สารออกฤทธิ์ทางชีวภาพจากต้นมะแข่น *Zanthoxylum limonella* Alston.

นางสาวจันทร์เพ็ญ ตั้งจิตรเจริญกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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BIOACTIVE COMPOUNDS FROM MA-KHAN *Zanthoxylum limonella* Alston.

Miss Janpen Tangjitjaroenkun

A Dissertation Submitted in Partial Fulfillment of the Requirements
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By	Miss Janpen Tangjitjaroenkun
Field of Study	Biotechnology
Thesis Advisor	Assistant Professor Warinthorn Chavasiri, Ph.D.
Thesis Co-Advisor	Assistant Professor Roongtawan Supabphol, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

S. Hannongbua
.....Dean of the Faculty of Science
(Professor Supot Hannongbua, Dr. rer. nat.)

THESIS COMMITTEE

Sirirat kokpol
.....Chairman
(Associate Professor Sirirat Kokpol, Ph.D.)

Warinthorn Chavasiri
.....Thesis Advisor
(Assistant Professor Warinthorn Chavasiri, Ph.D.)

Roongtawan Supabphol
.....Thesis Co-Advisor
(Assistant Professor Roongtawan Supabphol, Ph.D.)

Sirirat Rengpipat
.....Examiner
(Associate Professor Sirirat Rengpipat, Ph.D.)

Wandee Gritsanapan
.....External Examiner
(Associate Professor Wandee Gritsanapan, Ph.D.)

จันทร์เพ็ญ ตั้งจิตรเจริญกุล : สารออกฤทธิ์ทางชีวภาพจากต้นมะแข่น *Zanthoxylum limonella* Alston. (BIOACTIVE COMPOUNDS FROM MA-KHAN *Zanthoxylum limonella* Alston.) อาจารย์ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.ดร.วรินทร ชวศิริ, อาจารย์ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ.ดร.รุ้งตะวัน สุภาพผล, 178 หน้า.

ได้คัดกรองฤทธิ์ต้านเชื้อจุลินทรีย์และฤทธิ์ต้านออกซิเดชันของสิ่งสกัดไดคลอโรมีเทนและเมทานอลจากส่วนต่างๆ ของมะแข่น และน้ำมันหอมระเหยจากผล พบว่าน้ำมันหอมระเหยและสิ่งไดคลอโรมีเทนของลำต้นแสดงฤทธิ์ที่ดี จากการศึกษาองค์ประกอบทางเคมีของน้ำมันหอมระเหยประกอบด้วยมอนอเทอร์ปีน ได้แก่ sabinene (42.73%), limonene (39.05%) และ terpinen-4-ol (5.40%) น้ำมันหอมระเหยและ terpinen-4-ol แสดงฤทธิ์ที่ดีในการฆ่าราก่อโรคพืช และน้ำมันหอมระเหยและ sabinene ออกฤทธิ์ฆ่าแบคทีเรียได้ครอบคลุม นอกจากนี้ได้ศึกษาอัตราเร็วในการฆ่าแบคทีเรียสแตฟิโลคอกคัส ออเรียส สายพันธุ์ที่ตอบสนองต่อยาปฏิชีวนะเมทิซิลลิน (MSSA) และสายพันธุ์ดื้อยาปฏิชีวนะเมทิซิลลิน (MRSA), *E. coli* และสายพันธุ์ดื้อยาเซฟาโลสพอลิน (ESBL) พบว่าอัตราเร็วในการตายเพิ่มขึ้นเมื่อเพิ่มความเข้มข้นของน้ำมันหอมระเหยและ sabinene

การแยกสิ่งสกัดไดคลอโรมีเทนของลำต้นด้วยเทคนิคทางโครมาโทกราฟี สามารถแยกอัลคาลอยด์ใหม่ 1 ชนิด คือ 4-methoxy-3-(3-methyl-2-oxobut-3-enyl)quinolin-2(1H)-one (**6**) และสารที่มีรายงานมาแล้ว 5 ชนิด ได้แก่ (−)-asarinin (**2**), dictamnine (**3**), dihydroalatamide (**4**), *N*-nornitidine (**5**), และ (−)-tembamide (**7**) การศึกษาฤทธิ์ต้านราก่อโรคพืชพบว่าค่า IC_{50}, MIC และ MFC ของสาร **3** มีประสิทธิภาพในการฆ่าราทดสอบได้มากกว่าสิ่งสกัดไดคลอโรมีเทนจากลำต้น และสาร **3** แสดงฤทธิ์ในการต้านแบคทีเรียแกรมบวกและยีสต์ (*C. albicans*)

การศึกษาความสามารถต้านการเกิดออกซิเดชันของสิ่งสกัดไดคลอโรมีเทน น้ำมันหอมระเหยและองค์ประกอบหลักโดยทดสอบฤทธิ์ต้านอนุมูลอิสระ DPPH$^{\bullet}$, ABTS$^{\bullet+}$ และทดสอบฤทธิ์ต้านอนุมูลอิสระในเซลล์มะเร็งต่อมลูกหมากเพาะเลี้ยง (PC-3 และ DU-145) โดยวัดระดับ MDA, GSH และกิจกรรมของแคทาเลส โดยสิ่งสกัดไดคลอโรมีเทนจากลำต้นและสาร **3**, **4** และ **7** แสดงฤทธิ์ที่ดีในการต้านออกซิเดชันต่ออนุมูลอิสระ DPPH$^{\bullet}$ และ ABTS$^{\bullet+}$ ได้ดีกว่าน้ำมันหอมระเหยและองค์ประกอบหลักของน้ำมันหอมระเหย นอกจากนี้พบว่าสิ่งสกัดไดคลอโรมีเทนจากลำต้น, สาร **3**, **4** และ **7** แสดงฤทธิ์ต้านออกซิเดชันในเซลล์มะเร็งต่อมลูกหมากเพาะเลี้ยงโดยเพิ่มทั้งระดับ GSH และกิจกรรมของแคทาเลส ส่วนน้ำมันหอมระเหยจากผลแสดงฤทธิ์ต้านออกซิเดชันในเซลล์มะเร็งต่อมลูกหมากเพาะเลี้ยงทั้ง 2 ชนิด โดยเพิ่มกิจกรรมของแคทาเลสมากกว่าระดับ GSH

สาขาวิชา.....เทคโนโลยีชีวภาพ.....ลายมือชื่อนิสิต.....จันทร์เพ็ญ ตั้งจิตรเจริญกุล.....

ปีการศึกษา.....2553 .....ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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JANPEN TANGJITJAROENKUN: BIOACTIVE COMPOUNDS FROM MA-KHAN *Zanthoxylum limonella* Alston. THESIS ADVISOR : ASSISTANT PROFESSOR WARINTHORN CHAVASIRI, Ph.D., THESIS CO-ADVISOR : ASSISTANT PROFESSOR ROONGTAWAN SUPABPHOL, Ph.D., 178 pp.

The CH_2Cl_2 and MeOH extracts from different parts, and the essential oils of the fruits of *Z. limonella* were preliminarily screened for antimicrobial and antioxidant activities. The essential oil and the CH_2Cl_2 extract of the stems exhibited the most potent antimicrobial and antioxidant activities. The essential oils contained monoterpene as a major component such as sabinene (42.73%), limonene (39.05%), and terpinen-4-ol (5.40%). The essential oil and terpinen-4-ol exhibited strong activity against tested phytopathogenic fungi. In addition, the crude essential oil and sabinene possessed a broad spectrum antibacterial activity. The rate of time killing of the essential oil and sabinene appeared to be time and concentration dependent against meticillin sensitive *S. aureus* (MSSA), meticillin resistant *S. aureus* (MRSA), *E. coli*, and extend-spectrum β-lactamase producing *E. coli* (ESBL).

The chromatographic separation of the CH_2Cl_2 extract of the stems led to the isolation of a new quinoline alkaloid, 4-methoxy-3-(3-methyl-2-oxobut-3-enyl)quinolin-2(1H)-one (**6**), along with five known compounds, (−)-asarinin (**2**), dihydroalatamide (**4**) and (−)-tembamide (**7**), a furoquinolone alkaloid, dictamnine (**3**), and a benzophenanthridine alkaloid, *N*-nornitidine (**5**). The IC_{50}, MIC, and MFC values against tested fungi of compound **3** were more potent than the CH_2Cl_2 extract of the stems. On the other hand, the MIC and MBC values of the CH_2Cl_2 extract of the stems and compound **3** were active against tested Gram-positive bacteria and yeast (*C. albicans*). Compound **3** was determined as a major compound and possessed the antifungal and antibacterial activities.

The antioxidant capacity of the CH_2Cl_2 extract of the stems, essential oil and some isolated compounds were determined for primary antioxidative potential in cell-free system (DPPH and TEAC assay) and extended to prostate cancer cell lines, PC-3 and DU-145. The CH_2Cl_2 extract of the stems, compounds **3**, **4**, and **7** showed higher antioxidant capacity in cell free system than that of the essential oil of fruits and its major components. The CH_2Cl_2 extract of the stems and compounds **3**, **4**, and **7** seemed to act through GSH level and CAT activity. The essential oil of the fruits has high possibility of regulate CAT capacity than GSH level in both cell lines.

Field of Study : Biotechnology Student's Signature Janpen Tangjitjaroenkun

Academic Year : 2010 Advisor's Signature W Chavasiri

Co-Advisor's Signature Roongtawan Supabphol

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LIST OF ABBREVIATIONS

ABTS	=	2,2′-azinodi-3-ethylbenzthiazoline sulphonate
BHT	=	buthylated hydroxytoluene
^{O} C	=	degree Celsius
^{13}C NMR	=	carbon-13 nucler magnetic resonance
CAT	=	catalase enzyme
$CDCl_3$	=	deuterated chloroform
CD_3OD	=	deuterated methanol
CFU	=	colony forming unit
$CHCl_3$	=	chloroform
CH_2Cl_2	=	methylene chloride
COSY	=	homonuclear correlated spectroscopy
δ	=	chemical shift
d	=	doublet (for NMR spectral data)
dd	=	doublet of doublet (for NMR spectral data)
ddd	=	doublet of doublet of doublet (for NMR spectral data)
DMEM	=	Dulbecco's Modified Eagle Medium
DMSO-*d6*	=	deuterated dimethyl sulfoxide
DPPH	=	2,2-diphenyl-1-picryl-hydrazyl

DTNB	=	5,5′-dithiobis-2-nitrobenzoic acid
et al.	=	and other
ESIMS	=	electrospray ionization mass spectrometry
EtOAc	=	ethyl acetate
g	=	gram
GC	=	gas chromatography
GSH	=	glutathione
μg	=	microgram
h	=	hour
^{1}H NMR	=	proton nuclear magnetic resonance
HMBC	=	^{1}H-detected heteronuclear multiple bond correlation
HMQC	=	^{1}H-detected heteronuclear multiple quantum correlation
Hz	=	Hertz
IC_{50}	=	inhibitory concentration require for 50% inhibition of growth
J	=	coupling constant
L	=	liter
μL	=	microliter
MDA	=	malondialdehyde
MeOH	=	methanol

MH	=	Muller Hinton
μM	=	micromolar
m	=	multiplet (for NMR spectral data)
$[M+ Na]^+$	=	Molecular ion plus sodium
mg	=	milligram
MCC	=	minimum cidal concentration
MFC	=	minimum fungicidal concentration
MIC	=	minimum inhibitory concentration
min	=	minute
mL	=	milliliter
mm	=	millimeter
mM	=	millimolar
MHz	=	megahertz
MS	=	mass spectroscopy
m/z	=	mass to charge ratio
NaCl	=	sodium chloride
nm	=	nanometer
NMR	=	nuclear magnetic resonance
PDA	=	Potato Dextrose Agar

pent	=	pentet (for NMR spectral data)
ppm	=	part per million
psi	=	pound per square inch
q	=	quartet (for NMR spectral data)
rpm	=	round per minute
RT	=	retention time
s	=	singlet (for NMR spectral data)
SDS	=	sodium dodecyl sulfate
sp.	=	species
t	=	triplet (for NMR spectral data)
TEAC	=	trolox equivalent antioxidant capacity
TLC	=	thin layer chromatography
TNB	=	5-thio-2-nitrobenzoic acid
w	=	weight

CHAPTER I

INTRODUCTION

Thailand is well known as a rich bio-diversified country. Various medicinal plants have long been used as the primary source of medicine. Thai people have been using diverse herbs, vegetables and fruits for flavors, spices and condiments in cuisine. Several of these items have been used as traditional medicines (Manosori *et al.*, 2006). Traditional medicine is still the mainstay of health care and most of drugs and cures used come from plants. Medicinal plants contain several classes of phytochemicals and phytobiological natural products, including their potentials as therapeutic agents. Natural products from plants have been shown to exhibit a variety of biological activities such as antimicrobial, antioxidation, antimutagenic, anticarcinogenic, antiimmunodulatory properties *etc.*, making the plants of potential value in treating various diseases (Punturee *et al.*, 2004). Plants produce a large, diverse array of organic compounds that appear to have no direct function in growth and development. These substances are known as secondary metabolites, secondary products, or natural products. Thus, plant secondary metabolites are involved in versatile functions on different levels in plant organisms. The utilization of plant extracts as drug or supplements based on the biological activities from the scientific basis provides new choice of therapy.

1.1 Plant Metabolites

Phytochemistry deals with the chemistry of plant metabolites and their derivatives. The metabolic performance of living organisms can be distinguished into primary and secondary metabolism.

Primary metabolism is associated with fundamental life processes common to all plants. It comprises processes such as photosynthesis, pentose cycle, glycolysis, and tricarboxylic acid (TCA) cycle that are concerned with aerobic respiration and adenosine triphosphate (ATP) biosynthesis-the key energy molecule of the cell. Primary metabolites are produced and converted molecular entities, needed in

anabolic pathways to build, maintain and reproduce the living cell. In catabolic pathways, primary metabolites provide the chemical energy and precursors for biosynthesis. Therefore, basic or primary metabolism consists of biochemical pathways that are in general common to all cells. Additionally, plants produce a large, diverse array of organic compounds that appear to have on direct function in growth and development. These substances are derived biosynthetically from plant primary metabolites and are known as secondary metabolites.

Secondary metabolites generally show greater individuality and diversity in their molecular structures than primary metabolites. These are structurally diverse and many are distributed among a very limited number of species within the plant kingdom and so can be diagnostic in chemotaxonomic studies. Secondary metabolites comprise a range of chemically diverse compounds often specific to a particular species, which are not strictly essential for survival. Secondary metabolites are molecules that are not necessary for the growth and reproduction of a plant, but may serve some role in herbivore deterrence due to astringency or they may act as phytoalexins, killing bacteria that the plant recognizes as a threat. It was believed that secondary metabolites were irrelevant for the human diet. Secondary metabolites carry out a number of protective function in the human body. Plant secondary metabolites can boost the immune system, protect the body from free radicals, kill pathogenic germs and much more (Rosenthal *et al.*, 1991). In addition, the wide molecular diversity of secondary metabolites throughout the plant kingdom represents an extremely rich biogenic resource for the discovery of novel drugs and for developing innovative drugs. Plant secondary metabolites can be classified on the basis of chemical structure, composition, their solubility in various solvents, or the pathway by which they are synthesized. A simple classification includes three main groups: terpenes (made from mevalonic acid, composed almost entirely of carbon and hydrogen), phenolics (made from simple sugars, containing benzene rings, hydrogen, and oxygen), and nitrogen-containing compounds (extremely diverse, may also contain sulfur) (Agosta, 1996). **Figure 1.1** shows in simplified from the pathways involved in the biosynthesis of secondary metabolites and their interconnections with primary metabolism. That is, particular secondary metabolites are often found in only one plant species or related group of species, whereas primary metabolites are found

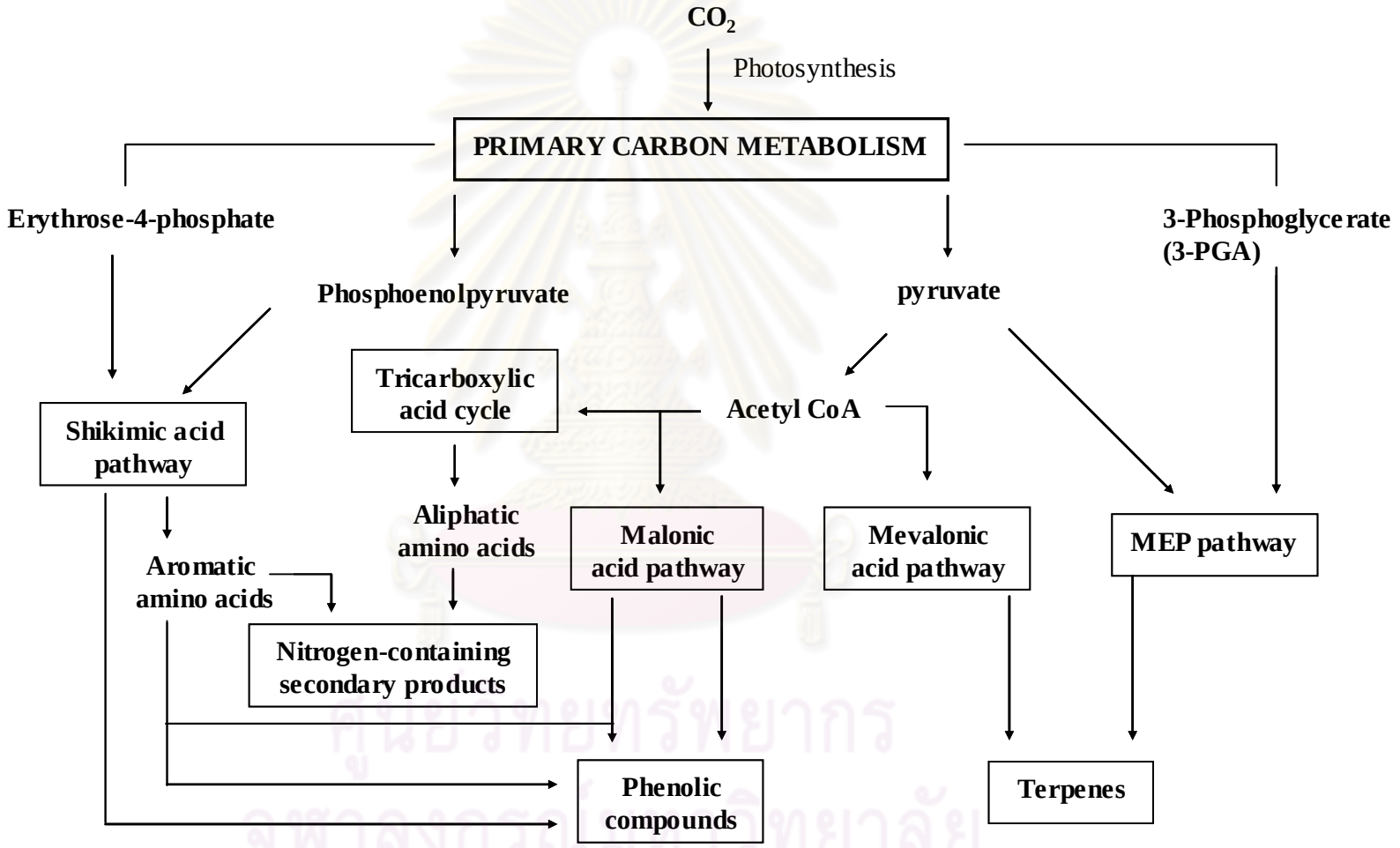


Figure 1.1 A simplified view of the major pathways of secondary-metabolite biosynthesis and their interrelationships with primary metabolism (Taiz and Zeiger, 2002)

throughout the plant kingdom. This research focused on bioactive compounds possessing antimicrobial and antioxidant activities.

1.2 Antimicrobial from Phytomedicines

An infectious disease is a clinically evident disease resulting from the presence of pathogenic microbial agents, including viruses, bacteria, fungi, protozoa, multicellular parasites and prions. These pathogens are able to cause disease in human, animal and/or plants. Number one worldwide infectious disease causes of death accounting for approximately one-half of all deaths in tropical countries (Westh *et al*., 2004). The clinical efficacy of many existing antibiotics is being threatened by the emergence of multidrug-resistant pathogens (Bandow *et al*., 2003). The emergence of pathogenic microbes with increased resistance to established antibiotics provides a major incentive for the discovery of new antimicrobial agents. Antimicrobial screening of plant extracts and phytochemicals represents a starting point for antimicrobial drug discovery (Setzer and Vogler, 2006). Many infectious diseases have been known to be treated with herbal remedies throughout the history of mankind.

