the extract is bactericidal whereas when MBC/MIC > 4, the extract was bacteriostatic. The MIC index of *P. odoratum* oil is shown in **Table 4.3**.

Depended on the MIC index, the indices of *Xoo* and *Xoc* were 4.00 and 16.02, respectively. For *Xoo*, this oil was bactericidal agent which could actively kill bacterial cells as cell wall synthesis inhibitors (Mayaud 2008). The bactericidal agents are generally not toxic, but they can be administered at high doses (Rahal 1979). For example, the β-lactam antibiotics (penicillin, cephalosporins, monobactams and carbapenems) and vancomycin against *Staphylococcus aureus*, phemococci, streptococci (*Streptococcus pneumoniae*) (Zurenko 1996) and some of essential oils may be organized into this group. The bactericidal effects against Gram-positive bacteria were lower than Gram-negative bacteria (Mayaud 2008). The mechanisms of action of bactericidal agents would be the inhibition in cell wall cross-linking, the interference with addition of new cell wall subunits (muramyl pentapeptides) and

the prevention of the addition of cell wall subunits by inhibiting recycling of membrane lipid carrier (Donnell 2012).

The MIC index on *Xoc* (16.02) revealed that the oil was bacteriostatic agent which inhibited and limited the growth of bacteria interfering with bacterial protein production, DNA replication, or bacterial cellular metabolism (Mayaud 2008), but keeping them in the secondary growth phase (Bernatova 2013). The bacteriostatic agents can inhibit bacteria with low concentrations. The groups of antibacterial agents and their mechanism of action have been reported. The inhibitors of protein synthesis included macrolides (binding to 50S ribosomal subunit), lincosamides and streptogamins (binding to 50S ribosomal subunit and blocking peptide chain elongation), chloramphenicol (binding to 50S ribosomal subunit and blocking amioacyl-*t*RNA attachment), tetracycline (binding reversibly to 30S ribosomal subunit and blocks binding of aminoacyl-*t*RNA), aminoglycosides (binding reversibly to 30S ribosomal subunit and inhibiting translocation of peptidyl-*t*RNA), mupriocin (inhibiting isoleucine *t*RNA synthase) and linezolid (binding to 50S ribosomal subunit and inhibiting initiation of protein synthesis). Rifampin and metronidazole appear to inhibit nucleic acid synthesis while quinolones and novobiocin will be inhibited activity of DNA gyrase (DNA synthesis) (Donnell 2012). These agents suggest that active on many Gram-positive and Gram-negative bacteria (Shetty 2009).

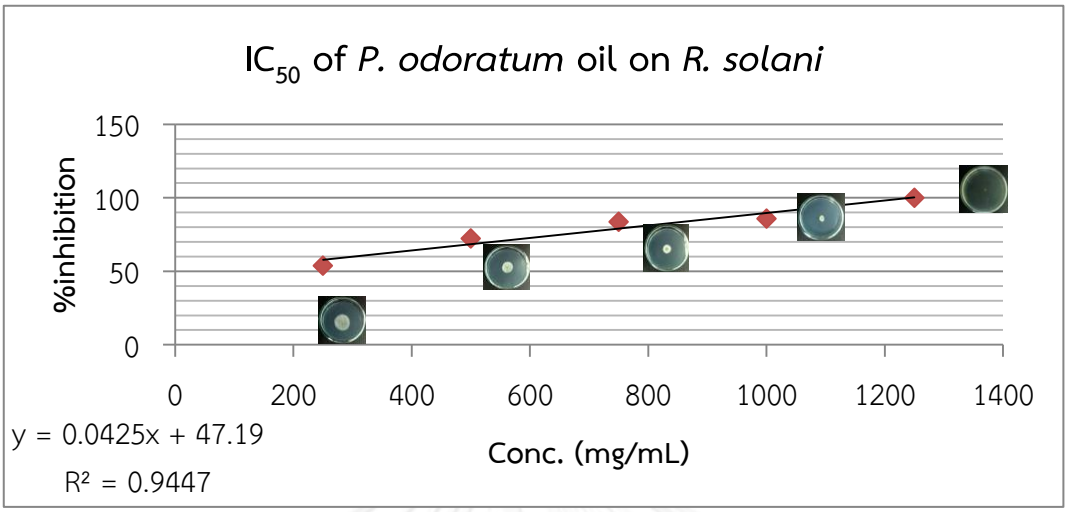
4.4.2.2 IC_{50}

The essential oil was evaluated for antifungal activity: *R. solani* and *B. oryzae* with IC_{50} 66 and 3,047 ppm by mycelia plate assay (**Table 4.3** and **Figure 4.5**). IC_{50} is half maximal inhibitory concentration which indicates the concentrations of agents to inhibit a biological or biochemical function by half *in vitro* (Cheng 1973).