Medicinal plants have provided a source of inspiration for novel drug compounds, as plant derived medicines have made large contributions to human health and well being. In addition, the merits of herbal medicine over orthodox drugs include minimal or no side effects on the organic functioning of the body, consistent potency, and the fact that they are well absorbed and distributed in the area of infection (Cheij, 1988; Nkere, 2003; Okigbo and Omodamiro, 2006) The investigation of certain indigenous plants for their antimicrobial properties may yield useful results. Many studies indicated that in some plants there are many substances such as phenols, coumarins, flavonoids, alkaloids, and terpenoids. These compounds may be lethal to microbial cells or they may simply inhibit various metabolic activities of bacteria, yeast, and molds (Beuchat, 1994; Davidson, 2001). The antimicrobial phytochemicals can be divided into several categories, summarized in **Table 1.1**.

Table 1.1 Major classes of antimicrobial compounds from plants (Cowan, 1999)

Class	Subclass	Example(s)	Mechanism
Phenolics	Simple phenols	Catechol	Substrate deprivation
		Epicathechin	Membrane disruption
	Phenolic acids	Cinnamic acid	Membrane disruption
	Quinones	Hypericin	Bind to adhesions, complexes with cell wall, inactivate enzymes
	Flavonoids	Chrysin	Bind to adhesions
	Flavones	Abyssinone	Complex with cell wall, Inactivate enzymes, Inhibit HIV reverse transcriptase
	Flavonols	Totarol	-
	Tannins	Ellagitannin	Bind to proteins
			Bind to adhesions
			Enzyme inhibition
			Substrate deprivation
			Complex with cell wall
			Membrane disruption
			Metal ion complexation
	Coumarins	Warfarin	Interaction with eukaryotic DNA (antiviral activity)
Terpenoids, essential oils		Capsaicin	Membrane disruption
Alkaloids		Berberine	Intercalate into cell wall and/or
		Piperine	DNA

1.2.1 Phenolics and Polyphenols

Plants produce a great variety of organic compounds that contain a phenol group. Phenolic compounds are secondary metabolites that are derivatives of the pentose phosphate, shikimate and phenylpropanoid pathways in plants (Randhir *et al*., 2004). Naturally occurring phenolic compounds are widespread in plants and may be found in a great variety of food systems, and as phenol derivatives they may have antimicrobial activity. These naturally occurring phenols and phenolic compounds may be classified into the following groups: simple phenols and phenolic acids, quinines, flavonoids (flavones, flavonols) and tannin (Cowan, 1999).

1.2.1.1 Simple Phenols and Phenolic Acids

Most of simple phenols are monomeric components of polyphenols and acids that make up some plant tissues. Some of the simplest bioactive phytochemicals consist of a single substituted phenolic ring. Cinnamic and caffeic acids are common representatives of a wide group of phenylpropane-derived compounds which possess broad spectrum of antimicrobial activities (**Figure 1.2**).

The site and number of hydroxyl groups on the phenolic group are thought to be related to their relative toxicity to microorganisms including enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins (Mason and Wasserman, 1987). Phenolic compounds possessing a C_3 side chain at a lower level of oxidation and containing no oxygen are classified as essential oil and often cited as antimicrobial as well. Eugenol, a well-characterized representative found in clove oil is considered bacteriostatic against both fungi (Duke, 1985) and bacteria (Thomson, 1978).

Several plants have been reported to possess antimicrobial activities of these compounds against microorganisms. The common herbs tarragon and thyme both contain caffeic acid, which is effective against viruses (Wild**,** 1994), bacteria (Brantner *et al*., 1996; Thomson, 1978), and fungi (Duke, 1985). The antibacterial activity of cinnamic acid against species of Corynebacteria, Enterococci, Staphylococci and Streptococci (Naz *et al*., 2006). Growth inhibition and decrease of respiration of *Rhodotorula minuta*, a basidiomycete fungus by cinnamic acid (Fujii *et al*., 2002). Additionally, the brewed coffee contained caffeic acid as an active substance which showed inhibitory effect against a strain of *Legionella pneumophila*, bacteria involved in respiratory infection (Dogazaki *et al*., 2002; Furahata *et al*., 2002). Apart from, catechol and pyrogallol are hydroxylated phenols exhibited toxicity to microorganisms. Their antimicrobial activities were investigated on *Pseudomonas putida*, *Pseudomonas pyocyanea*, *Corynebacerium xerosis* and two fungi (*Fusarium oxysporum, Penicillium italicum*) phytopathogenic species as test organisms using the disc diffusion method (Kocacaliskan *et al*., 2006).

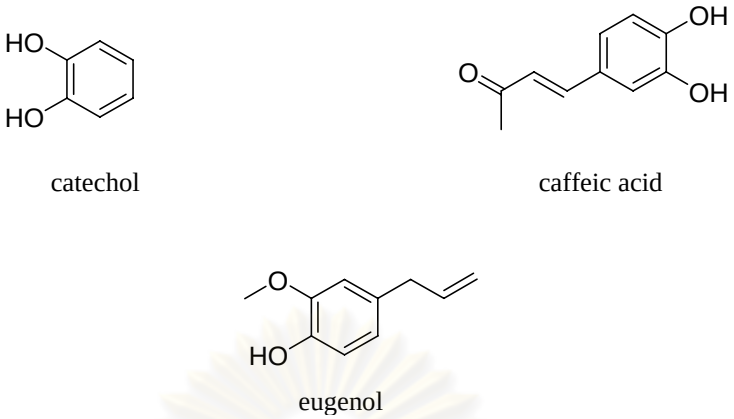
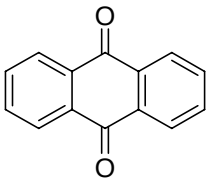


Figure 1.2 Structures of simple phenols and phenolic acids

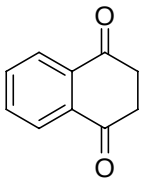
1.2.1.2 Quinones

Quinones are ubiquitious in nature and are characteristically highly reactive. These compounds, being colored, are responsible for the browning reaction in cut or injured fruits and vegetables and are an intermediate in the melanin synthesis pathway in human skin (Schmidt, 1988). More than 2000 naturally occurring quinones, for example–anthraquinones, naphthoquinones and benzoquinones (**Figure 1.3**) are widely distributed in nature as pigments and as intermediates in cellular respiration and photosynthesis (Morton, 1965; Nohl *et al*., 1986). These compounds provide a defense role as a result of their effectiveness at inhibiting the growth of bacteria, fungi, or parasites (Kerten, 1971; Olenick and Hahn, 1974; Martin *et al*., 1973). The mechanism of toxicity proposed to exhibit one or two mechanisms. Redox cycling is the concept in which compounds catalytically cycle and generate oxidative radicals, such as hydrogen peroxide and superoxide, which then damage the cell (Seung *et al*., 1998). Probable targets in the microbial cell are surface-exposed adhesions, cell wall polypeptides, and membrane-bound enzymes.

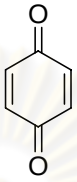
Quinones may also render substrates unavailable to the microorganism. As with all plant-derived antimicrobials, the possible toxic effects of quinones must be thoroughly examined. Kazmi *et al*., 1994 described an anthraquinone from *Cassia italica* which was bacteriostatic for *Bacillus anthracis*, *Corynebacterium pseudodiphthericum*, and *Pseudomonas aeruginosa* and bactericidal for *Pseudomonas*



anthraquinone



naphthoquinone



benzoquinone

Figure 1.3 Structures of some quinones

pseudomalliae. Hypericin, an anthraquinone from *Hypericum perforatum* reported that it had general antimicrobial properties (Duke, 1985).

Naphthoquinones are widely distributed in plants, fungi, and some animals. Plumbagone, juglone and lawsone are naturally occurring naphthoquinones of plant origin that have antibacterial effects on several species of both aerobic and anaerobic organisms (Didry *et al*., 1986). The antimicrobial activity of the natural naphthoquinones: alkannin and shikonin and their derivatives have been investigated. In general, they are active against Gram-positive bacteria such as *Staphylococcus aureus*, *Enterococcus faecium*, and *Bacillus subtilis*, but they are inactive against Gram-negative bacteria (Papageorgious *et al*., 1999). In addition, plumbagin was purified and identified from the leave of *Nepenthes ventricosa* x *maxima*. This compound exhibited antifungal activity against phytopathogenic fungi tested, *Alternaria alternata*, *Aspergillus niger*, *Bipolaris oryzae*, *Fusarium oxysporum*, *Phytophthora capsici*, *Rhizoctonia solani*, *Rhizopus stolonifer var. stolonifer* and *Sclerotinia sclerotiorum* (Shin *et al*., 2007).

1.2.1.3 Flavonoids

Flavonoids are phenolic substances widely distributed in all vascular plants. They determine the large variation in color occurring in vegetables and fruits, from yellow to red and dark purple and have been shown to have contributed to human

health through daily diet (Giulia *et al*., 1999). Flavonoids are naturally occurring substances possessing several biological properties. An ever increasing number of pharmacologic effects have become known through the discovery of new plant flavonoids and through variations of chemical structure of flavones and related derivatives (Garbor, 1986) (**Figure 1.4**).

Flavonoids are also hydroxylated phenolic substances but occur as a C_6-C_3 unit linked to an aromatic ring. They are known to be synthesized by plants in response to microbial infection (Dixon *et al*., 1983). The structures of flavonoids lacking hydroxyl groups on their β-rings are more active against microorganisms than are those with the -OH groups (Chabot *et al*., 1992). The activity of flavonoids are probably due to their ability to form complex with extra-cellular and soluble protein, as well as bacterial cell wall. This finding supports the idea that their microbial target is the permeable membrane. Lipophilic flavonoids would be more disruptive of microbial membrane structure (Tsuchiya *et al*., 1996). Quercetin and naringenin inhibited the growth of *Aspergillus niger, Bacillus subtilis, Candida albicans, Escherichia coli, Micrococcus luteus, Pseudomonas aeruginosa, Saccharomyces cerevisiae* and *Staphylococcus epidermidis* (Rauha *et al*., 2000). The dihydrofuranoisoflavones isolated from Lupinus species, family Leguminosae showed an antifungal activity against *Aspergillus flavus* and *Brotrytis cinerea* (Thara *et al*., 1984). Isoquercitrin and rutin from *Pelargonium radula* (Cav.) L´Hérit demonstrated strong inhibitory activity against *Staphylococcus aureus, Proteus rettgeri, Candida tropicalis* and *Microsporum gypseum* (Pepeljnjak *et al*., 2005).

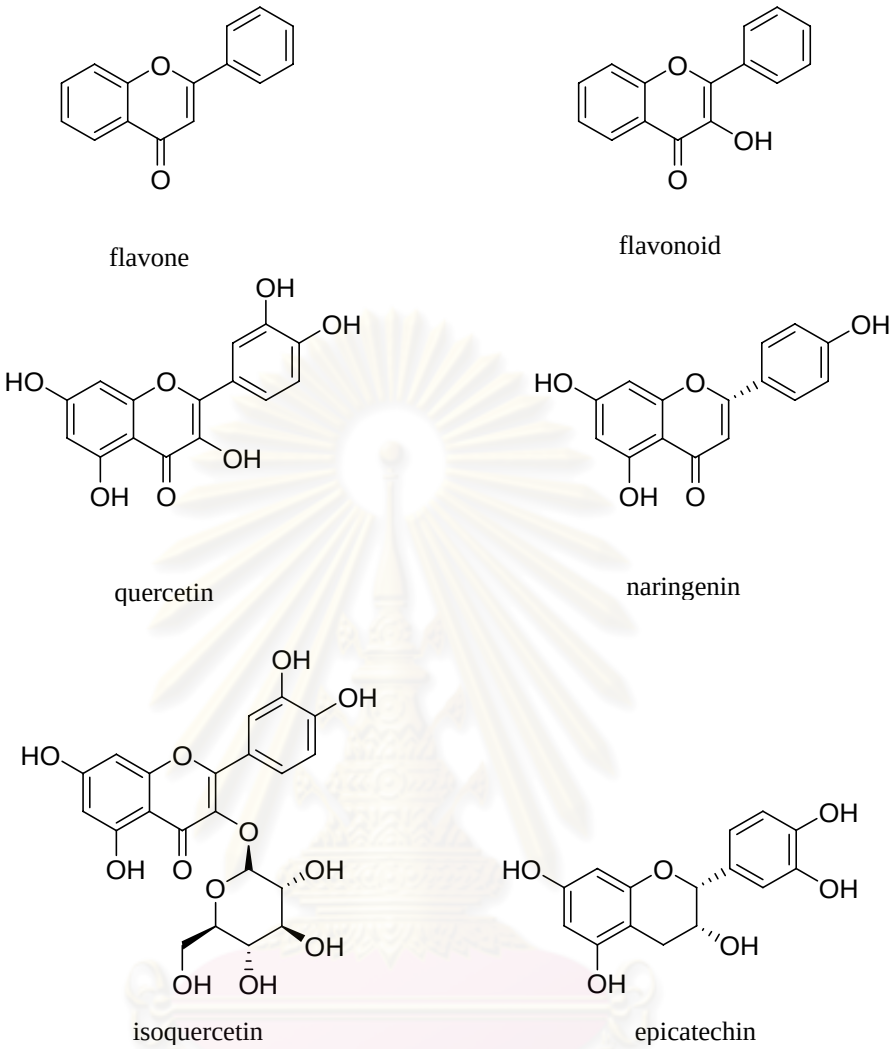


Figure 1.4 Structures of some flavonoids

Catechins, the most reduced form of the C_3 unit in flavonoids, deserve special mention. Recent studies have presented data that show a variety of biological activities of tea catechins, compounds which constitute about 15% (dry weight) of green tea (Hara, 1997). It has been reported that tea catechins have antibacterial activity against various foodborne pathogenic bacteria (Hara *et al*., 1989). These compounds inhibited *in vitro Vibrio cholerae* O1 (Borris, 1996), *Streptococcus mutans* (Batista *et al*., 1994; Sakanaka *et al*.,1989; Sakanaka *et al*., 1992; Tsuchiya *et al*., 1994), *Shigella* (Vijaya *et al*., 1995), and other bacteria and microorganisms (Sakanaka *et al*., 1992; Thomson, 1978). Ikigai *et al*., 1993 reported that the

mechanism of the bactericidal effects of catechins primarily involved acting on and damaging bacterial membranes of *Staphylococcus aureus* and *Escherichia coli*. The antibacterial activities of catechins were predominantly related to the gallic acid moiety and the hydroxyl group member. The mode of catechin action involves inducing rapid leakage of small molecules entrapped in the intraliposomal space and aggregation of the liposome. In addition, catechin has antifungal activity against plant pathogen. The infection-inhibiting activity of epicatechin against *Collectotrichum gloeosporioides* in the skin of avocado fruits. The existence of epicatechin in the skin of immature avocado fruits and the inhibition of pectate lyase by epicathechin (Wattad *et al*., 1994).

1.2.1.4 Tannins

Tannins are water soluble polyphenols which differ from most other natural phenolic compounds in their ability to precipitate proteins such as gelatin from solution, a property known as astringency (Spencer *et al*., 1988). Tannins are commonly found in a large array of higher plant species of both herbaceous and woody types. They are found in large amount (more than 10% of dry weight) in particular organs or tissues which can be almost any plant part: bark, wood, fruits and roots (Haslam, 1989). Their molecular weights range from 500 to 3,000 (Haslam, 1996).

Tannins can be classified into two categories: hydrolyzable and condensed tannins. Hydrolyzable tannins are esters of phenolic acids and a polyol which is usually glucose. The phenolic acids are either gallic acid in gallotannin or other phenolic acids deriving from the oxidation of gallic acid in ellagic tannin (**Figure 1.5**). On the other hand, condensed tannins are formed biosynthetically by the condensation of flavonols unit to form proanthocyanidins, the member of galloyl residues in galloyl esters or the eventual dimerization or polymerization of ellaglic tannin (**Figure 1.5**).

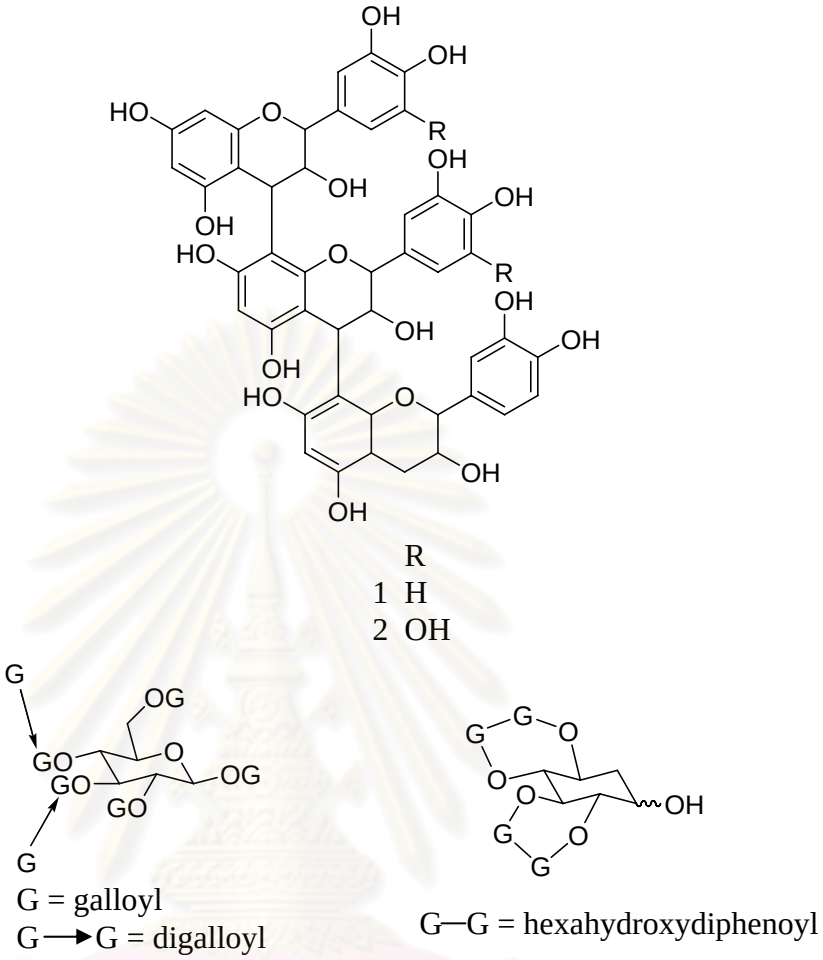


Figure 1.5 Structures of tannin

Several plants which are rich in tannins have been shown to possess antimicrobial activities against a number of microorganisms. Scalbert, 1991 reviewed the antimicrobial properties of tannins. Tannins can be toxic to filamentous fungi, yeasts, and bacteria. For example, hydrolysable tannins, tannic acid was found to be inhibitory to the growth of intestinal bacteria such as *Bacteroides fragilis*, *Clostridium perfringens*, *Escherichia coli* and *Enterobacter cloacae* (Avorn, 1996). Corilagin (hydrolysable tannin) isolated from the leaves of sumac (*Rhus* spp.) and eucalyptus (*Eucalyptus* spp.), and geraniin, from geranium (*Geranium* spp.) and *Phyllanthus* spp. (Buckingham, 2005). Both corilagin and geraniin show anti-human-immunodeficiency-virus (HIV) activity by inhibiting reverse transcriptase (Notka *et al*., 2004). In addition, Toda, 1990 reported that pyrogallol tannins, extracted from

green tea, showed a strong antibacterial activity against *Staphylococcus aureus* and *Vibrio cholerae*. Hydrolyzable tannins can suppress *Helicobacter pylori* without affecting intestinal bacterial flora (Funatogawa *et al.*, 2004). Quebracho tannin (condensed tannin) inhibited both polyphenol oxidase activity and growth of white rot fungi (Colin, 1991). The antimicrobial mechanisms of tannins can be summarized as follows: (i) the astringent property of the tannin may induce complexation with enzymes or substrates, (ii) tannin's toxicity may be related to its action on the membranes of the microorganisms, (iii) complexation of metal ions by tannins may account for tannin toxicity (Ahmad *et al.*, 1996).

1.2.1.5 Coumarins

Coumarins are phenolic substances made of fused benzene and α-pyrone ring. Coumarins are 2H-1-benzopyran-2-ones are widely distributed in plants, and are commonly found in families such as the Umbelliferae/Apiaceae and Rutaceae, both as free form and glycosides. Coumarins have extensive biological properties that promote human health and help reduce the risk of disease (O'Kennedy and Thornes, 1997).

Coumarins can be classified as the following: (i) simple-these are the hydroxylated, alkoxylated and alkylated derivatives of the parent compound, coumarin, along with their glycosides, (ii) furanocoumarins - these compounds consist of a five-membered furan ring attached to the coumarin nucleus, divided to linear and angular types with substituents at one or both of the remaining benzenoid position, (iii) pyranocoumarins-members of this group are analogous to the furanocoumarins, but containa six-membered ring, and (iv) coumarins substituted in the pyrone ring (**Figure 1.6**) (Kostova, 2007).