Table 4. 3 Antifungal activity of the oil on fungi and IC_{50}

Fungi	Concentration (ppm)	% inhibition (mean ± SD)	IC_{50} (ppm)
R. solani	1,250	100.00 ± 0.00	66
	1,000	85.93 ± 0.85	
	750	83.61 ± 4.37	
	500	72.22 ± 2.00	
	250	53.70 ± 4.63	
B. oryzae	1,500	13.29 ± 2.31	3,047
	2,000	24.07 ± 4.34	
	2,500	34.76 ± 4.41	
	3,000	54.42 ± 2.25	
	3,500	57.81 ± 6.25	

(a)



(b)

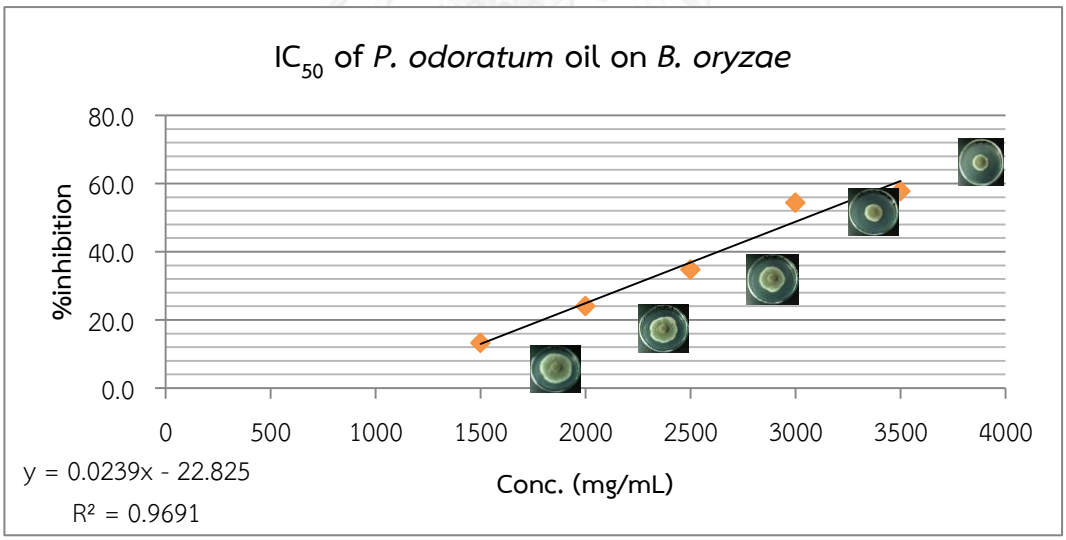


Figure 4. 5 Relationship between %inhibition and concentration of the oil on *R. solani* (a) and *B. oryzae* (b).

4.4.3 Determination of MIC, MBC, and IC_{50} of dodecanal (2)

4.4.3.1 MIC and MBC

The MIC and MBC of dodecanal (**2**) against *Xoo* and *Xoc* are given in **Table 4.4** and **Figures 4.6-4.7**.

Table 4. 4 The MIC, MBC and MIC index of dodecanal **(2)**

Bacterial strains	MIC mg/mL (mM)	MBC mg/mL (mM)	MIC index	Indication
Xoo	3.12 (16.93)	25 (135.63)	8.0	Bacteriostatic
Xoc	0.78 (4.23)	25 (135.63)	32.1	Bacteriostatic

The MIC of dodecanal (**2**) revealed the same activity as the essential oil, whereas the MBC of this compound possessed worse activity than the essential oil for two-folds. This may be because the essential oil contained the complex compounds which were synergistic anti-microbial activities on tested microorganisms (Harris 2002). Dodecanal (**2**) was bacteriostatic agent that was obstructed in the genetic products of bacterial nucleus (Pankey 2004). Thus, the oil and dodecanal (**2**) might be used for microcide.

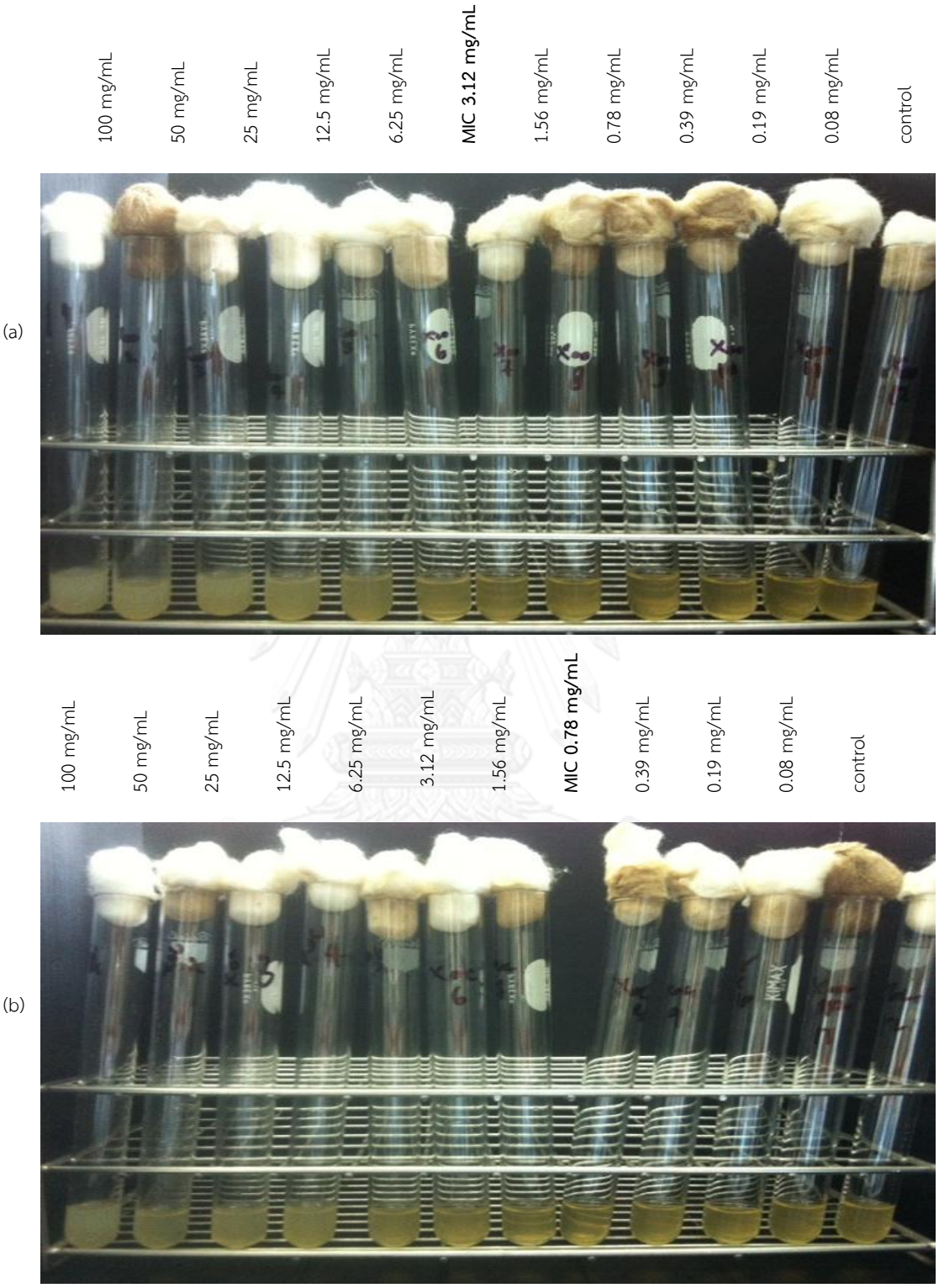
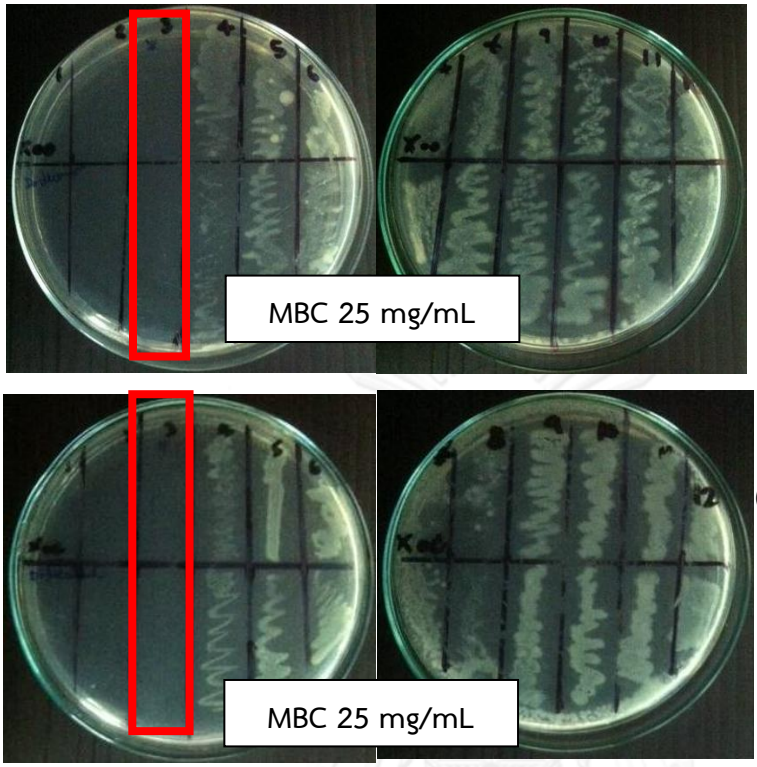


Figure 4. 6 The MIC of dodecanal (**2**) against Xoo (a) and Xoc (b).



(a) MBC = 25 mg/mL

(b) MBC = 25 mg/mL

Figure 4. 7 The plate where MBC for dodecanal (**2**) determined on Xoo (a) and Xoc (b).

4.4.3.2 IC_{50} of dodecanal (2)

The IC_{50}'s of the major compound (dodecanal, **2**) against rice pathogenic fungi were determined as shown in **Table 4.5**.

Table 4. 5 IC_{50} of dodecanal **(2)**

Strains	Concentration (mg/L)	% inhibition (mean ± SD)	IC_{50} (mg/L)	IC_{50} (mM)
R. solani	0	0.00	851[1]	4.62
	250	14.80±5.31		
	500	30.20±3.04		
	750	42.80±5.29		
	1,000	59.27±2.27		
B. oryzae	0	0.00	3,341[2]	18.13
	250	4.07±7.04		
	500	18.33±3.42		
	750	9.10±3.04		
	1,000	15.37±0.87		

[1] Linear equation is y = 0.586x + 0.1111

[2] Linear equation is y = 0.0143x + 2.2222

The IC_{50}'s of dodecanal (**2**) against *R.solani* and *B. oryzae* were found to be 851 and 3,341 mg/L, respectively. It should be mentioned that the essential oil could inhibit both fungi better than a single compound, dodecanal (**2**) (**Tables 4.3** and **4.5**). Thus, the essential oil might be used for anti-fungal agent in the future.