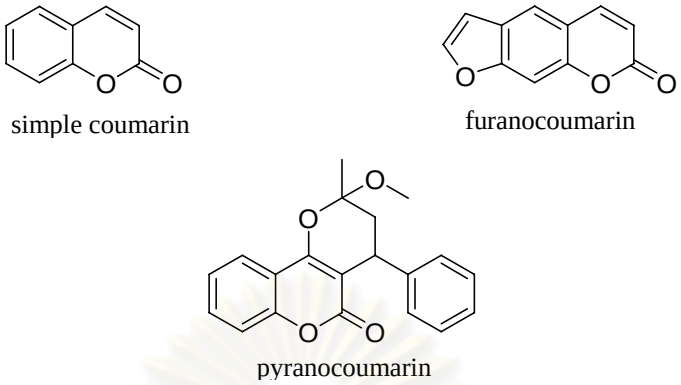


Figure 1.6 Structures of some coumarins

Naturally occurring coumarins possess a variety of pharmacological activities as well as for their antifungal, antimicrobial and antimutagenic properties (Kostova, 2005). Coumarins have been reported to accumulate on and/or under the surface of leaves, fruits, and seeds, where they may inhibit growth and sporulation of fungal pathogen (Matern *et al*., 1999). Coumarins show a better activity against plant microbial pathogens rather than against human pathogens (Ojala *et al*., 2000). Curir *et al*., 2007 reported that a prenylated coumarin, pavietin, isolated from the leaves of *Aesculus pavia* possessed significant activity against phytopathogenic fungal parasite, *Guignardia aesculi*. Osthol, a plant coumarin, could inhibit the hypha growth of *Fusarium graminearum* by decreasing hyphal absorption to reducing sugar (Shi *et al*., 2008). The mixture of prenyletin (70%) and prenyletin-methyl-ether (30%) isolated from aerial parts of *Pterocaulon polystachyum* showed activity against *Cryptococcus neoformans*, *Microsporum gypseum*, *Trichophyton rubrum* and *Trichophyton mentagrophytes* (Stein *et al*., 2006). The antibacterial effects of umbelliprenin, known *O*-prenylated coumarin, from the roots of *Ferula persic* var. *persica* was mostly active against *B. subtilis*, *B. cereus*, *E. coli*, *K. pneumoniae*, *S. Typhi*, *S. aureus* and *S. epidermidis* (Shahvedi *et al*., 2005).

1.2.2 Terpenoids and Essential oils

Essential oils are volatile, natural, complex compounds characterized by a strong odor and are formed by aromatic plants as secondary metabolites. Essential oils are extracted from various aromatic plants generally localized in temperate to warm

countries like Mediterranean and tropical countries. They can be synthesized by all plant organs, *i.e.* buds, flowers, leaves, stems, twigs, seeds, fruits, roots, woods or bark, and are stored in secretory cells, cavities, canals, epidermic cells or glandular trichomes (Bakkali *et al.,* 2008). They are liquid, volatile, lipid and rarely colored, lipid soluble and soluble in organic solvents with a generally lower density than that of water. Essential oils have been largely employed for their properties already observed in nature, *i.e.* for their antibacterial, antifungal and insecticidal activities (Perry *et al*., 2003). Essential oils are very complex natural mixtures which can contain about 20-60 components at quite different concentrations. They are characterized by two or three major components at fairly high concentrations compared to other components present in trace amounts. Generally, these major components determine the biological properties of the essential oils. The components include two groups of distinct biosynthetical origin (Croteau *et al*., 2000; Pichersky *et al*., 2006). The main group is composed of terpenes and terpenoids and the other of aromatic constituents, all characterized by low molecular weight (**Figure 1.7**).

Terpenes form structurally and functionally different classes. They are made from combinations of several isoprenes. The main terpenes are the monoterpenes (C_{10}) and sesquiterpenes (C_{15}), but hemiterpenes (C_5), diterpenes (C_{20}), triterpenes (C_{30}) and tetraterpenes (C_{40}) also exist. The monoterpenes are formed from the coupling of two isoprene units (C_{10}). They are the most representative molecules constituting 90% of the essential oils and allow a great variety of structure. Example of plants containing these compounds are angelica, bergamot, caraway, celery, citronella, coriander, eucalyptus, geranium, juniper, lavandin, lavender, lemon, lemongrass, mandarin, mint, orange, peppermint, petitgrain, pine, rosemary, sage and thyme (Bakkali *et al*., 2008).

Essential oils have long been recognized that some essential oils have antimicrobial properties and these have been reviewed in the past as have the antimicrobial properties of spice (Shelef, 1983; Nychas, 1995). These characteristics are possibly related to the function of these compounds in plants (Mahmoud and Croteau, 2002). For example, several studies have shown that thyme oils, particularly those of *Thymus vulgaris* and *Thymus zygis*, possess antimicrobial activity, those of the phenol type being the most active. The limited occurrence of these phenols in

nature is one of the reasons why Thymus oils containing thymol and carvacrol have been of great interest for some time (Pina-Vaz *et al*., 2004). Both substances appear to make the cell membrane permeable (Lambert *et al*., 2001). Studies with *B. cereus* have shown that carvacrol interacts with the cell membrane, where it dissolves in the phospholipids bilayer and is assumed to align between the fatty acid chains (Ultee *et al*., 2000a). Anise oil exhibited strong inhibitory activity against *Candida blanki* and *Candida glabrata*. *Trans*-anethole is the predominant active component of anise oil (Juglal, 2002). In addition, Pruthi, 1976 reported that asafoetida oil showed inhibitory activity toward *Candida tropicalis*, *Candida albicans* MTCC-227, *Saccharomyces cerevisiae*, and *Aspergillus.* Because of the great number of constituents, essential oils seem to have no specific cellular targets (Carson *et al*., 2002). Essential oils can coagulate the cytoplasm and damage lipids and proteins (Burt, 2004). Damage to the cell wall and membrane can lead to the leakage of macromolecules and to lysis (Cox *et al*., 2000).

Terpenes

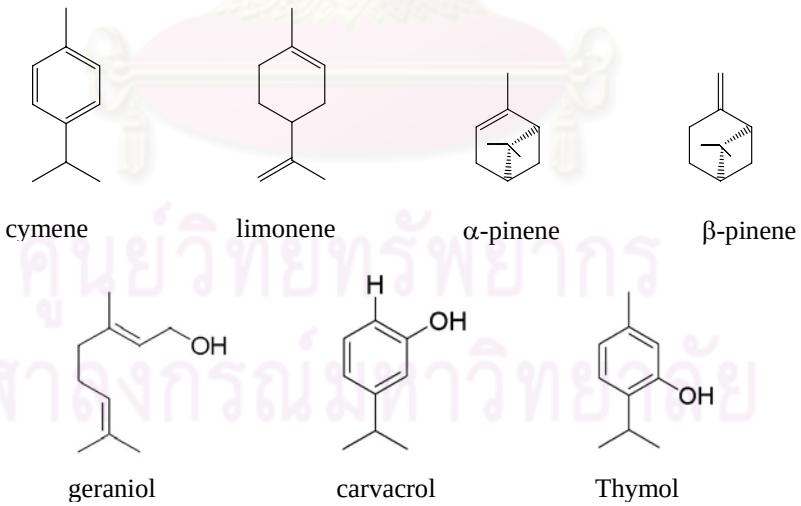
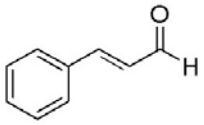
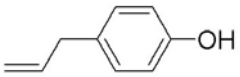


Figure 1.7 Chemical structures of selected compounds of essential oils

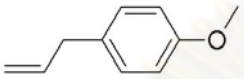
Aromatic compounds



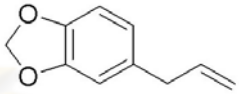
Cinnamaldehyde



chavicol

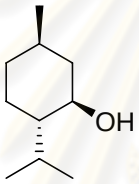


anethole



safrole

Terpenoides (Isoprenoides)



Menthol

Figure 1.7 (continued)

Oussalah *et al*., 2006). In bacteria, the permeabilization of the membranes is associated with loss of ions and reduction of membrane potential, collapse of the proton pump and depletion of the ATP pool (Sikkema *et al*., 1994; Di Pasqua *et al*., 2006; Turina *et al*., 2006). In eukaryotic cells, essential oils can provoke depolarization of the mitochondrial membranes by decreasing the membrane potential, affect ionic Ca^{2+} cycling and other ionic channels and reduce the pH gradient, affecting (as in bacteria) the proton pump and the ATP pool (Novgorodov and Gudz, 1996). They change the fluidity of membranes, which become abnormally permeable resulting in leakage of radicals, cytochrome C, calcium ions and proteins, as in the case of oxidative stress and bioenergetic failure. Permeabilization of outer and inner mitochondrial membranes leads to cell death by apoptosis and necrosis (Yoon *et al*., 2000; Armstron, 2006).

1.2.3 Alkaloids

The alkaloids are nitrogen-containing compounds widely distributed in different plant groups. Alkaloids are often classified according to the nature of the nitrogen-containing structure, *e.g.* pyrrolidine, piperidine, quinoline, isoquinoline, indole, *etc.* (**Figure 1.8**) (Dewick, 2002). Plants containing these alkaloids have been used throughout recorded history as poison, but many alkaloids do have valuable pharmaceutical properties. The first medically useful example of an alkaloid was morphine, isolated in 1805 from the opium poppy *Papaver somniferum* (Fessenden *et al*., 1982). In addition, atropine, the racemic form of hyoscyamine, comes from *Atropa belladonna* and is used to dilate the pupils of the eye. Atropine is also a CNS stimulant and is used as a treatment for nerve gas poisoning (Rang, 2007).

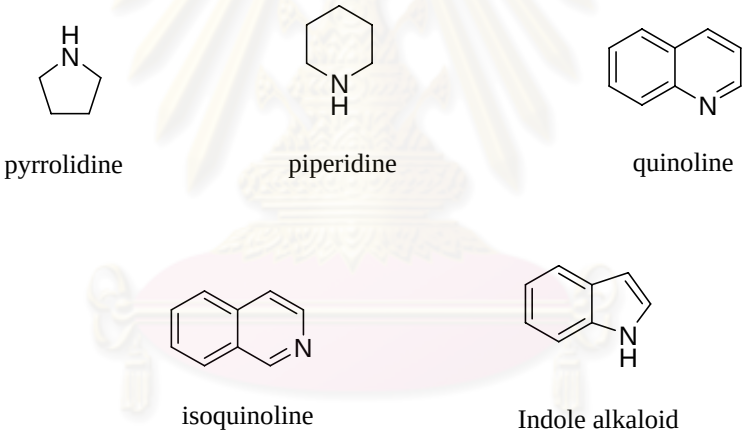


Figure 1.8 Chemical structures of alkaloids

Alkaloids are known to affect biological functions at very low concentration. Many alkaloids are known to be antimicrobial (Rahman *et al*., 1997; Singh *et al*., 1994). The antimicrobial activity of compounds isolated from the leaves of *Plumeria acutifolia* revealed that Grandine A, B, C and Phoebegrandine B posses intermediate activity against *B. subtilis* and *S. aureus* (Almahy *et al*., 2007). The indoloquinoline alkaloid, cryptolepine, is obtained from the shrub *Cryptolepis sanguinolenta* (Periplocaceae). Cryptolepine causes cell lysis and morphological changes of *S. aureus* (Sawer *et al*., 2005). The alkaloid holarrifine-24-ol from the stem bark of

Holarrhena antidysenterica (Wall.) was found to be active against *S. dysenteriae* and *S. typhi* (Raman *et al*., 2004). Additionally, ramiflorines A and B isolated from the stem bark of *Aspidosperma ramiflorum* showed significant activity against *S. aureus* (MIC 25 μg/mL) and *E. faealis* (MIC 50 μg/mL), with EC_{50} of 8 and 2.5 μg/mL for ramiflorines A and B, respectively, against *S. aureus* (Tanaka *et al*., 2006). Singh *et al*., 1999 reported that a plant quaternary alkaloid Δ^3-Alstovenine (3-hydro-alstovenine) isolated from the water-soluble base fraction of the bark of *Alstonia venenata*. Δ^3-Alstovenine completely inhibited the spore germination of *Cercospora* sp. at 250 mg/L. The antimicrobial effects of the alkaloid may be through another mechanism, since the compound is known to be a DNA intercalator and an inhibitor of DNA synthesis through topoisomerase inhibition (Bonjean *et al.*, 1998; Dassonneville *et al*., 2000; Guittat *et al.*, 2003).

1.3 Antioxidants in Phytomedicines

1.3.1 Free Radicals

A free radical is any atom or molecule containing a single unpaired electron in its outermost shell of electron. It is highly reactive because of its extremely unstable configuration (Karlsson, 1997). Several types of free radicals can be formed both exogenously and endogenously. The major free radical species of interest have been those with an oxygen center referred as reactive oxygen species (ROS) that are more strongly oxidizing than normal oxygen molecules. The example of ROS in cells include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and some free radical such as the hydroxyl radical ($^{\bullet}OH$), superoxide ($O_2^{\bullet}$), alkoxyl radical ($RO^{\bullet}$), and peroxyl radical ($ROO^{\bullet}$) (Valko *et al*., 2007). During normal metabolism two percent of oxygen consumed for energy production lose an electron and left a single unpaired electron as an oxygen free radical. It tries to capture an electron from the adjacent molecules to make itself stabilized and a new radical is formed. The consequence of this repeated activity as the chain reaction can produce ROS to interact with other intracellular molecules and cause oxidative to many biomolecules, proteins, membranes, lipids and genes (Sies, 1997). Cell accumulates these damage overtime and lead to many diseases such as cardiovascular disease, cancer, Alzheimer's

disease, and Parkinson's disease. External factors, pollution, smoking and ionizing radiation also trigger the production of new free radicals.

Generally, body has an ability to counteract oxidative stress by antioxidant defense system containing superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX). SOD converts superoxide to hydrogen peroxide, while CAT and GPX catalyze hydrogen peroxide to water and oxygen (Choi, *et al.*, 2007). Additionally, food based antioxidants including vitamins, trace elements, and some non-nutrients from plant will be very useful, especially when endogenous antioxidants are not sufficient to neutralize free radicals.

1.3.2 Endogenous Antioxidants

An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Antioxidants work to protect lipids from peroxidation by radicals. Antioxidants are effective because they are willing to give up their own electrons to free radicals. When a free radical gains the electron from an antioxidant it no longer needs to attack the cell and the chain reaction of oxidation is broken (Dekkers *et al*., 1996). After donating an electron an antioxidant becomes a free radical by definition. Antioxidants in this state are not harmful because they have the ability to accommodate the change in electrons without becoming reactive (Kaczmarski *et al*., 1999).

The human body has several mechanisms to counteract damage by free radicals and other reactive oxygen species. These act on different oxidants as well as in different cellular compartments. There are two lines of antioxidant defense within the cell. The first line of defense is a system of essential enzymes, including superoxide dismutases, catalases and, glutathione peroxidases which decrease concentrations of the most harmful oxidants in the tissue. Several essential minerals including selenium, copper, manganese and zinc are necessary for the formation or activity of these enzymes (Vouldoukis *et al*., 2003). The second line of defense against free radical damage is the electron trapping molecules. These antioxidants, including glutathione, ubiquinol and uric acid, are produced during normal metabolism in the body. Under normal conditions, there is a balance between both

activities and intracellular levels of these antioxidants. This balance is essential for the survival of organisms and their health (Bagchi and Puri, 1998).

Natural antioxidant enzymes manufactured in the body provide an important defense against free radicals. The superoxide released by processes such as oxidative phosphorylation is first converted to hydrogen peroxide and then further reduced to give water. This detoxification pathway is well known that superoxide ion (O_2^-) is the starting point in the chain production of free radicals. At this early stage, superoxide dismutase (SOD) inactivates the superoxide ion by transforming it into hydrogen peroxide (H_2O_2). The latter is then quickly catabolized by catalase (CAT) and glutathione peroxidases (GPx) into dioxygen (O_2) and water (H_2O) (**Figure 1.9**) (Ames, 1993). Different studies have confirmed that the production of H_2O_2 under the action of SOD is the triggering factor in the natural antioxidant defense mechanisms. SOD therefore seems to be the key enzyme in the natural defense against free radicals (**Figure 1.10**) (Menvielle-Bourg, 2005).

SOD has been found in almost all organisms living in the presence of oxygen, including some anaerobic bacteria. SOD protects oxygen-metabolizing cells against harmful effects of superoxide free radicals, the most common free radical in the body. The O_2^- ion, which has been considered important in aging, lipid peroxidation and the peroxidative hemolysis of red blood cells (Fee and Teitelbaum, 1972). SOD's are enzymes that catalyze the conversion of two superoxides into hydrogen peroxide and oxygen (equation A). The benefit here is that hydrogen peroxide is substantially less toxic than superoxide. SOD accelerates this detoxifying reaction roughly 10,000-fold over the non-catalyzed reaction (Marklund, 1985).

$$O_2^{-\bullet} + O_2^{-\bullet} \longrightarrow O_2 + H_2O_2 \qquad \text{(equation A)}$$

SOD is a powerful enzyme and acts as a cellular antioxidant. SOD helps the body use zinc, copper, and manganese. There are two types of SOD: copper/zinc (Cu/Zn) SOD and manganese (Mn) SOD. Each type of SOD plays a different role in keeping cells healthy. In mammal, Mn SOD is the most abundant in mitochondria, while the Cu/Zn forms predominant in cytoplasm. SODs are inducible enzyme – exposure of bacteria or vertebrate cells to higher concentrations of oxygen results in

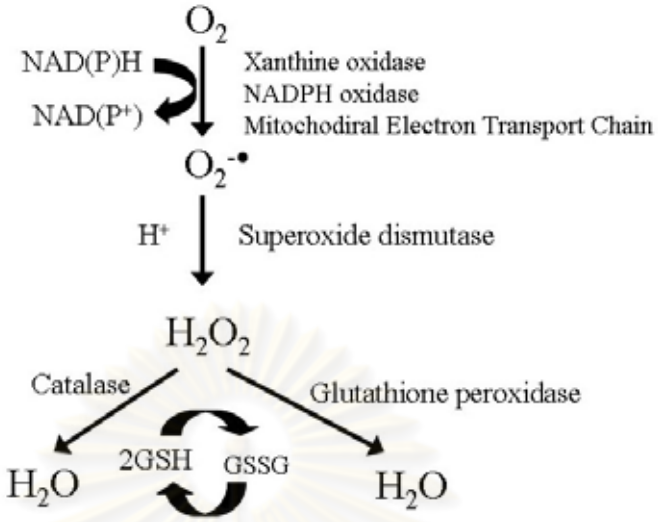
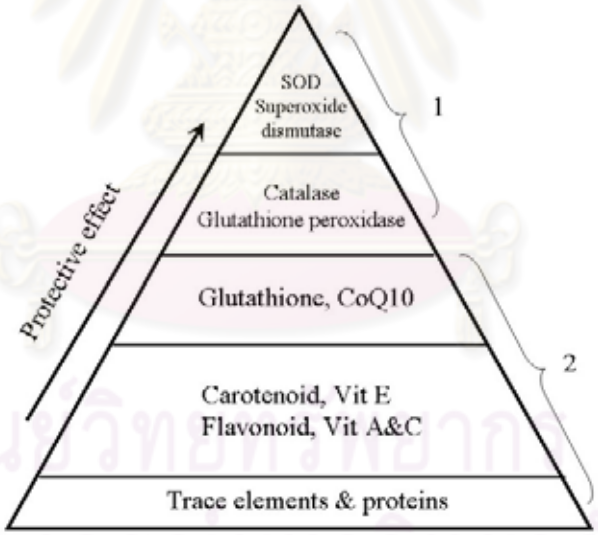


Figure 1.9 Detoxification of reactive oxygen species by the primary antioxidant enzyme (Ames *et al*., 1993)



1) The main essential enzymes

Superoxide Dismutase (SOD), catalase, glutathione peroxidase (GPx)

2) The electron trapping molecules

Vitamins A, E, C, beta-carotene, flavonoids, trace elements

Figure 1.10 SOD among the other electron trappers: vitamins A, E, C, *beta*-carotene, flavonoids, trace elements (Joanny, 2005)

rapid increases in the concentration of SOD. SOD can help neutralize free radicals and in doing so may limit or stop some of the damage they cause. This is a very important function of SOD because free radicals can be unstable and highly active. They have the ability to damage any molecule in the body. The result can be tissue and/or organ damage especially where the production of scavenger enzymes like SOD is exceeded by the production of free-radicals. SOD is the body's primary defense system for tissues and organs against free radicals. SOD has also been used to treat arthritis, prostate problems, corneal ulcer, burn injuries, inflammatory diseases, inflammatory bowel disease, and long-term damage from exposure to smoke and radiation, and to prevent side effects of cancer drug. In its topical form, it may help to reduce facial wrinkles, scar tissue, heal wounds and burns, lighten dark or hyperpigmentation, and protect against harmful UV rays.