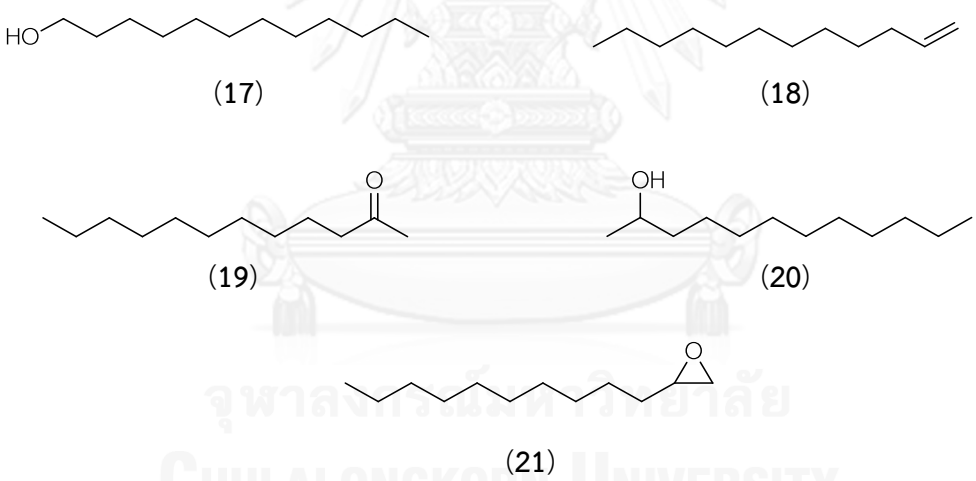
To summarize, dodecanal (**2**) showed the strong antimicrobial activity on *Xoc* and *R. solani* with MIC/MBC of 0.28/25 mg/mL and IC_{50} 0.851 mg/mL, respectively. In addition, it had moderate inhibition on *X. oryzae* pv. *oryzae* (3.12/25 mg/mL) and *B. oryzae* (> 3.0 mg/mL).

The antimicrobial activities of dodecanal (**2**) were consistent with the data reported by Ho (2012) against Gram–positive bacteria with MIC (125–250 µg/mL),

Gram–negative bacteria (250–1,000 μg/mL), and anti–wood decay fungi (25–75 μg/mL). Kubo (2004) reported that dodecanal (**2**) possessed antibacterial activity against *S. choleraesuis* with MIC/MBC of 100/100 μg/mL. Moreover, dodecanal (**2**) was an effective agent with MIC of 200 μg/mL on yeast (*Saccaromyces cerevisiae*) (Kubo 2012).

4.5 Structure-anti rice pathogenic microbial activity relationship of dodecanal (2)

Dodecanal (**2**), 1-dodecanol (**17**), 1-dodecene (**18**), 2-dodecanone (**19**), 2-dodecanol (**20**), and 1,2-dodecene oxide (**21**) have been known to be active with different specificity and levels of activity. For fungal activity, 2-dodecanone (**19**) and 2-dodecanol (**20**) displayed significant antifungal activity against *R. solani* at a concentration of 1 mM (**Table 4.6**) comparing with Nativo 750 WG and only DMSO as a positive and negative control, respectively (**Figure 4.8**).



(**17**) (**18**)

(**19**) (**20**)

(**21**)

Table 4. 6 Structure activity relationship (SAR) of dodecanal **(2)** on pathogenic rice at 1mM concentration

Compound	Fungal growth assay	Agar diffusion assay	
	%inhibition[1]	Mean of clear zone (cm)[2]	
	R. solani	*Xoo*	*Xoc*
Dodecanal	56.94 ± 0.74^{e,3}	0.23 ± 0.04^{c,3}	0.45 ± 0.21^{b,3}
1-Dodecanol	70.37 ± 1.16^{d}	0.35 ± 0.07^{b}	0.48 ± 0.04^{b}
1-Dodecene	30.74 ± 1.95^{f}	0.00 ± 0.00^{d}	0.00 ± 0.00^{c}
2-Dodecanone	85.19 ± 5.01^{b}	0.00 ± 0.00^{d}	0.00 ± 0.00^{c}
2-Dodecanol	83.52 ± 1.70^{b}	0.33 ± 0.04^{b}	0.45 ± 0.07^{b}
1,2-Dodecene oxide	78.71 ± 1.95^{c}	0.00 ± 0.00^{d}	0.00 ± 0.00^{c}
Nativo 750 WG (Positive control)	100.00 ± 0.00^{a}	1.25 ± 0.07^{a}	1.30 ± 0.14^{a}
DMSO (Negative control)	0.00^{g}	0.00^{d}	0.00^{c}

[1] Values were determined by: (control – treatment)/control x 100% and represented the mean values of 3 replicates of 1 mM concentration.