CAT is an enzyme present in the cells of plants, animals and aerobic (oxygen requiring) bacteria. It is located in a cell organelle called the peroxisome. Peroxisomes in animal cells are involved in the oxidation of fatty acids, and the synthesis of cholesterol and bile acids (Alberts, 2002). H_2O_2 is a byproduct of fatty acid oxidation. White blood cells produce H_2O_2 to kill bacteria. In both cases, CAT prevents the H_2O_2 from harming the cell itself. H_2O_2 is produced as an intermediate during these chemical processes and must be removed to prevent damage to cellular machinery. CAT is frequently used by cells to rapidly catalyze the decomposition of H_2O_2 into less reactive gaseous oxygen and water molecules (equation B) (Gaetani, 1996).

$$2H_2O_2 \longrightarrow 2H_2O + O_2 \quad \text{(equation B)}$$

Each molecule of CAT is a tetramer of four polypeptide chains. Each chain is composed of more than 500 amino acids. Located within this tetramer are four porphyrin heme groups that are very much like the familiar hemoglobins, cytochromes, chlorophylls and nitrogen-fixing enzymes in legumes. The heme group is responsible for CAT's enzymatic activity (Boon, 2007). CAT has one of the highest turnover rates for all enzymes: one molecule of CAT can convert 6 million molecules of H_2O_2 to water and oxygen each minute. CAT works closely with SOD to prevent

free radical damage to the body. SOD converts the dangerous superoxide radical to H_2O_2, which CAT converts to harmless water and oxygen (Goodsell, 2007).

GPx is a group of enzyme, the most abundant of which contain selenium. These enzymes, like CAT, decompose H_2O_2 and various hydro─ and lipid peroxides (Kinnula *et al*., 1995). The classical form of GPx is cellular and dispersed throughout the cytoplasm, but GPx activity is also found in mitochondria (Buettner, 1998). Extracellular form of GPx is genetically distinct from cellular GPx (Yoshimura *et al.*, 1994) and has been detected in several human tissues, including lung (Chu *et al.*, 1992). The Km (Michealis constant) value for GPx is lower than that for catalase, and GPx is considered more important in physiologic conditions (Kinnula *et al*., 1995). Selenium is essential for the protein synthesis and enzymatic activity of GPx. It is a tetrameric protein 85,000 Daltons. It has 4 atoms of selenium (Se) bound as seleno-cysteine moieties that confers the catalytic activity. One of the essential requirements is glutathione as a cosubstrate. There are several isozymes encoded by different genes, which vary in celullar location and substrate specificity. Glutathione peroxidase 1 (GPx1) is the most abundant version, found in the cytoplasm of nearly all mammalian tissues, whose preferred substrate is hydrogen peroxide. Glutathione peroxidase 4 (GPx4) has a high preference for lipid hydroperoxides; it is expressed in nearly every mammalian cell, though at much lower levels. Glutathione peroxidase 2 is an intestinal and extracellular enzyme, while glutathione peroxidase 3 is extracellular, especially aboundant in plasma (Muller *et al*., 2007).

GPx reduces H_2O_2 to H_2O by oxidizing glutathione (GSH) (equation C). Reduction of the oxidized form of glutathione (GSSG) is then catalysed by glutathione reductase (equation D). These enzymes also require trace metal cofactors for maximal efficiency, including selenium for glutathione peroxidase; copper, zinc, or manganese for SOD; and iron for catalase (Halliwell, 1995).

$$H_2O_2 + 2\ GSH \longrightarrow GSSG + 2\ H_2O \text{ (equation C)}$$

$$GSSG + NADPH + H^+ \longrightarrow 2\ GSH + NADP^+ \text{ (equation D)}$$

1.3.3 Exogenous Antioxidants

Natural antioxidants occur in all parts of plants. These antioxidants include carotenoids, vitamins, phenols, flavonoids. Plant-derived antioxidants have been shown to function as singlet and triplet oxygen quenchers, free radical scavengers, peroxide decomposers, enzyme inhibitors, and synergists (Manach *et al.*, 1998). Fruits and vegetables contain different antioxidant compounds, such as vitamins C, E and carotenoids. Many phytochemical substances in plants, generally phenolic compounds, display antioxidant properties and, thus, may be important for health (Vouldoukis *et al*., 2004). The most current research on antioxidant action focuses on phenolic compounds such as flavonoids.

1.3.3.1 Dietary Antioxidant

Vitamins C, E, and β-carotene are among the most widely studied dietary antioxidants. These antioxidants have specific activities and they often work synergistically to enhance the overall antioxidant capability of the body (Bagchi, Puri, 1998) (**Table 1.2**).

Table 1.2 Antioxidant function of vitamins E and C and β-carotene (Bagchi and Puri, 1998)

Nutrient	Function
Vitamin E	Chain breaking antioxidant
	Free radical scavenger
	Singlet oxygen quencher
Vitamin C	Free radical scavenger
	Singlet oxygen quencher
	Regeneration of vitamin E
β-carotene	Singlet oxygen quencher
	Chain breaking antioxidant
	Free radical scavenger

L-ascorbic acid ($C_6H_8O_6$) is the trivial name of vitamin C. The chemical name is 2-oxo-L-theo-hexono-1,4-lactone-2,3-enediol. L-ascorbic and dehydroascorbic acid are the major dietary forms of vitamin C (Moser and Bendich, 1990). The body does not produce vitamin C. Ascorbic acid is widely distributed in fresh fruits and vegetables. It is present in fruits like orange, lemons, grapefruit, watermelon, papaya, strawberries, cantaloupe, mango, pineapple, raspberries and cherries. It is also found in green leafy vegetables, tomatos, broccoli, green and red peppers, cauliflower and cabbage (Naidu, 2003). Ascorbic acid is well known for its antioxidant activity. Ascorbate acts as a reducing agent to reverse oxidation in aqueous solution. This vitamin neutralizes potentially harmful reactions in the watery parts of the body, such as the blood plasma, between cells, eye fluid and lung fluid. It can fight aqueous radicals before they get an opportunity to damage body lipids (McGregor and Biesalski, 2006). The formation of dangerous free radicals is prevented by vitamin C by preventing lipid peroxidation. Vitamin C may help decrease total and LDL cholesterol and triglycerides, as well as increase HDL levels. Vitamin C's antioxidant activity may be helpful in the prevention of some cancers and cardiovascular disease (Frei, 1999). The antioxidant properties of vitamin C are thought to protect smokers, as well as people exposed to secondhand smoke, from the harmful effects of free radicals (Kallner *et al.*, 1981). As a powerful antioxidant, vitamin C may help to fight cancer by protecting healthy cells from free-radical damage and inhibiting the proliferation of cancerous cells (Eckert-Maksic *et al*., 1991).

Vitamin E is the collective name for eight compounds such as α-, β-, γ-, and δ-tocopherols and the corresponding four tocotrienols (Herrera and Barbas, 2001). Of these, α-tocopherol has been most studied as it has the highest bioavailability (Packer *et al*., 2001). Vitamin E is a fat-soluble substance present in all cellular membranes and is mainly stored in adipose tissue, the liver and muscle. It is an antioxidant that prevents free radical damage in the body and protects cell membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction (Traber and Atkinson, 2007). It is a singlet oxygen quencher, neutralizing these highly reactive and unstable singlet oxygen molecules. Free radicals can cause cell damage that may contribute to the development of cardiovascular disease and cancer. Vitamin

E helps protect against heart disease by limiting the oxidation of LDL-cholesterol (Rimm *et al*., 1993). Vitamin E is a very effective option for the reduction of hot flashes in menopausal women (Ziaei *et al*., 2007). Vitamin E helps prevent oxidation of lipoproteins, particularly in smokers, and reduces the stickiness of platelets in the bloodstream. Vitamin E as an antioxidant helps to stabilize cell membranes and protect the tissues of the skin, eyes, liver, breast, and testes, which are more sensitive to oxidation. Due to the ability of vitamin E to work at higher oxygen pressures, free radicals are scavenged and tissue injury is minimized. Besides its anti-aging properties, vitamin E is known to afford protection against cancer, ischaemia and reperfusion injury, cataract, arthritis and certain neurological disorders. Vitamin E is found in many common foods, including vegetable oils (such as soybean, corn, cottonseed and safflower) and products made from these oils (such as margarine), wheat germ, nuts and green leafy vegetables.

Carotenoids are a group of red, orange and yellow pigments found in plant foods, particularly fruits and vegetables. β-Carotene, a member of carotenoids could be converted by the body into vitamin A (retinol) and acts a powerful antioxidant and helps support the immune system. One molecule of β-carotene can be cleaved by a specific intestinal enzyme into two molecules of vitamin A (Wang *et al*., 2000). Other members of the antioxidant carotenoid family include cryptoxanthin, α-carotene, zeaxanthin, lutein, and lycopene. However, unlike β-carotene, most of these antioxidants are not converted to vitamin A in significant amounts (Olson, 1999). β-Carotene is an effective antioxidant as it is one of the most powerful singlet oxygen quenchers. It can dissipate the energy of singlet oxygen, thus preventing this active molecule from generating free radicals. Its other antioxidant properties include the scavenging of free radicals. Unlike other nutrient antioxidants, β-carotene is efficient at low oxygen pressure. Individuals with the highest levels of β-carotene intake have lower risks of lung cancer, coronary artery heart disease, stroke and age-related eye disease than individuals with lowest levels of β-carotene intake (Tanvetyanon and Bepler, 2008). The richest sources of β-carotene are yellow, orange, and green leafy fruits and vegetables (such as carrots, spinach, lettuce, tomatoes, sweet potatoes, broccoli, cantaloupe, and winter squash).

1.3.3.2 Phytochemical Antioxidants

Many plant-derived substances, collectively termed “phytochemicals”, are becoming increasingly known for their antioxidant activity. Phenolic compounds are secondary metabolites that are derivatives of the pentose phosphate, shikimate, and phenylpropanoid pathways in plant (Randhir *et al*., 2004). Phenolics are compounds possessing one or more aromatic rings with one or more hydroxyl groups and generally are categorized as flavonoids, phenolic acid, stilbenes, coumarins, and tannins. Their structures are categorized according to the number and position of hydroxyl group and the presence of other substituents. The antioxidant activity of phenolic compounds is due to their ability to scavenge free radicals, donate hydrogen atoms or electron, or chelate metal cations (Amarowiez *et al.*, 2004). The structure of phenolic compounds is a key determinant of their radical scavenging and metal chelating activity, and this is referred to as structure-activity relationships. The antioxidant activity depends on the number and position of the hydroxyl groups in relation to the carboxy furnction group. Phenolic compounds could be a major determinant of antioxidant potentials of foods, and could therefore be a natural source of antioxidants (Parr and Bolwell, 2000). The most important sources and the classes of phenols they contain are briefly presented in **Table 1.3**.

Flavonoids are a group of phenolic compounds with antioxidant acitivity that have been identified in fruits, vegetables, and other plant foods and that have been linked to reducing the risk of major chronic diseases. Flavonoids have a generic structure consisting of two aromatic rings (A and B rings) linked by 3 carbons that are usually in an oxygenated heterocycle ring, or C ring (**Figure 1.11**). Difference in the generic structure of the heterocycle C ring classify them as flavonols, flavones, catechins, flavanones, anthocyanidins, and isoflavonoids (**Figure 1.12**). These compounds are well known that flavonoids possess antioxidant properties *in vitro* and *in vivo*. The flavonoids contain a number of phenolic hydroxyl groups attached to ring structures, which confer the antioxidant activity. Catechin and their epimers serve as powerful antioxidants for directly eliminating superoxide anion radicals (Chen and Chan,

Table 1.3 Sources of antioxidant plant phenols

Sources	Antioxidant compounds	References
Fruits		
Berries	Flavonols, hydroxycinamic acids, hydroxybenzoic acids, anthocyanins	Hakkinen *et al*., 1998; Belitz and Grosch, 1999; Wang and Lin, 2000; Manach *et al*., 2004
Cherries	Hydroxycinnamic acids, anthocyanins	Belitz and Grosch, 1999; Yanishlieva-Maslarova *et al*., 2001; Manach *et al*., 2004
Blackgrapes	Anthocyanins, flavonols	Blitz and Grosch, 1999; Yanishlieva-Maslarova *et al*., 2001; Manach *et al*., 2004
Citrus fruits	Flavones, flavonols, phenolic acids	Yanishlieva-Maslarova *et al*., 2001; Beecher, 2003; Manach *et al*., 2004
Plums, prunes, apples, pears	Hydorxycinnamic acids, catechins	Blitz and Grosch, 1999; Yanishlieva-Maslarova *et al*., 2001; Manach *et al*., 2004
Pasley	Flavones	Manach *et al*., 2004
Beans	Flavanols	Manach *et al*., 2004
Spinach	Flavonoids, *p*-coumaric acid	Bergman *et al*., 2001
Teas		
Black, green	Flava-3-ols, flavonols	Beecher, 2003; Manach *et al*., 2004
Alcoholic drinks		
Red wine	Flavan-3-ols, flavonols, anthocyanins	Beecher, 2003; Manach *et al*., 2004
Cider	Hydroxycinnamic acids	Manach *et al*., 2004
Herb and spices		
Rosemary	Carnosic acid, carnosol, rosmarinic acid rosmanol	Yanishlieva-Maslarove *et al*., 2001; Ibanez *et al*., 2003
Oregano	Rosmarinic acid, phenolic acids, flavonoids	Yanishlieva-Maslarove *et al*., 2001; Exarchou et al., 2002
Thyme	Thymol, carvacrol	Yanishlieva-Maslarove *et al*., 2001

1996). In addition, the antioxidant activities of individual constituent catechin and catechin-gallate esters of green tea have shown that *in vitro* they are more effective antioxidants on a molar basis than vitamin C. This ranking of reactivity follows the order: epicatechin gallate = epigallocatechin gallate > epigallocatechin > epicatechin = catechin. These finding are in agreement with the hierarchies of antioxidant activities against the $DPPH^{\bullet+}$ and superoxide radical reduction (Chen and Ho, 1995). Proanthocyanidins from grape seeds are apparently responsible for the action on the cardiovascular system (Pekié *et al*., 1997). Kaempferol 3-O-α-rhamnoside from *Licania licaniaeflora* exhibited DPPH radical scavenging activity and quercetin derivative from this plant showed strong antiradical activity (Braca *et al*., 2002). Epicatechin, epigallocatechin, epicatechin gallate and procyanidin B1 and B2 from grape seed extract showed strong DPPH radical scavenging activity (Guendez *et al*., 2005).

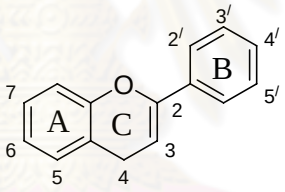


Figure 1.11 Generic structure of flavonoids

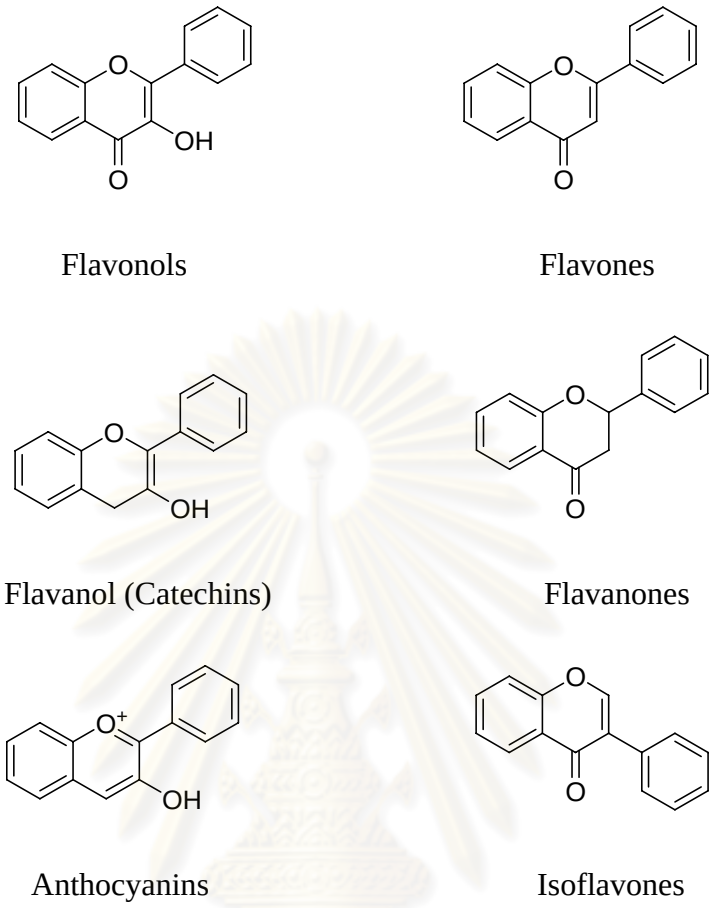


Figure 1.12 Structures of main classes of flavonoids

Phenolic acids can be divided into two major groups, hydroxybenzoic acids and hydroxycinnamic acids (**Figure 1.13**). Hydroxybenzoic acid derivatives include *p*-hydroxybenzoic acid derivatives include *p*-hydroxybenzoic, protocatechuic, vannilic, syringic, and gallic acid. They are commonly present in the bound form are typically a component of a complex structure like lignins and hydrolysable tannins. They can also be found in the form of sugar derivatives and organic acids in plant foods (Sudjaroen, 2009). The antioxidant activity of phenolic acids depends on the number of hydroxyl groups in the molecule and the activity can be strengthened by steric hindrance. The electron withdrawing properties of the carboxylate group in benzoic acids has negative influence on the H-donating abilities of the hydroxyl benzoates. Hydroxylated cinnamates are more effective than benzoate counterparts (Rice-Evan *et al*., 1996). Neochlorogenic acid and cryptochlorogenic acid isolated from prunes can

scavenge superoxide anion radicals and inhibit oxidation of the methyl linoleate system (Nakatani *et al*., 2000)

(a) (b)

Figure 1.13 Structures of common phenolic acids: (a) benzoic acid and derivative; (b) cinnamic acid and derivatives

1.4 Prostate Cancer

Prostate cancer is a malignant (cancerous) tumor (growth) that consists of cells from the prostate gland. The tumor usually grows slowly and remains confined to the gland for many years. During this time, the tumor produces little or no symptoms or outward signs (abnormalities on physical examination). As the cancer advances, however, it can spread beyond the prostate into the surrounding tissues (local spread). Moreover, the cancer also can metastasize (spread even farther) throughout other areas of the body, such as the bones, lungs, and liver. Symptoms and signs, therefore, are more often associated with advanced prostate cancer (Schmid *et al*., 1993) (**Figure 1.14**).

Prostate cancer is the most prevalent type of internal malignancy found in men over the age of fifty. It is the second leading cause of cancer-related deaths among men in Western nations, especially African-American men (Jemal *et al*., 2007). The causes of prostate cancer are not completely understood. Age is found as the strongest risk factors and some other risk factors are also studied. Some epidemiologic studies have suggested that dietary fat closely associated with lipid peroxidation maybe the important factor for prostate cancer (Vaca *et al*., 1988). Wang, *et al*., 1995 suggested that dietary fat content can influence the tumor growth of androgen-sensitive, human prostatic adenocainoma cells (LNCaP cells) in nude mice. The highest PSA levels were found in the high-fat diet group and lowest in the low-fat group which indicated

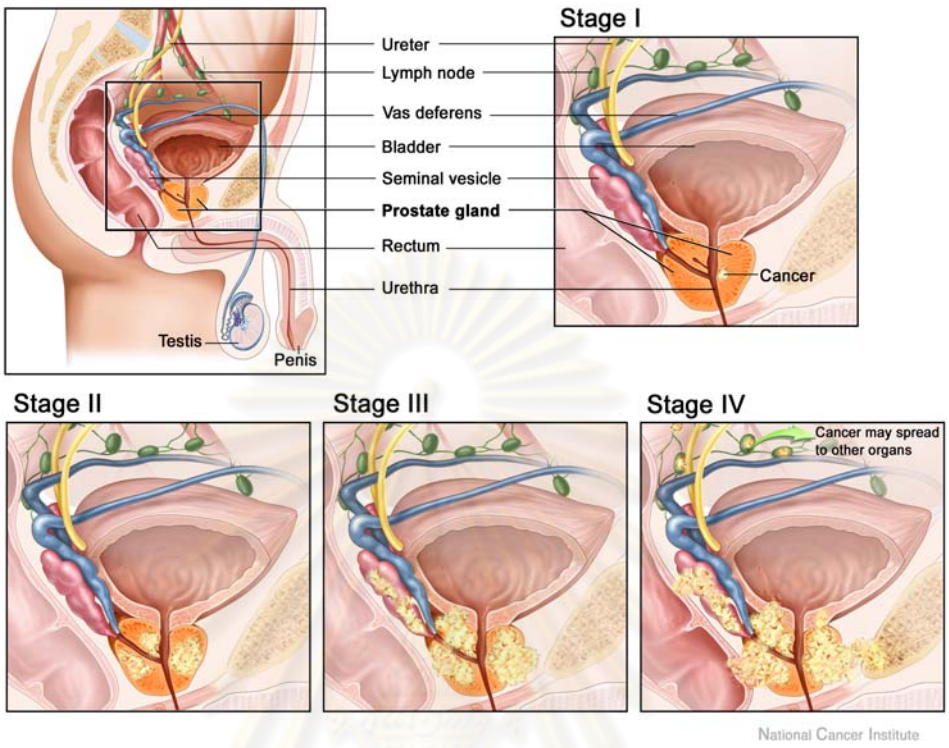


Figure 1.14 The location of the prostate cancer, in front of the rectum and just below the bladder

to prostate cancer progression due to dietary fat. Extensive research has established the strong relationship between ROS generation and carcinogenesis including cancer progression. ROS can act as secondary messengers to monitor several signaling cascades. They can induce the mutation and alter gene function resulting in carcinogenesis *via* oxidation processes (Halliwell, 1994). The mutation of p53 protein has been found associated with the progression of prostate cancer exhibiting various degrees of aggressiveness (Navone *et al*., 1993). ROS plays the essential role for migratory/invasiveness phenotypes of prostate cancer. Elimination of excessive ROS maybe very effective choice decrease prostate cancer formation and metastasis. This can be extended to some other malignancies due to the strong relation between ROS and tumor formation. Several studies have investigated the natural agents or chemopreventive agents from dietary substances to prevent and possible to cure of cancer (Syed *et al*., 2008). Many reports mentioned to some natural compounds and

dietary agents such as selenium, vitamins E and D, lycopene, soy and isoflavone, green tea, low-fat diet, epigallocatechin-3-gallate (EGCG) from green tea, a few compound from pomegranate were tested to reduce the possibility of prostate cancer both growth and progression (Liao *et al*., 2000).