[2] Values, an average ± standard deviation of 2 replicates, of the mean inhibition zones of compounds as 1 mM concentration.

[3] Row values followed by the same letter were not significantly (P > 0.05) differences according to Duncan's multiple comparison tests.

In case of anti-bacterial activity, 1-dodecanol (**17**) revealed moderate anti-bacterial activity against *Xoo* (0.35 cm) and *Xoc* (0.48 cm), followed by 2-dodecanol (**20**) (*Xoo*, 0.33 cm; *X*oc, 0.45 cm) and dodecanal (**2**) (*Xoo*, 0.23 cm; *Xoc*, 0.45 cm), while 1,2-dodecene oxide (**21**), 2-dodecanone (**19**), and 1-dodecene (**18**) were inactive. In addition, 1-dodecanol (**17**) and 2-dodecanol (**20**) gave no significant differences of activity (*P>0.05*) for *Xoo*. 1-dodecanol (**17**), 2-dodecanol (**20**), and dodecanal (**2**) revealed not significant differences for *Xoc*. The position of hydroxyl group showed not anti-bacterial activity differences. The alcohols that the carbon number more than EtOH will increase effective in killing bacteria as well. Therefore, 1-dodecanol and 2-dodecanol has equal number of carbon was not effective in killing microbes differ. The effect of alcohol is to make proteins and enzymes to break them down and remove the fat cell membrane. Therefore, the structure of the

cell membrane is destroyed. It is also draw water out of the cell. Actives of cell disruptions can hinder (Suwanpinit 2010).

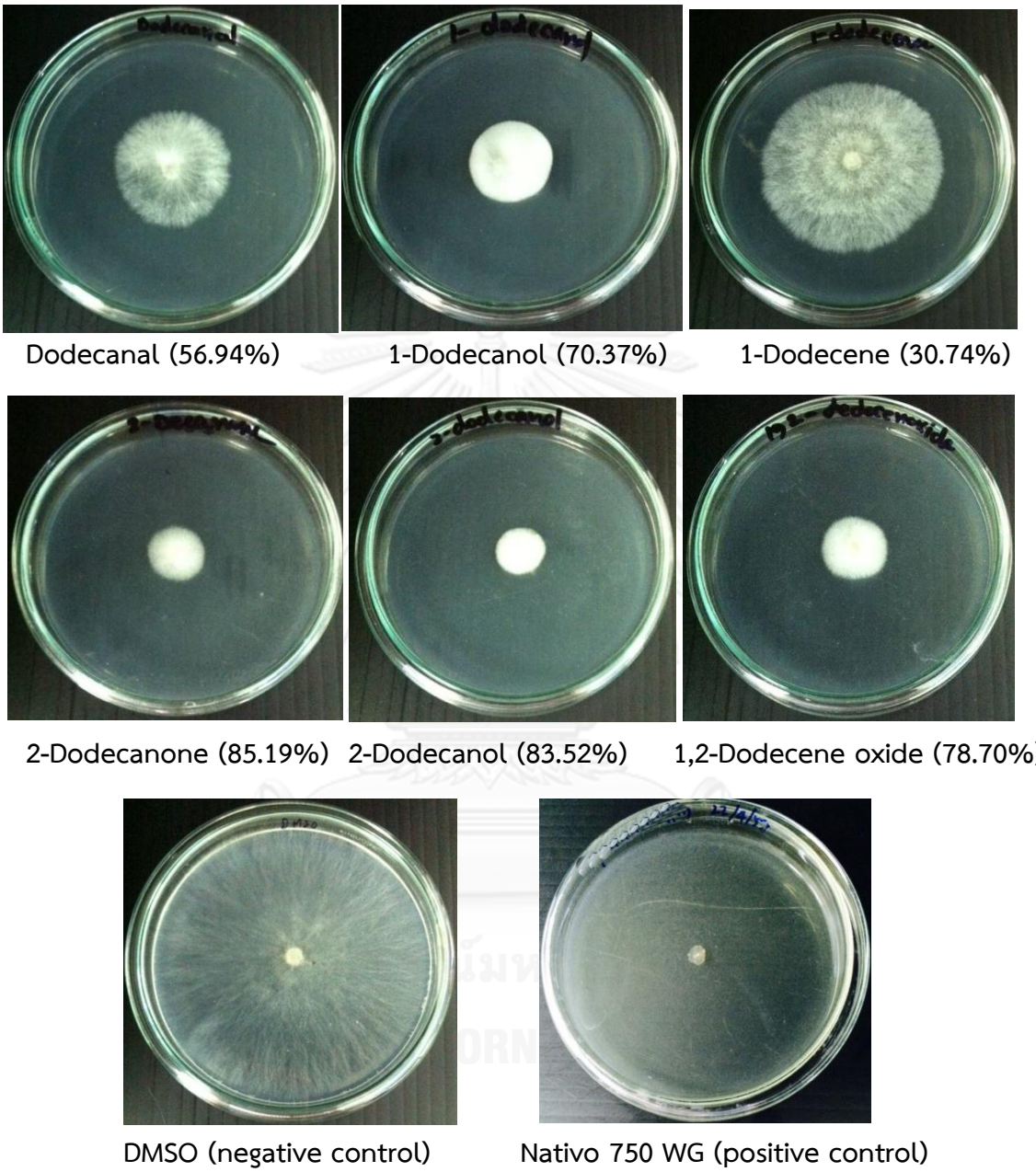


Figure 4. 8 Structure activity relationships (SAR) of dodecanal on pathogenic rice at 1mM concentration