1.5 Ethnomedical Information and Biological Activities

The *Zanthoxylum* (Rutaceae) is distributed worldwide from the topics and the temperated zone. There are over 200 species from small shrubs to large tree. Throughout the world, *Zanthoxylum* is recognized as having medicinal qualities such as treating stomach ache, toothache, intestinal worms, rheumatism, scabies, snakebites, fever and cholera (Pongboonrod, 1979). *Zanthoxylum limonella* Alston is widely distributed in the northern part of Thailand. Only one species has been reported in Thailand (Smitinard, 1980) which is commonly known as Ma-khan (*Zanthoxylum limonella* Alston.). Every part of *Z. limonella* such as root, bark, stem, fruit and seed is used for medicinal purpose. This plant is used traditionally in food, especially in ripe fruit used as spice and vitamin E is detected in the seed oil (Fish *et al*., 1975). The essential oil from the fruit possessed stimulation effect on different smooth muscle preparation by non-specific mechanism (Ittipanichpong *et al*., 2002). The bark is noted for its febrifugal, sudorific and diuretic properties (Pongboonrod, 1979). The stem bark has been reported to contain several alkaloids, lupeol, rutaecarpine, xanthoxyletin, osthol and scopoletin (Somanabandhu *et al.*, 1992). Previous reports of another species of *Zanthoxylum* demonstrated that the chloroform extract from the bark of *Z. budranga* showed highly potent antibacterial and antifungal activity, while the most significant cytotoxic activity was found from the methanol extract of the bark (Islam *et al.*, 2001). In addition, the methanol extract of *Z. rhetsa* Roxb. stem bark orally treated to mice at dose 250 and 500 mg/kg significantly reduced the abdominal contraction induced by acetic acid and the diarrhoeal episodes induced by caster oil in mice (Rahman *et al.*, 2002). The ripe fruit is used as a condiment in curries. In addition, vitamin E was detected in the seed oil (Fish *et al.*, 1975) and aromatic components isolated from the essential oil (Thappa *et al.*, 1976). It also possessed an antibacterial effect against cholera in rabbits (Nayak, 1961). The major constituent of the essential oil from dried fruits of *Z. rhesta* has been reported as sabinene 35.7-

67.7%. (Shankaracharya *et al.*, 1994 and Shafi *et al.*, 2000) and showed anti-imflammatory, anesthetic and hypotensive activities (Ahsan *et al.*, 2000). Examination of chemical composition of essential oil distilled from the fruit of *Z. limonella* Alston. revealed the presence of 33 components. Evaluation of the oil composition was achieved by GC/MS analysis. Limonene (31.09%), terpinen-4-ol (13.94%) and sabinene (9.13%) were found to be the major component. In addition, the essential oil from the fruit of *Z. limonella* possessed stimulation effect on different smooth muscle preparation by non-specific mechanisms (Itthipanichpong *et al.*, 2002). The compounds containing in the essential oil of seed from *Z. rhetsa* were analyzed using GC/FID and GC/MS. Monoterpene: sabinene, limonene, pinenes, *p*-cymene and terpinene were identified as the main components of this oil. In addition, terpinen-4-ol and α-terpineol are also dominant constituents. (Jirovertz *et al.*, 1998). The essential oil from the fruit of *Zanthoxylum limonella* was the most effective against the larvae of *Aedes aegypti*, which are the main vector of dengue hemorrhagic fever (Pitasawat *et al.*, 2007).

In addition, limited sciencetifically proven information is available on the antioxidative effects on this plant. However, the antioxidant activity of *Zanthoxylum* in other species was investigated in previous research, Yamazaki *et al.*, 2007 reported that hyperoside (quercetin-3-*O*-galactoside) and quercitrin (quercetin-3-O-rhamnoside) were found antioxidant compounds of the methanol extract from Japanese pepper (*Z. piperitum* DC.) fruit. The DPPH radical scavenging activities hyperoside and quercitin were as strong antioxidant. In addition, the antioxidative and hepatoprotective potential of glycoprotein isolated from *Z. piperatum* DC fruit (ZPDC glycoprotein). ZPDC glycoprotein has a strong scavenging activity against DPPH radical, superoxide anion and hydroxyl radical in the cell-free system and has an inhibitory effect on hypoxanthine/xanthine oxidase- or glucose/glucose oxidase-induced cytotoxicity in a dose-dependent manner (Lee and Lim, 2008).

Figure 1.15 Stems, leaves and fruits of *Z. limonella* Alston.

1.6 Objective of this Research

1. To screen for antioxidant and antimicrobial activities from various parts of *Z. limonella*

2. To separate phytochemical compositions from active fractions using bioassay guides

CHAPTER II

EXTRACTION AND PRELIMINARY BIOASSAY SCREENING OF *Zanthoxylum limonella* Alston.

2.1 Plant Materials

Stem barks, stems, leaves, roots and ripe fruits were collected from Phrae province, Thailand in January 2007 and voucher specimen (BKF No.152276) was submitted to the Herbarium of the Royal Forest Department of Thailand. In addition, stem barks and stems were collected from Chiang-Mai province, Thailand in April 2007.

2.2 Extraction for Preliminarily Biological Screening Test

All parts of plant materials were separately milled into coarsely power and then macerated with CH_2Cl_2 and MeOH at room temperature. The extracts were fitered and evaporated under reduced pressure. In addition, dried and powdered fruits were hydro-distilled for essential oil. The percentage yield of each extract was calculated as collected in **Table 2.1**.

Table 2.1 The percentage yield of the CH_2Cl_2 and MeOH extracts from various parts of *Z. limonella* and the percentage yield of essential oil from the fruits.

Parts	% Yield		
	CH_2Cl_2 extract	MeOH extract	Hydrodistillation
Chiang-Mai province			
Stem-bark	2.08	5.02	-
Stem	0.66	4.62	-
Phrae province			
Stem-bark	0.82	2.52	-
Stem	0.32	1.59	-
Leave	5.49	6.52	-
Root	0.39	1.71	-
Fruit	19.19	15.23	11.63

Table 2.1 reveals that the yields of MeOH extracts were higher than those of CH_2Cl_2 extracts. The highest percentage yield of the CH_2Cl_2 and MeOH extracts could be achieved from the fruits. These data showed that the different solvents have different capacity to extract chemical constituents from plants. The stem-bark and stem extracts from Chiang-Mai province provided higher yield than those obtained from Phrae province. This strongly shows that the plant growing in different geographical locations have the variation in the amounts of phytochemicals present (Binns, 2002). All *Z. limonella* extracts were preliminarily screened for antimicrobial and antioxidant activities.

2.3 The Preliminary Bioassay Screening for Antimicrobial Activity

The CH_2Cl_2 and MeOH extracts from different plant parts and the essential oil from the fruits were preliminarily evaluated for antibacterial and anticandidal activities using paper disc diffusion method and agar incorporation method for screening of antifungal activity.

2.3.1 Antibacterial and Anticandidal Activities Screening Test of Extracts and Essential Oil

The antibacterial and anticandidal bioassays of the CH_2Cl_2 and MeOH extracts and the essential oil of the fruits were tested against (i) two Gram-positive bacteria, *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 25923 and two Gram-negative bacteria, *Esherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 (ii) the pathogenic yeast like fungi *Candida albicans* ATCC 10231, all at the final concentration of 5 mg per disc by paper disc diffusion method. The diameter of the inhibition zone was evaluated as presented in **Table 2.2**.

As the results presented in **Table 2.2** and **Figure 2.1**, all CH_2Cl_2 and MeOH extracts and the essential oil of the fruits displayed antibacterial and anticandidal activities with different extents. Among the inhibitory effect of these extracts, the essential oil from the ripe fruits exhibited the most potent antibacterial and anticandidal activity against all tested bacteria and yeast, with a maximum inhibition zone against *S. aureus* (30 mm) and minimum against *P. aeruginosa* (7.6 mm) at 5 mg/disk. The CH_2Cl_2 extract of the stem from Chiang-Mai province was more

Table 2.2 Antibacterial and anticandidal activities of CH_2Cl_2 and MeOH extracts from different parts and the essential oil from fruits of *Z.limonella*

Parts	Diameter of inhibition zone*(mm)				
	B. subtilis	***S. aureus***	***E. coli***	***P. aeruginosa***	***C. albicans***
Chiangmai Province					
Stem bark CH_2Cl_2 extract	10.8±0.8	10.7±0.8	7.8±0.4	6.9±0.5	8.0±0.0
Stem bark MeOH extract	NA	6.3±0.2	NA	NA	NA
Stem CH_2Cl_2 extract	11.0±0.00	12.9±0.5	8.4±0.2	7.2±0.4	8.9±0.3
Stem MeOH extract	NA	NA	NA	NA	NA
Phrae Province					
Stem bark CH_2Cl_2 extract	NA	14.3±0.6	NA	7.2±0.3	NA
Stem bark MeOH extract	NA	6.1±0.2	NA	NA	NA
Stem CH_2Cl_2 extract	11.2±0.3	13.2±0.3	NA	NA	8.5±0.0
Stem MeOH extract	NA	6.4±0.2	NA	NA	NA
Leave CH_2Cl_2 extract	NA	NA	NA	NA	NA
Leave MeOH extract	NA	NA	NA	NA	NA
Root CH_2Cl_2 extract	11.0±0.3	11.5±0.5	7.8±0.3	7.2±0.4	8.5±0.4
Root MeOH extract	6.7±0.3	6.6±0.4	NA	NA	8.2±0.3
Fruit CH_2Cl_2 extract	7.2±0.1	11.5±1.0	NA	NA	7.8±0.6
Fruit MeOH extract	NA	NA	NA	NA	NA
Fruit (Hydrodistillation)	17.9±1.9	30.4±1.2	8.7±0.4	7.6±0.3	11.6±0.6
Penicillin G (10 unit/disc)	25.2 ± 1.4	29.9 ± 1.7	NA	NA	NA
Chloramphenical (30 µg/disc)	25.3 ± 2.3	19.0 ± 1.4	NA	NA	NA
Amphotericin B (25 µg/disc)	NA	NA	NA	NA	10.5 ± 0.8

[a] Values, an average ± standard error of 3 replicates, of the mean inhibition zone, NA = Not active

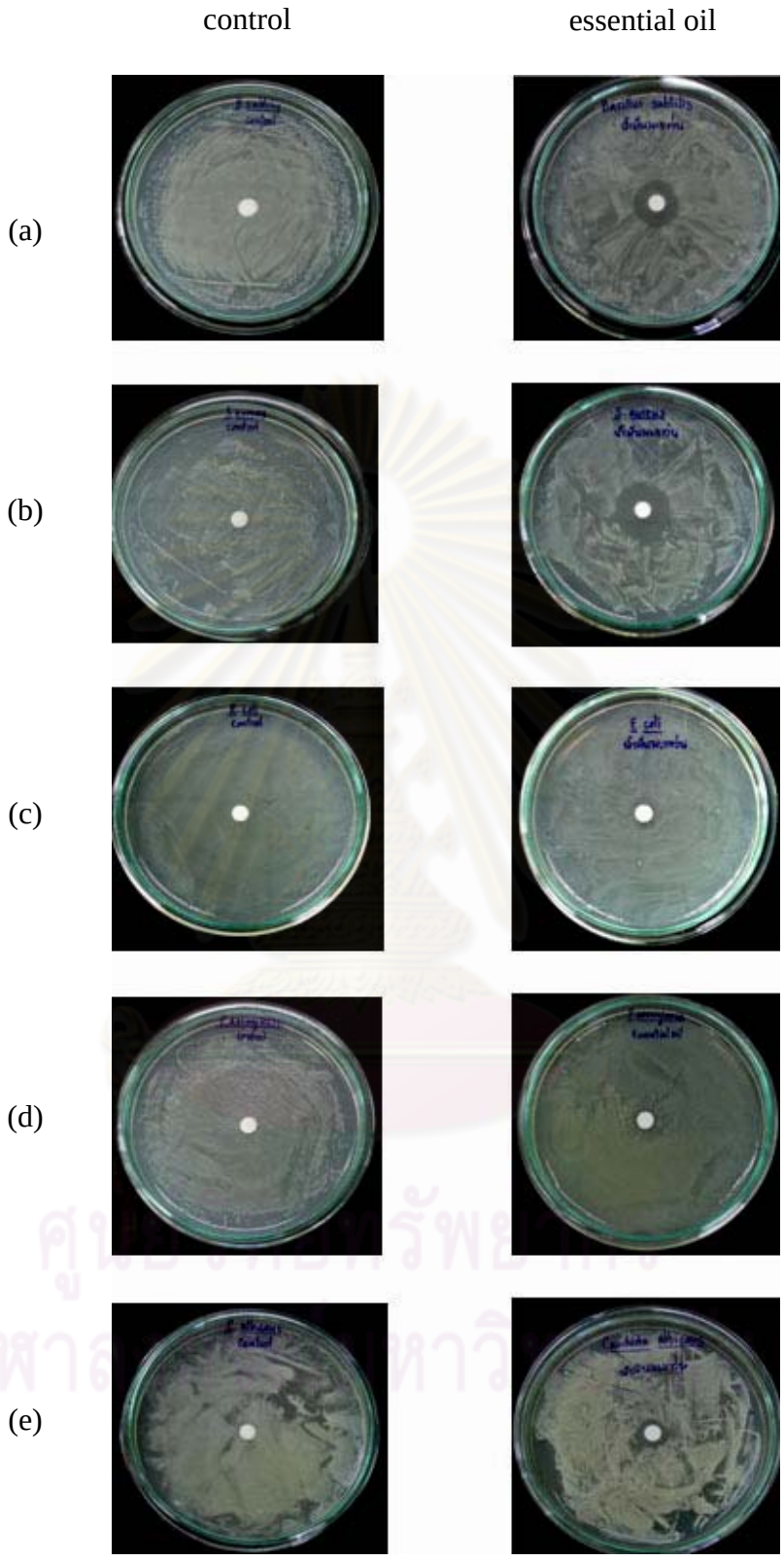


Figure 2.1 The inhibition zone of the control (left hand) and essential oil (right hand) from *Z. limonella* against (a) *B. subtilis* , (b) *S. aureus*, (c) *E. coli* (d) *P. aeruginosa* and (e) *C. albicans*

effective than those attained from other extracts and showed moderated activity in comparison with the essential oil of the fruits with maximum inhibition (12.9 mm) against *S. aureus* and minimum against *P. aeruginosa* (7 mm). These data indicated that the essential oil of the fruits and the CH_2Cl_2 extract of the stem from Chiang-Mai province had more antibacterial constituents which were less or absent in the CH_2Cl_2 and MeOH extracts from stem-barks, roots, leaves, and fruits. Previously, antibacterial and anticandidal activities of Zanthoxylum were investigated. The essential oil of the dried fruits of *Z. xanthoxyloides* showed the most extensive zone for *B.cereus* and *E. faecalis* (Ngassoum *et al*., 2003). These data indicated that the extraction using different solvents would lead to various antibacterial compounds. For instance, the $CHCl_3$ extract of the stem-barks of *Z. budrunga* showed the more potent antibacterial activity than that from the petroleum ether and MeOH extracts (Islam *et al*., 2001). In addition, Bhattacharya *et al.*, 2009 performed the *in vitro* antibacterial activity of aqueous and EtOH extracts from the roots and stem-barks of *Z. nitidum* and found that the EtOH extract of the root was the most potent with maximum concentration dependent and the aqueous extract of the stem-bark was the least active. Gram-positive bacteria were more sensitive to the extracts. The essential oil of the fruits and almost extracts inhibited the growth of Gram-positive bacteria and pathogenic yeast more selectively than Gram-negative bacteria. This may be due to general physiological differences in the membrane constitution of Gram-negative and Gram-positive bacteria. Lack of activity against Gram-negative bacteria can be accounted for the presence of their thick membrane layer, which prevents the entry of inhibitions (Suffredini *et al*., 2006).

2.3.2 Antifungal Activity Screening of the CH_2Cl_2 and MeOH Extracts and the Essential Oil of the Fruits

The antifungal activity of the CH_2Cl_2, MeOH extracts and the essential oil of the fruits were tested against five phytopathogenic fungi: *Fusarium oxysporum, Corynespora cassiicola, Exserohilum turcicum, Fusarium oxysporum f.* sp. *vasinfectum* and *Curvularia lunata* at the final concentration of 5,000 ppm by agar incorporation method. The percentage inhibition was calculated as represented in **Table 2.3** and the antifungal activity was depicted as shown in **Figure 2.2**.