The results indicated that the antimicrobial activity was influenced by functional groups (R-OH, $R_2C=O$, R-COH, -CH-CH- and -C=C-), their position, and its water solubility. The solubility in water of functional groups were followed alcohols > ketones ≈ aldehydes > alkanes > alkenes which are effected to cell wall and cell

membrane binding site of microbial cells. Moreover, the high polar functional groups (alcohol and ketone) could be attached with microbial cell wall and cell membrane which controlled mechanism of microbial growth (Jagessar 2011). The hydroxyl group (alcohol), the role of second position is performed to more antifungal activity than the first position because the 2nd position (Nidiry 2005). Cell wall and cell membrane between bacteria and fungi are different structures; thus, the effective of functional groups was differences (Suwanpinit 2010).

4.6 Comparative anti-fungal study of *Polygonum* oil with other plant essential

The comparison of IC_{50} of *Polygonum* essential oil and other plant oils including *Piper sarmentosum* and *Limophia aromatica* are shown in **Table 4.7** and **Figure 4.9**.

Table 4. 7 IC_{50} of the essential oils from *P. odoratum, L. aromatica* and *P. sarmentosum*

Plants	IC_{50} (mg/L)	
	R. solani	***B. oryzae***
P. odoratum	66	3,047
L. aromatica	146	484
P. sarmentosum	48	15

Table 4.7 represents that the essential oil of *P. sarmentosum* exhibited the highest anti-fungal activity against *R. solani* and *B. oryzae* with IC_{50} of 48 and 15 mg/L, respectively; followed by *P. odoratum* (66 and 3,047) and *L. aromatica* (146 and 484). The linear equation of each oil is depicted in **Figure 4.9 a, b** and **c**. Although the *Polygonum* oil revealed good activity, it did not display better activity than *P. sarmentosum* oil. Although the *Piper* oil was the most effective, but the amount of it extracted minimal back making it unsuitable in adoption. The oil from *P. odoratum* and *L. aromatica* shift in effective antifungal inhibition was less than *P. sarmentosum* oil, but the amount extracted oils were over. So, these oils should be used for new fungicides in the future.