Table 2.3 The percentage of mycelial growth inhibition of the CH_2Cl_2 extracts and the essential oil at 5,000 ppm final concentration of five fungi tested

Parts	*F.oxysporum f. sp.vasnifectum*		*F.oxyspolum*		*C. lunata*		*C. cassiicola*		*E. turcicum*	
	Growth[a] (mm)	Inh (%)	Growth[a] (mm)	Inh (%)	Growth[a] (mm)	Inh (%)	Growth[a] (mm)	Inh (%)	Growth[a] (mm)	Inh (%)
Chiangmai Province										
Stem bark CH_2Cl_2 extract	62.2 ± 3.8	17.8 ± 5.0	27.2 ± 1.0	69.8 ± 1.2	36.2 ± 0.6	60.6 ± 1.9	35.2 ± 2.9	55.4 ± 0.4	13.7 ± 0.6	84.8± 0.7
Stem bark MeOH extract	90.0 ± 0.0	0.0 ± 0.0	81.0 ± 1.0	10.0 ± 1.1	42.8 ± 2.1	18.0 ± 0.7	64.7 ± 0.6	18.1 ± 0.7	90.0 ± 0.0	0.0± 0.0
Stem CH_2Cl_2 extract	30.1 ± 1.0	64.0 ± 1.1	13.0 ± 1.0	85.6 ± 1.1	0.0 ± 0.0	100.0 ± 0.0	21.6 ± 0.7	71.8 ± 0.9	0.0 ± 0.0	100.0 ± 0.0
Stem MeOH extract	90.0 ± 0.0	0.0 ± 0.0	90.0 ± 0.0	0.0 ± 0.0	65.2 ± 1.7	27.5 ± 1.9	62.8 ± 1.0	20.5 ± 1.3	90.0 ± 0.0	0.0± 0.0
Phrae Province										
Stem bark CH_2Cl_2 extract	90.0 ± 0.0	0.0 ± 0.0	53.3 ± 5.4	40.8 ± 6.0	55.8 ± 0.8	38.0 ± 0.9	55.12 ± 1.5	31.1 ± 1.9	30.5 ± 0.5	66.1 ± 0.6
Stem bark MeOH extract	90.0 ± 0.0	0.0 ± 0.0	90.0 ± 0.0	0.0 ± 0.0	43.9 ± 1.4	51.2 ± 1.6	57.7 ± 1.5	27.0 ± 1.9	54.7 ± 3.8	39.3 ± 4.2
Stem CH_2Cl_2 extract	35.7 ± 1.2	60.3 ± 1.3	41.4 ± 2.4	54.0 ± 2.6	0.0 ± 0.0	100.0 ± 0.0	27.5 ± 0.9	68.1 ± 3.2	0.0 ± 0.0	100.0 ± 0.0
Stem MeOH extract	90.0 ± 0.0	0.0 ±0.0	90.0 ± 0.0	0.0 ± 0.0	72.2 ± 0.4	19.8 ± 0.4	63.7 ± 3.8	19.4 ± 4.8	90.0 ± 0.0	0.0 ± 0.0
Leave CH_2Cl_2 extract	90.0 ± 0.0	0.0 ± 0.0	90.0 ± 0.0	0.0 ± 0.0	90.0 ± 0.0	0.0 ± 0.0	72.8 ± 2.0	9.0 ± 2.5	74.8 ± 2.0	16.9 ± 2.3
Leave MeOH extract	90.0 ± 0.0	0.0 ± 0.0	90.0 ± 0.0	0.0 ± 0.0	90.0 ± 0.0	0.0 ± 0.0	90.0 ± 0.0	0.0 ± 0.0	43.0 ± 2.0	52.2 ± 2.2
Root CH_2Cl_2 extract	37.9 ± 0.6	54.6 ± 0.7	22.3 ± 0.0	75.1 ± 4.6	18.8 ± 1.8	79.1 ± 2.0	33.9 ± 1.1	55.82 ± 1.4	0.0 ± 0.0	100.0 ± 0.0
Root MeOH extract	41.8 ± 1.3	53.5 ± 1.4	55.3 ± 1.5	38.5 ± 1.7	23.5 ± 1.0	73.9 ± 1.1	53.0± 1.0	32.5 ± 1.3	53.0 ± 2.7	41.1 ± 2.9
Fruit CH_2Cl_2 extract	51.6 ± 2.0	42.7 ± 2.2	23.6 ± 3.5	73.8 ± 3.9	21.1 ± 2.9	76.6 ± 3.2	28.3± 2.9	64.2 ± 3.6	0.0 ± 0.0	100.0 ± 0.0
Fruit MeOH extract	90.0± 0.0	0.0 ± 0.0	90.0 ± 0.0	0.0 ± 0.0	77.9 ± 2.0	13.4 ± 2.2	79.0±0.0	0.0 ± 0.0	90.0 ± 2.7	0.0 ± 0.0
Fruit (Hydrodistillation)	9.0± 0.0	90.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	0.0± 0.0	100.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0
Benomyl (100 ppm)	0.0 ± 0.0	100.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	19.1 ± 2.7	78.8 ± 2.9	0.0 ± 0.0	100.0 ± 0.0	71.6 ± 2.8	18.9 ± 3.1

[a] Values, an average ± standard deviation of 3 replicates of the mean growth inhibition of fungi species, Inh = Inhibition

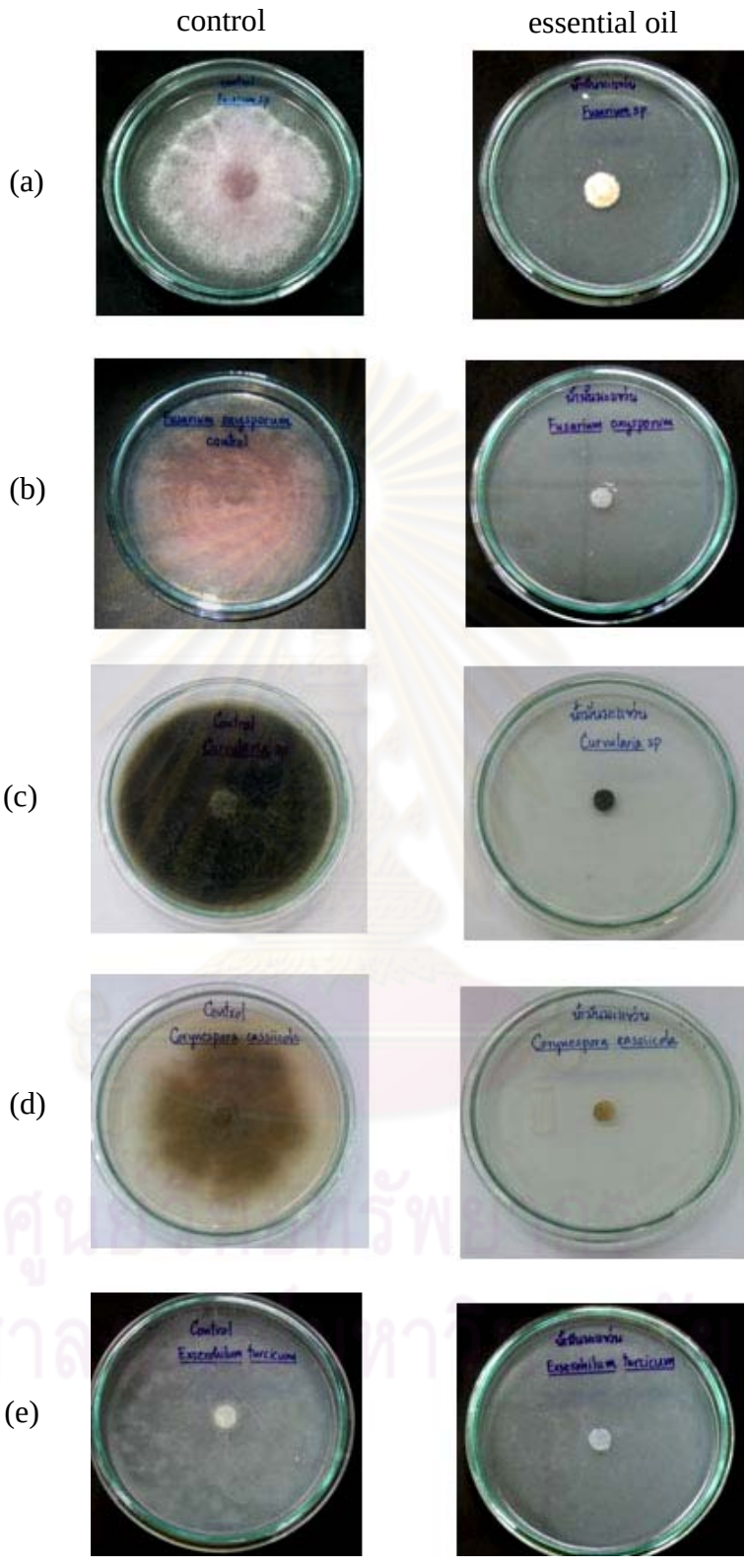


Figure 2.2 The mycelial growth inhibition zone of the control (left hand) and essential oil (right hand) from *Z. limonella* against (a) *F. oxysporum f.* sp. *vasinfectum* (b) *F. oxysporum* (c) *C. lunata* (d) *C. cassiicola* and (e) *E. turcicum.*

The data from **Table 2.3** still displayed that the essential oil exhibited the most effective antifungal activity against the mycelial growth of almost phytopathogenic fungi tested by 100% inhibition higher than those observed from the other extracts. The CH_2Cl_2 extracts of the stem-barks, stems, and roots showed more potent activity than other CH_2Cl_2 and MeOH extracts. The lowest activity was exhibited by the MeOH extract of the leaves and fruits. In addition, the CH_2Cl_2 extracts of the stem-barks and stems from Chiang-Mai province expressed higher percentage inhibition than the CH_2Cl_2 extracts of stem-barks, stems, and roots from Phrae province. From the data present, the inhibition effect of the CH_2Cl_2 extract displayed more potent activity than the MeOH extract. Antifungal activity of *Zanthoxylum* was previously addressed. The 90% aqueous-EtOH extract of the roots of *Z. xanthoxyloides* at 4 mg/mL inhibited *Botrytis cinerea* to a fair extent in solid medium and the stem-bark extract at 0.5 mg/mL had a fungicidal effect on *B. cinerea* in liquid medium (Ngane *et al.*, 2000).

In summary, the constituents of the essential oil of ripe fruits and the CH_2Cl_2 extracts of the stems and stem-barks from Chiang-Mai province could serve as a source of phytochemical constituents useful to cure and to eliminate some phytopathogenic fungi.

2.4 The Preliminary Screening for Antioxidant Activity of the CH_2Cl_2, MeOH Extracts and the Essential Oil of the Fruits

The CH_2Cl_2 extracts and the essential oil of the fruits of *Z. limonella* were in addition preliminarily evaluated for antioxidant activity using TLC autographic method with 2,2-diphenyl-1picryl-hydrazyl (DPPH) in MeOH (Hostettmann *et al.*,1997).

Generally, DPPH, a purple-colored stable free radical could be reduced into the yellow colored diphenylpicryl hydrazine. Thus, if the tested samples could exhibit antioxidant activity, after spraying DPPH solution for 30 min, yellow spots against purple background should be observed. The antioxidant activity test results are collected in **Table 2.4**.

It was obviously seen that the CH_2Cl_2, MeOH extracts of the stems, roots and stem barks either from Phrae or Chiangmai exhibited the highest level of antioxidant activity, while that of the essential oil and the CH_2Cl_2 extract from the fruits showed

moderate activity and the CH_2Cl_2 extract from leave did not reveal free radical scavenging effect on DPPH.

According to the antimicrobial and antioxidant activity screening tests, the essential oil displayed the most effective fraction inhibiting the growth of bacteria, yeast like fungi and phytopathogenic fungi and good antioxidant activity. In addition, the CH_2Cl_2 extract from the stems showed good antimicrobial activity and exhibited the highest level of antioxidant activity. Therefore, the essential oil and the CH_2Cl_2 extracts of the stems were selected for further study to evaluate for chemical compositions and biological activity.

Table 2.4 Antioxidant screening test of different parts of *Z. limonella*

Parts	**Radical scavenging effect on DPPH**		
	CH_2Cl_2 extract	**MeOH extract**	**Hydrodistillation**
Chiang-Mai Province			
Stem barks	+++	+++	ND
Stems	+++	+++	ND
Phrae Province			
Stem barks	+++	+++	ND
Stems	+++	+++	ND
Leaves	-	+++	ND
Roots	+++	+++	ND
Fruits	++	+++	++

- negative result; + positive results observed within 30 min; + positive results observed within 15 min; ++ positive results observed immediately; ND = Not detectable

2.5 Experimental Section

2.5.1 Extraction of Plant Materials

Each part of plant was air-dried, minced and extracted with CH_2Cl_2 by maceration method for three times. The marc was then similarly extracted with MeOH for three times. Each extract was filtered and evaporated by rotary evaporator and kept in the dark until used.

Dried fruits of *Z. limonella* was powdered with a blender and essential oil was obtained by hydrodistillation using a Clevenger-type apparatus. Essential oil was stored at 4^{o}C until analyzed its compositions and bioassays.

2.5.2 The Preliminary Bioassay Screening for Antibacterial and Anticandidal Activities

Antibacterial and anticandidal activities of the CH_2Cl_2 extract are individually tested against (i) two Gram-positive bacteria *B. subtilis* and *S. aureus* and two Gram-negative bacteria *E. coli* and *P. aeruginosa* (ii) the pathogenic yeast like fungi *C. albicans*. Bacteria strains were grown on nutrient agar, whereas those of yeast were on yeast malt extract agar. Paper disc diffusion technique (CLSI, 2005) was used for this screening test. The cultures were adjusted with sterile saline solution to obtain turbidity comparable to that of McFarland No 0.5 standard ($1.0x10^8$ CFU/mL). A suspension of the tested microorganism 100 μL of 10^8 cells per mL is spread on the Mueller Hinton agar and Sabouraud dextrose agar plate. Filter paper discs (6 mm in diameter) were impregnated with 10 μL of each assayed extract and placed on the agar surface. Discs impregnated with suitable solvent were used as negative controls. Discs with standard antibiotic were used as positive controls. These plates were incubated at 37°C, 24 h for bacteria and at 37°C, 48 h for yeast. The diameters of the inhibition zones are measured in mm. All tests were performed in triplicate.

2.5.3 The Preliminary Bioassay Screening for Antifungal Activity

Antifungal activity of crude extract was individually tested against five phytopathogenic fungi: 3 strains (*F. oxysporum*, *C. cassiicola* and *E. turcicum*) supplied by the Division of Plant Disease and Microbiology, Department of Agriculture, Ministry of Agriculture and Cooperative, Bangkok, Thailand and 2 strains (*F.* oxysporum *f.* sp. *vasinfectum*, *Curvularia lunata*.) from naturally infected pineapple disease. Cultures of each fungus were maintained on potato dextrose agar (PDA) slants stored at 4°C. Antifungal activity was modified by the method of agar incorporation (dilution on a solid medium) described by Taudon *et al.*, 1990 and Dwivedy and Dubey, 1993. The fungal strains were cultivated on PDA medium in Petri dish. Tested samples including essential oil and its major components were

diluted with DMSO for CH_2Cl_2, MeOH crude extracts and 1% tween 80 for essential oil at various concentrations and 1,000 μL of each solution was aseptically mixed with 9 mL of PDA (liquefied and maintained at melting point in water bath). After cooling and solidification, the seeding was carried out by inoculation 7 days old mycelium culture in the middle of Petri dish. Three replicates for each concentration and fungal species were conducted. The Petri dishes were then incubated at 25°C for 7 days. PDA plates treated with DMSO and 1% tween 80 without samples were used as a negative control, whereas those with benomyl were also used as positive control.

The antifungal activity of the extract was evaluated according to the method of Singh *et al.* (1993) by calculating the percentage inhibition (%I) using the following formula.

$$\% \text{ inhibition} = \frac{(d_C - d_E)}{d_C} \times 100$$

d_C: colony diameter of control plate (mm)

d_E: colony diameter of treatment (mm)

2.5.4 The Preliminary Bioassay Screening for Antioxidant Activities (TLC Autographic Assay)

To detect for antioxidants present, the extracts were carried out using TLC. After drying, the TLC plate was sprayed with 0.2% DPPH in MeOH. The presence of antioxidants was revealed within 30 min as pale yellow zone against a purple background (Hostettmann *et al.*, 1997).

CHAPTER III

CHEMICAL COMPOSITION AND BIOACTIVE COMPOUNDS FROM THE ESSENTIAL OIL OF *Zanthoxylum limonella* Alston.

3.1 Chemical Compositions of the Essential Oil from the Fruits, Leaves and Stems of *Z. limonella*

According to aforementioned results, the essential oil from the fruits of *Z. limonella* showed the most effective antibacterial, anticandidal and antiphytopathogenic fungi and exhibited good antioxidant activity. Therefore, the further investigation on the essential oils from the fruits, leaves and stems of *Z. limonella* were performed by hydrodistillation using a Clevenger apparatus. The %yield of each essential oil was calculated as presented in **Table 3.1** and the GC/MS analysis results are given in **Table 3.2**.

Table 3.1 The %yield of the essential oil from various parts of *Z. limonella*

Parts	plant (g)	essential oil (g)	%yield
fruits	1,000	116.32	11.63
leaves	1,500	1.55	0.10
stems	1,600	0.13	0.0081

Table 3.2 Chemical composition of the essential oil from *Z. limonella*

Compounds	R_t (min)	%Area		
		fruits	leaves	stems
1. α-pinene	6.13	0.97	8.04	-
2. α-thujene	6.21	-	-	3.42
3. topilidene	6.73	-	0.48	-
4. sabinene	7.54	42.73	18.63	-
5. α-phellandrene	7.90	0.70	7.50	3.17
6. limonene	8.22	39.05	5.17	-
7. β-*trans*-ocimene	8.46	0.12	0.28	-
8. 3-carene	8.81	2.70	6.62	-
9. terpinolene	9.13	0.35	-	-
10. 1,8-cineol	9.21	-	27.20	0.80
11. β-cymene	9.47	1.18	2.04	-
12. β-pinene	12.13	0.54	0.07	-
13. linalool	12.38	0.12	2.94	-
14. cyclopentene	12.72	0.09	-	-
15. (5*E*,9*E*)-1,5,9,13-tetradecatetraene	12.94	-	0.13	-
16. 5-isopropyl-2-methyl bicyclo-[3.1.0]-hexan-2ol	13.18	0.40	0.11	-
17. β-caryophyllene	13.56	0.27	7.14	-
18. terpinen-4-ol	13.70	5.40	2.63	-
19. thujol	14.24	0.10	0.06	-
20. α-caryophyllene	14.33	0.17	1.25	-
21. *cis*-*p*-meth-2,8-dienol	14.41	0.09	-	-
22. α-terpineol	14.57	0.98	5.91	-

Table 3.2 (continued)

Compounds	R_t (min)	%Area		
		fruits	leaves	stems
23. dihydromyrcene	14.78	-	0.07	-
24. *cis*-geraniol	14.90	0.36	0.22	-
25. *trans*-carveol	15.22	0.19	-	-
26. *cis*-carveol	15.80	0.29	-	-
27. butylatedhydroxytoluene	16.13	-	-	86.62
28. cedr-8-en-15-ol	16.23	0.43	-	-
29. (*S*)-carvone	16.35	0.33	-	-
30. cuminaldehyde	16.71	0.04	-	-
31. γ-elemene	17.25	-	1.54	-
32. 5-hydroxy-methyl-1,3,3-trimethyl-2-(3-methyl-buta-1,3-diemyl-cyclopentanol)	17.82	-	-	0.50
33. (9*Z*)-9,17-octadecadienol	18.63	-	0.08	-
34. valencene	19.01	-	0.38	-
35. α-gurjunene	19.68	-	0.28	-
36. γ-cadinene	19.98	-	0.09	-
37. spathulenol	20.16	0.52	-	-
38. aromadendrene oxide	20.24	-	0.37	-
39. heptadecanoate (C17:0)	20.53	-	-	1.02
40. *p*-anisaldehyde	21.04	-	-	-
41. α-bulnesene	21.32	-	0.17	-
42. decanedioic acid	21.79	-	-	1.12
43. 1H-indene	22.57	-	0.17	-
44. 9,12,15-octadecatrienoate (C18:3n3)	27.95	-	-	3.55

Table 3.2 (continued)

Compounds	R_t (min)	%Area		
		fruits	leaves	stems
45. 3-chloro-5-hydroxy cholestan-6-yl acetate	38.61	-	-	-
46. dictamine	40.64	1.84	-	-
Total		99.96	99.57	100

From **Table 3.1**, %yield of the essential oil from leaves, fruits and stems were 0.10, 11.63 and 0.0081%, respectively. For the essential oil from the fruit, 27 compounds (representing 99.96% of the total oil) could be identified (**Table 3.2** and **Figure 3.1**). Among these, sabinene (42.73%) and limonene (39.05%) appeared to be the major components followed by terpinen-4-ol (5.40%). The fruit essential oil compositions could be classified into 6 groups including 9 monoterpenes, 10 oxygenated monoterpenes, 4 sesquiterpenes, 1 cyclic hydrocarbon, 1 phenylpropanoid and 1 furanoquinoline alkaloid. Quantitatively, monoterpenes were the main constituent, accounting for 88.34% of the essential oil. Oxygenated monoterpenes and sesquiterpenes were present in a lesser amount of 8.26% and 1.39%, respectively (**Figure 3.2**). Structurally, the major components: sabinene and limonene were classified into thujane and menthane monoterpenoids, whereas terpinen-4-ol was belonged to the oxygenated menthane monoterpenoid.

These obtained results correlate with those reported by Ittipanichpong *et al*., 2002 which revealed that the chemical analysis of the essential oil from the fruits of *Z. limonella* by GC/MS presence of 33 chemical components and 67% of compound was attributed to the monoterpene group including limonene (31.09%) and sabinene (9.13%) as the major components. Another major component, terpinen-4-ol belonged to the oxygenated monoterpene group. These data indicated that the classes of major components were similar to other varieties of *Z. limonella*, but with different quantities. It was possible that the plant growing in different geographical locations may have the variation in the amounts of phytochemicals present (Binns, 2002).

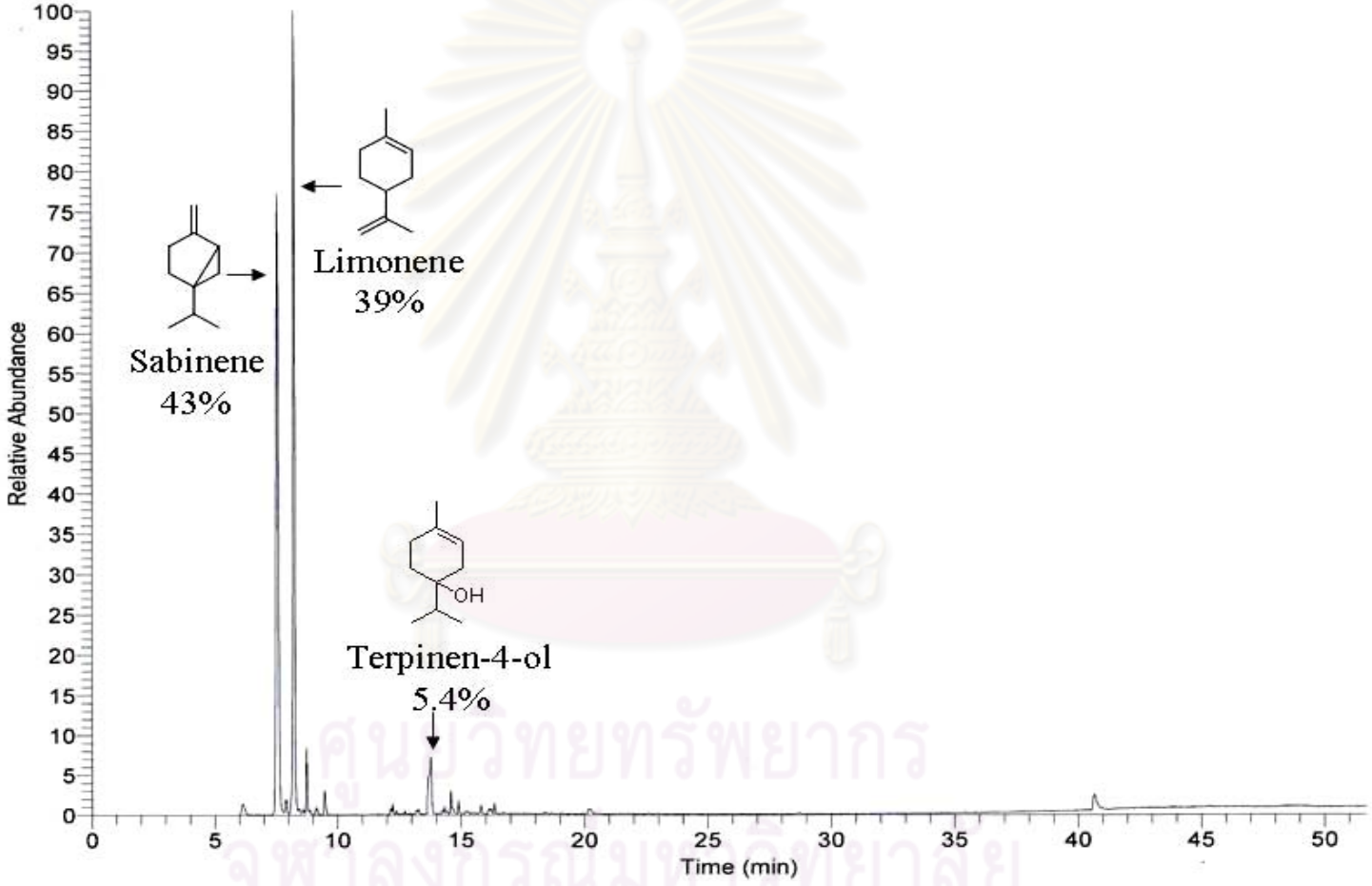


Figure 3.1 GC chromatogram of essential oil from fruits of *Z. limonella*

52

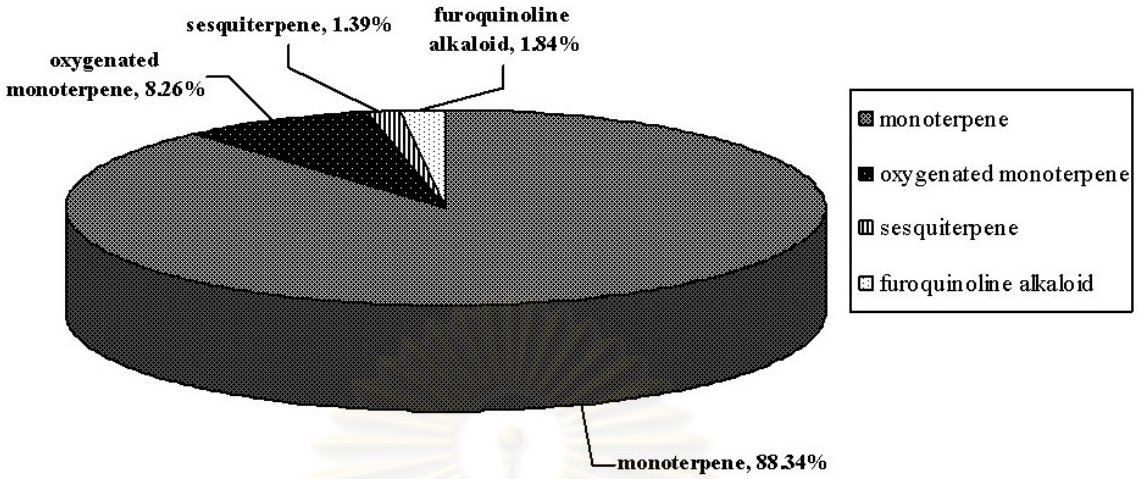


Figure 3.2 The percentage composition of compound groups present in the essential oil of *Z. limonella* fruits

The essential oil from the leaves of *Z. limonella* composed of 28 compounds (representing 99.57% of the total oil) (**Figure 3.3**). The main compositions were identified as 1,8-cineol (27.20%) and sabinene (18.63%) followed by α-pinene (8.4%), α-phellandrene (7.50%) and β-caryophyllene (7.14%), respectively. The essential oil compositions from the leaves could be classified into 4 groups: 11 monoterpenes, 7 oxygenated monoterpenes, 8 sesquiterpenes and 1 polycyclic hydrocarbon. In terms of the relative amount, monoterpene appeared to be the major group accounting for 49.03% of the essential oil. Oxygenated monoterpenes and sesquiterpenes were present in lesser amount of 39.07% and 11.22%, respectively (**Figure 3.4).** In terms of structure, 1,8-cineol and α-phellandrene belonged to the menthane monoterpenoid group, whereas sabinene and β-caryophyllene could be classified into thujane monoterpene and caryophylane sesquiterpenoid group, respectively. To the best our knowledge, there is no report in the literature concerning the chemical compositions of essential oil from the leaves of *Z. limonella*.

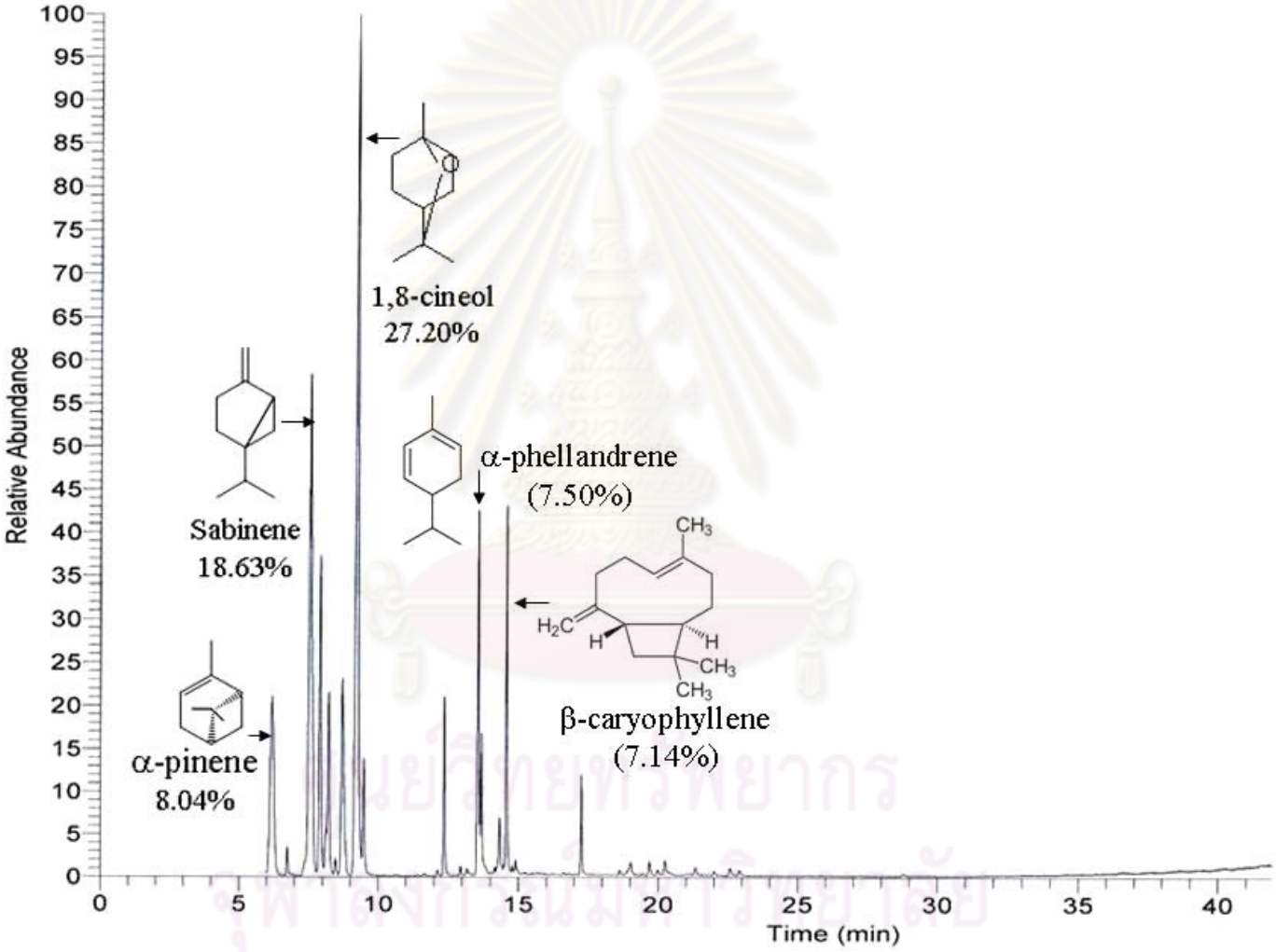


Figure 3.3 GC chromatogram of essential oil from the leaves of *Z. limonella*

54

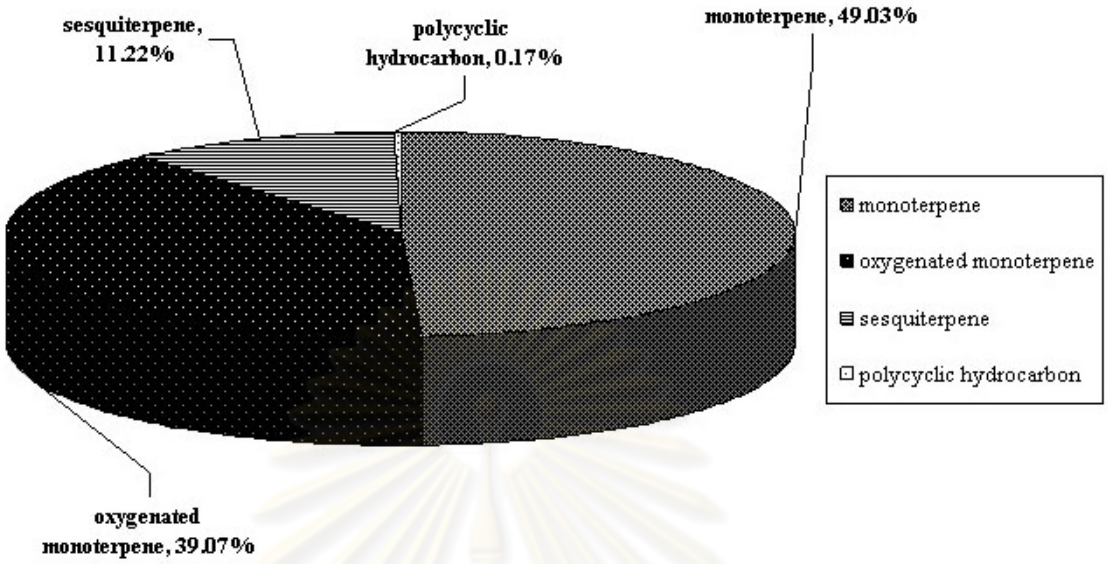


Figure 3.4 The percentage composition of compound groups present in the essential oil of *Z. limonella* leaves

Therefore this result can be evaluated as the first report about the classification and identification of phytochemical components of this essential oil.

The essential oil from the stems of *Z. limonella* composed of 8 compounds based on the GC/MS analysis result (**Figure 3.5**). Surprisingly, butylated hydroxytoluene (BHT) (86.62%), the well-known antioxidant, was identified as the major component, followed by α-thujene (3.42%) and α-phellandrene (3.17%). The major component, BHT belonged to the lipophilic compound, whereas α-thujene and α-phellandrene could be grouped to thujane and menthane monoterpenoid group, respectively. Based on the GC/MS analysis, it was found that the essential oil from Phrae and Chiang-Mai provinces contained natural BHT as a major component. (**Figiure 3.5** and **Figure 3.6**). In addition, the presence of BHT was confirmed by comparing the ^{1}H-NMR spectra of the crude essential oil from the stem and the synthetic BHT (**Figure 3.7**). The results from this study revealed that

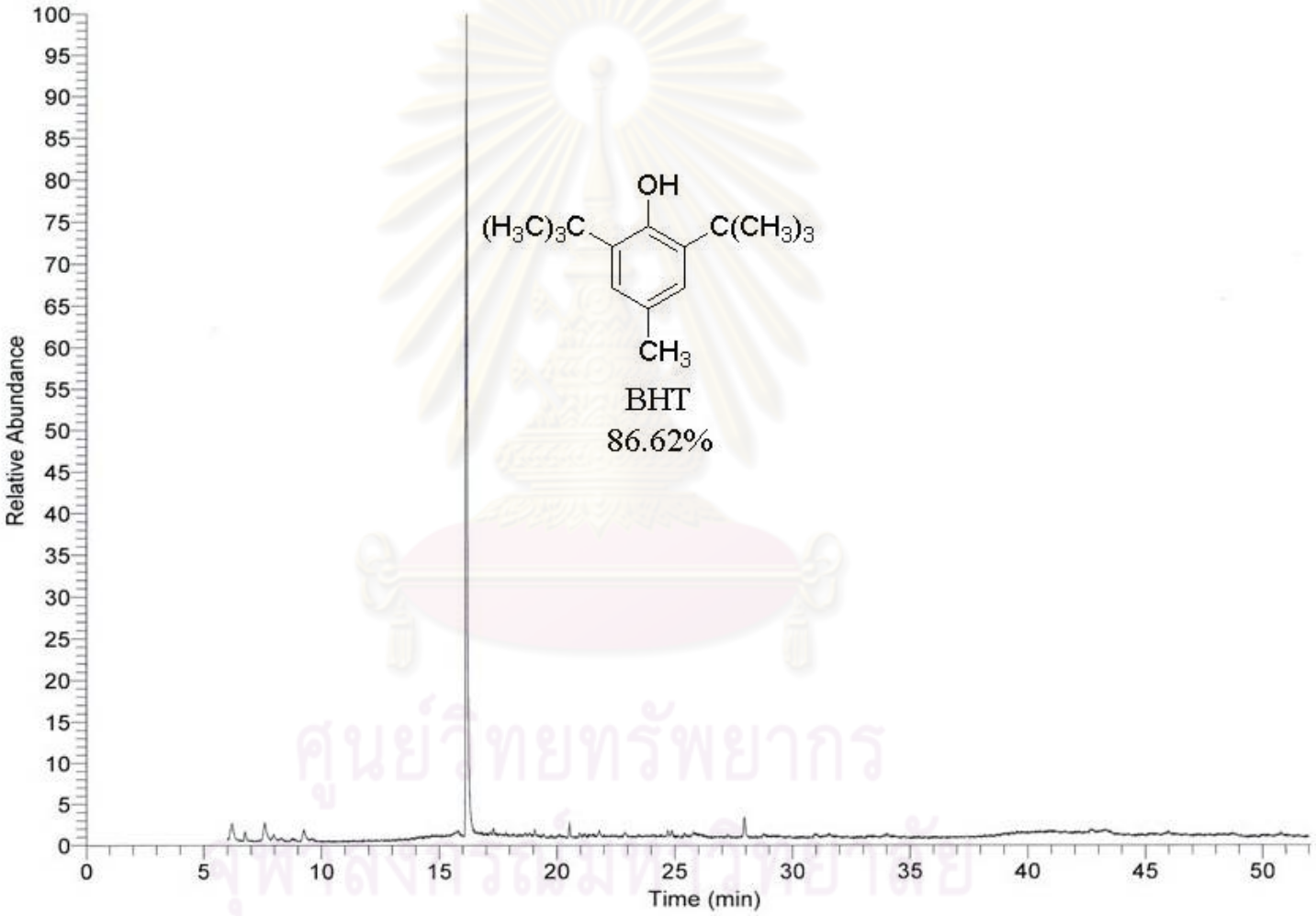


Figure 3.5 GC chromatogram of essential oil from stems of *Z. limonell*a (Chiang-Mai province)