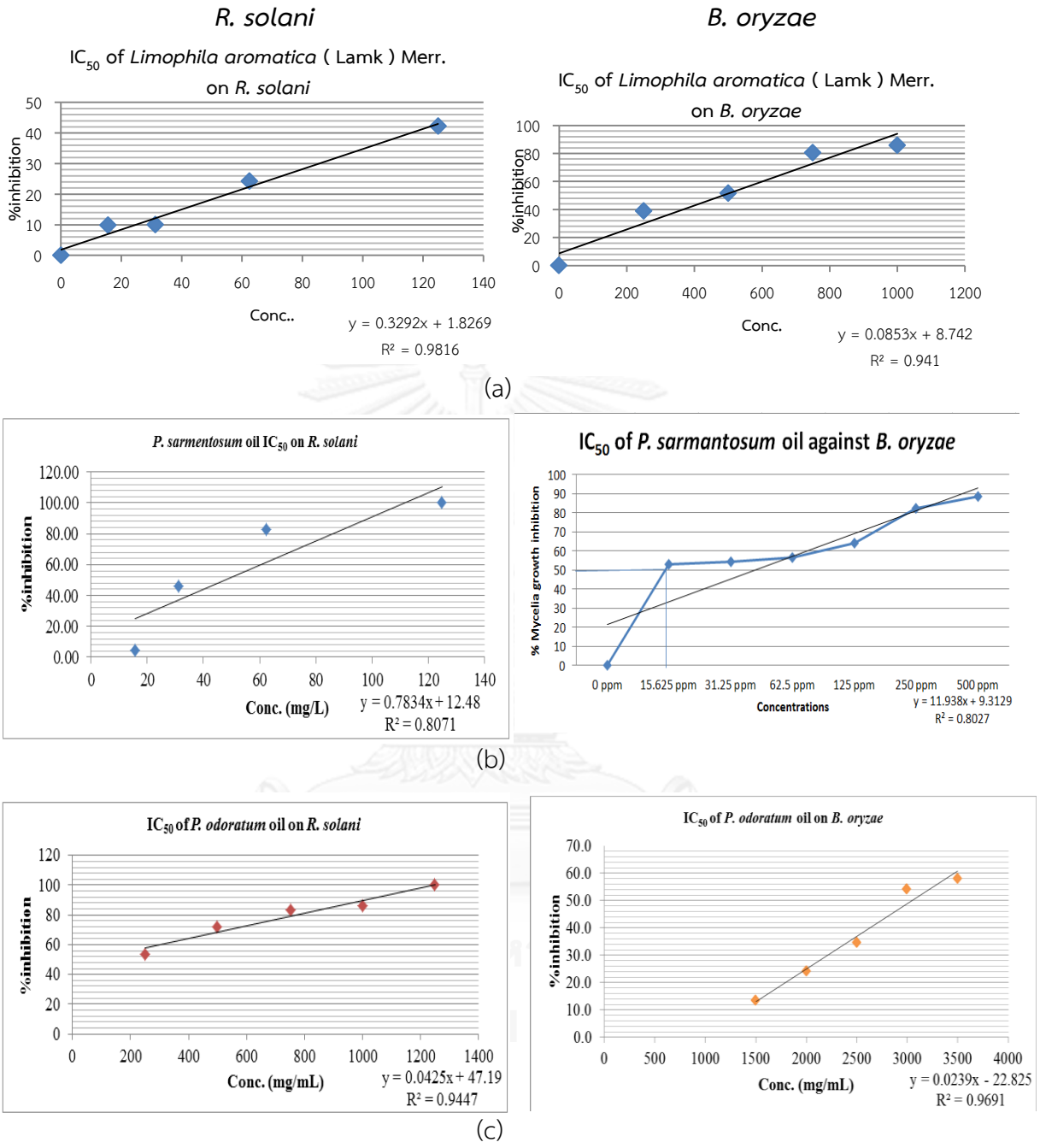


Figure 4. 9 Relationship between the concentration and %inhibition of *L.aromatica* (a), *P. sarmentosum* (b) and *P. odoratum* (c) on *R. solani* and *B. oryzae*

4.7 Experimental section

4.7.1 Hydrodistillation of essential oil

The fresh plants of *P. odoratum* (200 g) or *L. aromatica* (200 g) were cut and hydro-distilled in a modified Dean-Stark apparatus. After extraction with Et_2O, the extract was evaporated under reduced pressure in rotary evaporator. The oil was further used for antimicrobial activity (Ho 2012).

4.7.2 Antibacterial assays

The agar diffusion method was modified from Barry (1999) and used to screen the antibacterial activity of plant essential oil and all compounds. Initially, two bacterial strains (*Xoo* and *Xoc*) were cultivated in nutrient agar (NA) and incubated at 37°C for 18–24 h. Selected 4–5 single colonies of each tested bacteria were cultivated in nutrient broth (NB) and incubated at 37°C for 2-5 h. Freshly cultured bacterial suspensions in NB were standardized to cell density of $1.5x10^8$ CFU/mL (McFarland No. 0.5) and adjusted by 0.85% sterile NaCl. Each tested NA media (19 mL) was mixed with adjusted bacterial suspension (1 mL), poured into petri plates, and then allowed to set. The tested culture plates created the wells using 6 mm cork border, added 40 µL of dissolved extract and DMSO (control) into these wells, incubated the plates at 37°C overnight, and recorded the zones of inhibition in triplication.

4.7.3 Minimum inhibitory concentration (MIC)

MIC's were measured by the macro-dilution broth susceptibility assay recommended by NCCL (McClatchey 2002). Stock solutions of the oil were prepared in DMSO. Ten-fold serial dilutions of oil or major constituent were prepared from 100 mg/mL to 0.08 mg/mL in 10 test tubes with sterile NB (1 mL) in as diluents. Each dilution was pipetted with 1 mL of test bacteria to the standard concentration (1.5×10^8 CFU/mL). Oil or major constituent were used as experimental positive control and only medium as a negative control. The tubes were incubated at 37°C for 24 h. The least concentration of the oil extract or major constituent showing no visible growth was taken as the MIC. After 24 h of incubation period, mean MIC values were calculated. The test was performed in triplicates for each bacterium used and the final results were expressed as the arithmetic average of triplicate experiments. 100 µL test media from each MIC broth tube was spread over the NA plates. Plates were

incubated at 37°C for 24 h. The test MIC concentration showing no bacterial growth on agar plates was considered as MBC of the extract (Singh 2011).

4.7.4 Anti-fungal activity assay

The anti-fungal activities of the extract and major constituent were performed by inhibition of mycelia growth tests. The extract and major constituent were dissolved in DMSO and 100 µL was added to PDA to execute a final concentration at 1,000 mg/L for plant extract and 1 mM for pure constituent. A 5 mm agar disc containing mycelia was transferred to the center of the PDA plate containing the extract or compounds. Plates were incubated at 25°C, 3 days for *R. solani* and 8 days for *B. oryzae*. Nativo 750 WG was used as a positive control and 1% DMSO was used as a negative control. When the mycelium of fungi had reached the edges of the control petri dishes (those without essential oil), the antifungal indices were calculated. The formula of antifungal indices is shown as **Eq 4.1**.

$$\text{Percentage inhibition} = \frac{(C - T) \times 100}{C} \quad \% \qquad \textbf{(Eq 4.1)}$$

Where, C = colony diameter (cm) of the control.

T = colony diameter (cm) of the test plate.

4.7.5 Sclerotia and spore germination

The effects of essential oil on sclerotia germination of *R. solani*, batches of ten sclerotia were each placed on three replicate PDA plates (9 cm diam). Germination of sclerotia was determined after 72 h incubation at 25°C by viewing hyphae under a stereo binocular microscope at x 45 magnification. A sclerotium was considered to have germinated when outgrowing hyphae were equal to or greater than the diameter of sclerotium (Ritchie 2006).

Spores of *B. oryzae* were used as the tested fungus. Stock spores preparation of *B. oryzae* was increased by cultivated mycelium on PDA for 14 days or the highest yield of spores. After that, the 9 mL of sterile water added on filled mycelia agar and scraped by needle. The spore concentration of this fungus was adjusted to approximately 10^6 cfu mL^{-1} by 0.5 McFaland standards. Batches of 0.1 mL spore suspension were each placed on three replicate PDA plates (9 cm diam) using auto pipette.

4.7.6 GC-MS analysis.

The GC-MS analysis was performed by Agilent 6890 gas chromatograph in electron impact (EI, 70eV) mode coupled to an HP 5973 mass selective detector and fitted with a fused silica capillary column (HP-5MS) (30 m x 0.25 mm x 0.25 µm film thickness). Helium (1.0 mL/min) was used as a carrier gas. Samples were injected in the split less mode at ratio of 1:10-1:100. The injector was kept at 250 °C and the transfer line at 280 °C. The MS was EM mode at 1,576.5 EM Voltage, in the *m/z* range 50-550. The identification of the compounds was performed by comparing their retention indices and mass spectra with those found in the literature and supplemented by the Wiley 7n GC/MS libraries.

4.7.7 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.

CHAPTER V
CONCLUSION

Anti-rice pathogenic agents were isolated from *P. sarmentosum* leaves and fruits, and essential oil of *P. odoratum*. The effective CH_2Cl_2 extract of *P. sarmentosum* leaves revealed the major active compound against *R. solani* and *B. oryzae* including myristicin (**21**). The antimicrobial agents from the CH_2Cl_2 extract of *P. sarmentosum* fruits were identified as brachystamide B (**7**), sarmentine (**8**), brachyamide B (**10**) and piperonal (**21**). The anti-rice pathogenic microbial agent of *P. odoratum* was dodecanal (**2**). The comparison of these agents is shown in **Table 5.1**.

Table 5. 1 The comparison of anti-rice pathogenic agents

Compounds	Concentrations (mM)			
	Rice pathogenic fungi (IC_{50})		Rice pathogenic bacteria (MIC/MBC)	
	R. solani	*B. oryzae*	*Xoo*	*Xoc*
Myristicin (21)	**0.69**	0.42	-	-
Sarmentine (8)	1.02	0.92	14.76/59.15	7.38/29.57
Brachystamide B (7)	2.87	1.81	15.19/15.19	15.19/30.37
Brchyamide B (10)	0.97	**0.12**	**7.62/1.90**	30.52/1.90
Piperonal (29)	1.11	0.26	41.57/41.57	**2.59/20.75**
Dodecanal (1)	4.62	18.13	16.93/135.63	4.23/135.63

Myristicin (**21**) displayed the most anti-fungal activity against *R. solani* because this compound structure might be only inhibited of eukaryotic cell (fungi). Brachyamide B (**10**) displayed more anti-*B. oryzae* than the other compounds. In case of antibacterial activity, brachyamide B (**10**) revealed the most anti-*Xoo* activity and piperonal (**29**) appeared more antimicrobial activity than the aldehyde compound from *P. odoratum* (dodecanal, **1**) against *Xoc*. The amide compound may control the growth and mechanisms of prokaryotic and eukaryotic cell. However, the aldehyde

compound (dodecanal) should be used for the new generation microcides than other compound because this compound was less toxicity for humans and animals.

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APPENDIX

Media

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 20 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 20 min.

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

VITA

Mr. Pragatsawat Chanprapai was born on April 1st, 1981 in Samutprakarn, Thailand. He was the first son of Lieutenant Colonel Weera and Mrs. Narumol Chanprapai. He graduated high school at Pibulwittayalai School (Lopburi) in 1999, Bachelor degree on Applied Biology at Phranakhon Si Ayutthaya Rajabhat University in 2003 and Master degree on Botany at Chulalongkorn University in 2008. He was admitted to the Doctoral degree of Science in Biotechnology, Faculty of Science and Chulalongkorn University.

Academic presentation;

1) Chanprapai, P. and Chavasiri, W. 2012. Antifungal compounds from Zanthoxylum limonella Alston. fruits against Rhizoctonia solani. Poster presentation of the 38th Congress on Science and Technology of Thailand at Chaingmai Province.

2) Chanprapai, P. and Chavasiri, W. 2013. Antifungal activity of essential oil and its compositions from Polygonum odoratum Lour. against rice pathogenic fungi. Poster presentation of the International Chemical Ecology Conference 2013 (ICEC 2013), 19-23 August 2013, Melbourne Convention and Exhibition Centre, Victoria, Australia.

3) Chanprapai, P. and Chavasiri, W. 2014. Anti-fungal activity of Piper sarmentosum Roxb. against rice diseases. Oral presentation of the 18th Biological Sciences Graduate Congress (BSGC), 6-8 January 2014, University of Malaya, Kuala Lumpur, Malaysia.

Scholarships:

1) The 90th anniversary of Chulalongkorn University fund (Ratchadaphiseksomphot Endowment Fund). 2013.

2) Conference Grant for Ph.D. Student, Chulalongkorn University. 2013-2014.