56

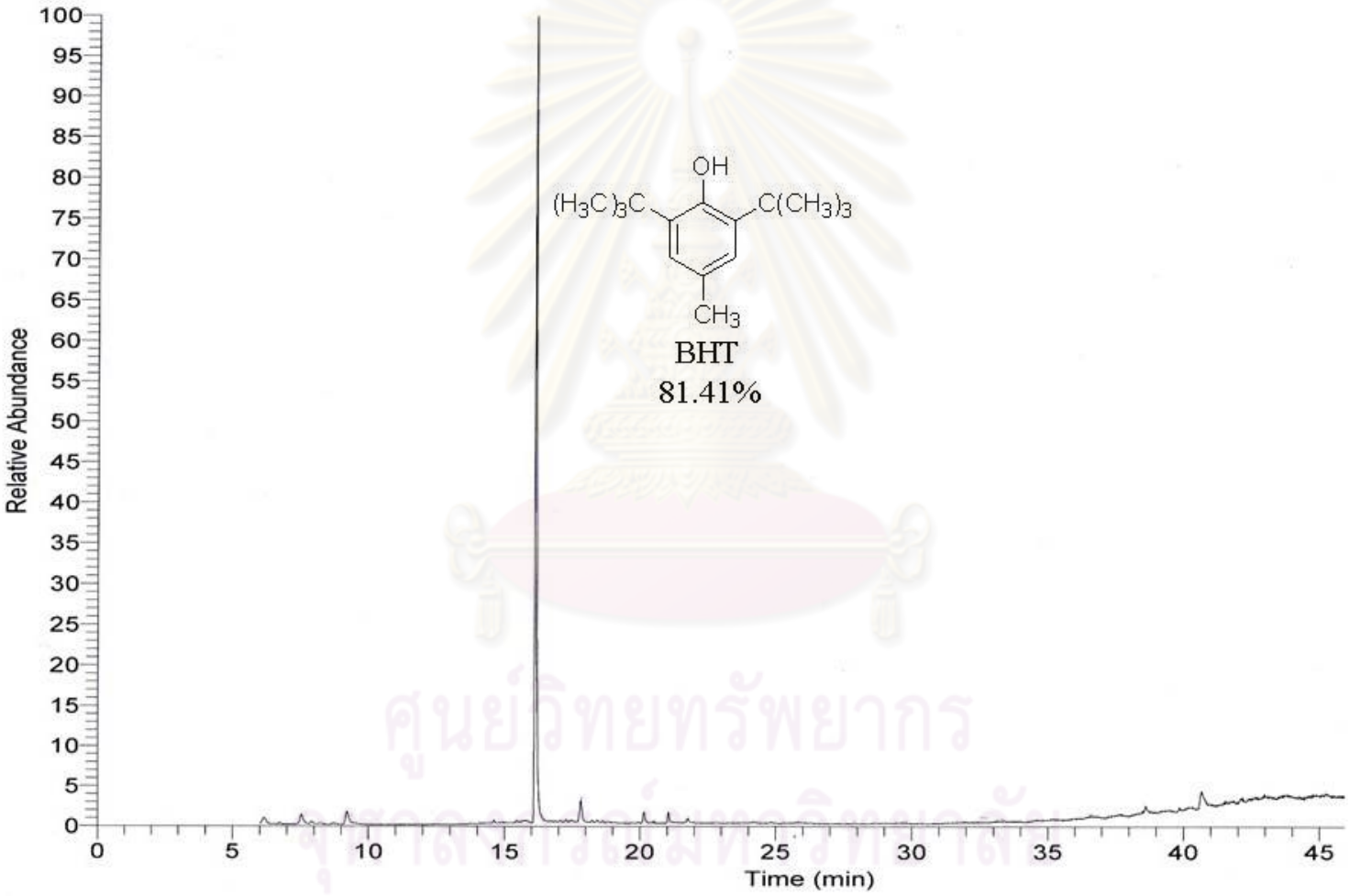
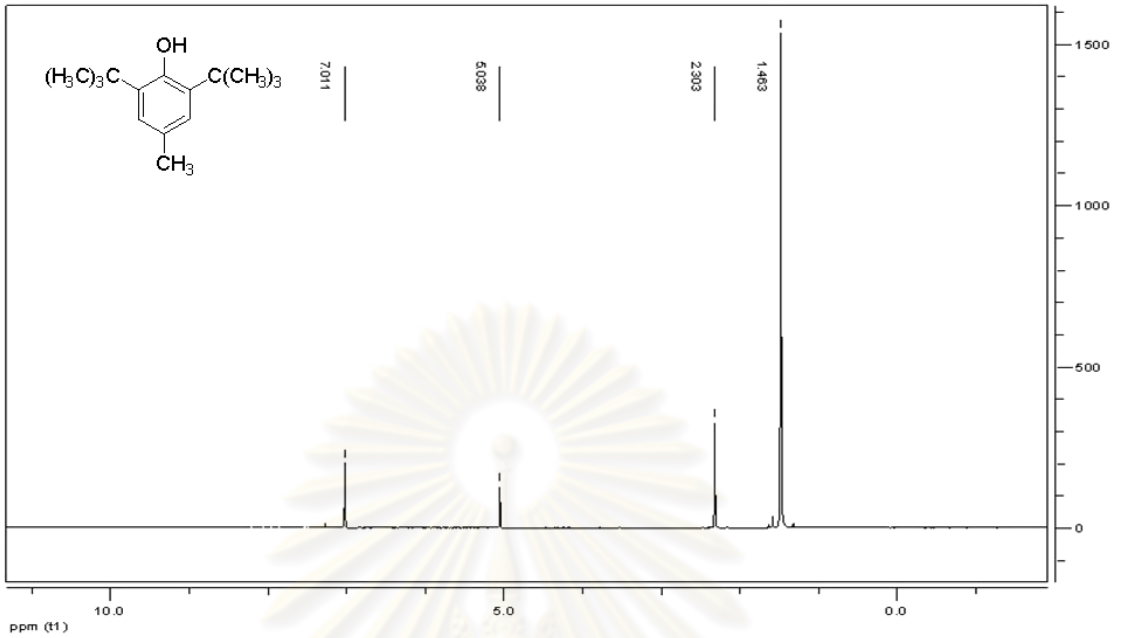
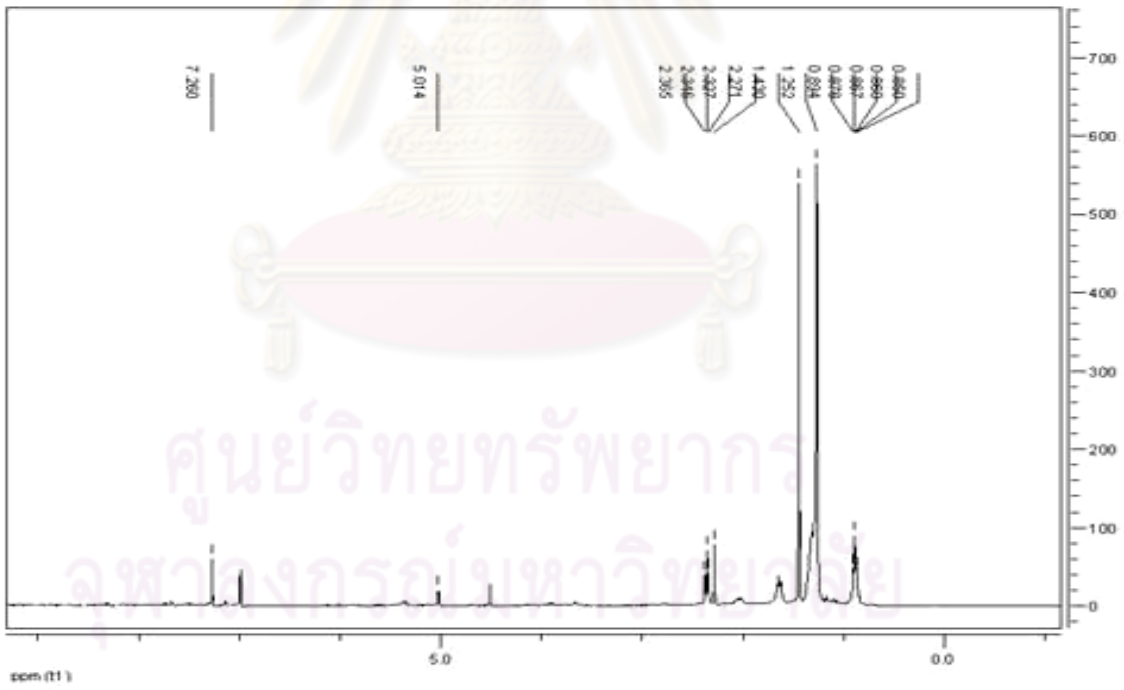


Figure 3.6 GC chromatogram of essential oil from stems of *Z. limonella* (Phrae provience)

57



(a)



(b)

Figure 3.7 The ^{1}H-NMR spectra of (a) synthetic BHT and (b) the crude essential oil from the stem

the photosynthetic organisms are exposed to a combination of light and high oxygen concentrations, which leads to the formation of free radicals and other strong oxidizing agents, suggesting that their cells contain protective antioxidative mechanisms and compounds (Sukenik *et al*., 1993; Matsukawa *et al*., 1997; Sinha *et al*., 2003; Park *et al*., 2005; Sigaud-Kutner *et al*., 2005). Thus, the production of BHT exhibits a light dependency and is considered to protect the cells from photooxidation. Babu and Wu, 2008 reported that four freshwater phytoplankton, including a green algae (*Bootryococcus braunii* Kütz.) and three cyanobacteria (*Cylindrospermopsis raciborskii* (Wollosz.) Seenaya et Sabba Raju, *Microcystis aeruginosa* (Kütz.) and *Oscillatoria* sp.) were capable of producing natural BHT. These data were confirmed by GC/MS analysis, the antioxidant compound observed in the algal species is identical to that of a synthetic BHT, as it thus not only had the same retention time, but also had similar mass spectral data.

3.2 Distribution of Essential Oil Components in Various Parts of *Z. limonella*

The GC chromatogram shows the distribution of essential oil components in *Z. limonella*. Monoterpenoids are easily noticeable from these GC chromatograms in all crude essential oils. Among these crude essential oils, α-phellandrene is predominant.

Among various parts of crude essential oils, the one from the stem seems to be the most unique. It contains very high content of BHT (86.62%). Unlike the other crude essential oils, that from the fruits and the leaves contained terpenoid as major components.

As previously reported, the highest yield of the essential oil could be achieved from the fruit. Based on the preliminary biological activity test, this fraction gave good activity against bacteria, yeast and phytopathogenic fungi. Thus, the essential oil of the fruits was selected to further investigate for its bioactive compounds against tested microorganisms.

3.3 Antiphytopathogenic Fungi Activities of the Crude Essential Oil from the Fruits of *Z. limonella* and its Major Components

The essential oil of the fruits was selected for further investigation. Five phytopathogenenic fungi (*F. oxysporum* DOAC 1258, *C. cassiicola* DOAC 1357, *E. turcicum* DOAC 0549, *F. oxysporum f.* sp. *vasinfectum*, *C. lunata*) were tested at 5,000 ppm dose compared with a commercial available fungicide, benomyl. The results are demonstrated as shown in **Table 3.3** and **Figure 3.8**.

The data from **Table 3.3** pointed out that the native essential oil showed the fully inhibition effect on the growth of almost fungal strains except for *F. oxysporum f.* sp. *vasinfectum*. Among three major components, terpinen-4-ol displayed the most potent activity. It should be noted that the percentage inhibition of terpinen-4-ol was close to the essential oil. Benomyl displayed the complete inhibition against *F. oxysporum f.* sp. *vasinfectum*, but not fully inhibition for *E. turcicum* DOAC 0549 and *C. lunata*. Thus, it should be possible to conclude that terpinen-4-ol, a major component of essential oil from the ripe fruits displayed antifungal activity against all phytopathogenic fungi species. Therefore, various concentrations of the essential oil and terpinen-4-ol: 500, 1,000, 2,500, 5,000 ppm on sterile PDA medium phytopathogenic fungi were experimented to examine IC_{50} values of each fungus. The percent growth inhibition and IC_{50} values (ppm) were calculated as shown in **Table 3.4**.

Table 3.4 exhibits that the IC_{50} values of the essential oil against almost phytopathogenic fungi were lower than those of terpinen-4-ol. These results indicated that almost fungal strains were sensitive to essential oil more than terpinen-4-ol, except for *Exserohilum turcicum* DOAC 0549. With *Curvularia lunata*, the sensitive strain, the essential oil and terpinen-4-ol displayed IC_{50} 218.9, 324.7 ppm, respectively compared with that against other tested fungi.

The *in vitro* antifungal activity of native essential oil and terpinen-4-ol were the next step to explore by a broth macrodilution method. Antifungal activity was expressed as minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC). The results are summarized in **Figure 3.9** and **Table 3.5**.

Table 3.3 Growth inhibition of fungal species in agar incorporation method by the essential oil, major components of *Z. limonella* and positive control (benomyl).

Fungi	Essential oil (5,000 ppm)		Terpinen-4ol (5,000 ppm)		sabinene (5,000 ppm)		limonene (5,000 ppm)		benomyl (100 ppm)		control	
	Growth[a] (mm)	Inh (%)	Growth[a] (mm)	Inh (%)	Growth[a] (mm)	Inh (%)	Growth[a] (mm)	Inh (%)	Growth[a] (mm)	Inh (%)	Growth[a] (mm)	Inh (%)
F. oxysporum	0 ± 0.0	100.0*	0 ± 0.0	100.0*	77.5 ± 2.1	13.9*	78.2 ± 0.9	13.1*	0 ± 0.0	100.0*	90.0 ± 0.0	0.0*
F. oxysporum f. sp. *vasinfectum*	9 ± 0.0	90.0*	16.2 ± 0.2	82.0*	73.0 ± 2.0	18.7*	65.2 ± 0.2	27.6*	0 ± 0.0	100.0*	90.0 ± 0.0	0.0*
C. cassiicola	0 ± 0.0	100.0*	0 ± 0.0	100.0*	73.2 ± 0.2	8.8*	74.0 ± 0.6	7.7*	0 ± 0.0	100.0*	80.0 ±0.0	0.0*
C. lunata	0 ± 0.0	100.0*	0 ± 0.0	100.0*	78.2 ± 0.4	13.1*	73.2 ± 1.2	7.7*	74.2 ± 0.8	77.8*	90.0 ± 0.0	0.0*
E. turcicum	0 ± 0.0	100.0*	0 ± 0.0	100.0*	0 ± 0.0	100.0*	39.3 ± 2.9	56.3*	20.0 ± 0.0	17.6*	90.0 ± 0.0	0.0*

Inh.; Inhibition *significant difference at $p<0.001$

[a]Values, an average ± standard deviation of 3 replicates, of the mean growth inhibition of fungi species

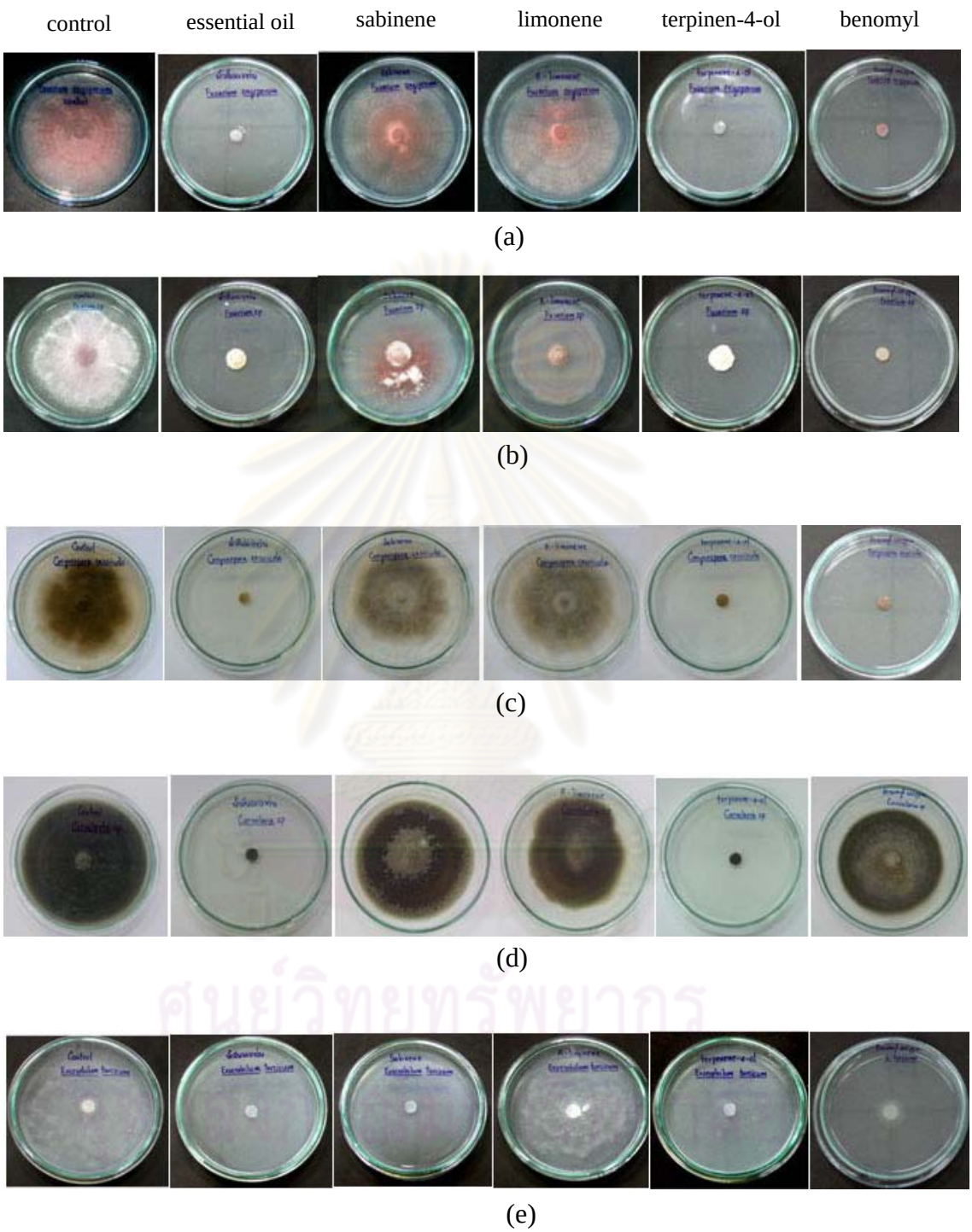


Figure 3.8 The mycelial growth inhibition zone of the essential oil from *Z. limonella*, sabinene, limonene, terpinen-4-ol and antifungal standard benomyl against (a) *F.oxysporum f.* sp. *vasinfectum* (b) *F.oxysporum* (c) *C. Lunata* (d) *C.cassiicola* and (e) *E. turcicum*

Table 3.4 IC_{50} value of the essential oil of *Z.limonella* and terpinen-4-ol

Fungi	Essential oil					terpinen-4-ol				
	% Inhibition[a]				IC_{50}	% inhibition[a]				IC_{50}
	5,000 ppm	2,500 ppm	1,000 ppm	500 ppm	(ppm)	5,000 ppm	2,500 ppm	1,000 ppm	500 ppm	(ppm)
F. oxysporum	100.0±0.0	75.8±2.0	36.1±1.4	15.1±1.3	1,489.4	90.0±0.4	68.3±1.7	36.1±1.3	10.2±2.7	1,671.4
F. oxysporum f. sp. *vasinfectum*	90.0±0.6	63.7±2.8	41.9±2.2	8.4±1.2	1,846.3	81.7±0.6	32.4±2.2	11.0±2.4	7.4±1.1	3,038.3
C. cassiicola	100.0±0.0	31.8±1.0	13.2±1.3	11.4±1.8	2,794.2	100.0±0.0	40.9±1.9	14.8±2.5	0.0±0.0	2,755.3
C. lunata	100.0±0.0	100.0±0.0	75.6±1.1	57.3±2.3	218.9	100.0±0.0	89.3±0.6	71.9±1.7	50.7±0.8	324.7
E. turcicum	100.0±0.0	85.2±1.7	68.9±2.3	38.3±0.8	671.8	100.0±0.0	87.8±1.2	41.5±1.8	28.2±3.4	1,159.6

[a]Values, an average ± standard deviation of 3 replicates

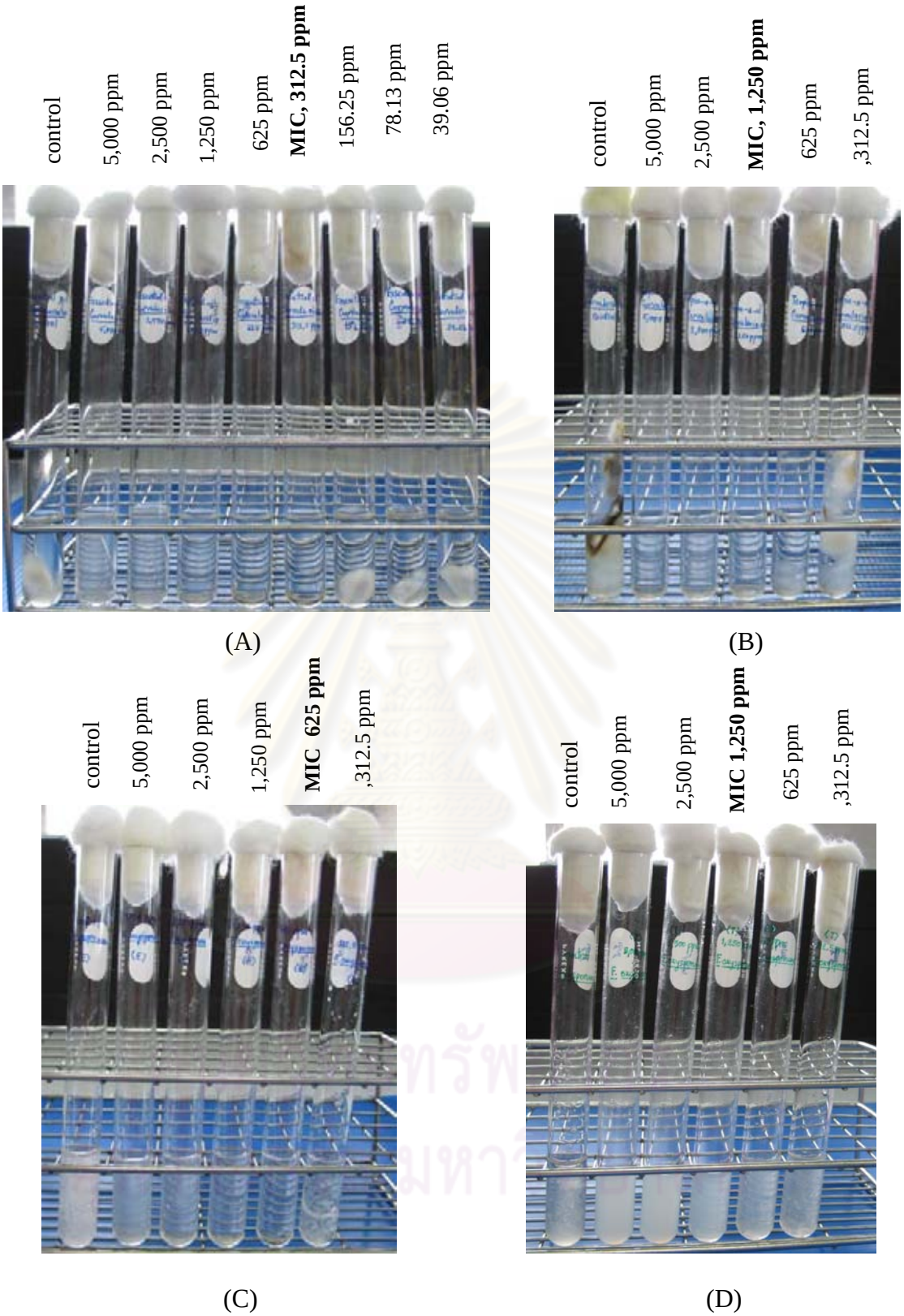


Figure 3.9 The MIC of essential oil and terpinen-4-ol against *C. lunata* (A and B) and *F. oxysporum* (C and D)

Table 3.5 MIC and MFC values of the essential oil of *Z. limonella* and terpinen-4-ol

Fungi	essential oil (ppm)		terpinen-4-ol (ppm)		benomyl (ppm)		mancozeb (ppm)	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
F. oxysporum	625	625	1,250	2,500	3.90	3.90	3.90	3.90
F. oxysporum f. sp. *vasinfectum*	1,250	2,500	2,500	5,000	3.90	3.90	3.90	3.90
C. cassiicola	2,500	5,000	1,250	2,500	0.39		31.25	62.50
C. lunata	312.5	312.5	1,250	1,250	125	250	7.80	7.80
E. turcicum	1,250	1,250	1,250	1,250	62.50	125	15.60	15.60

Table 3.5 indicates that the essential oil and terpinen-4-ol exhibited various levels of antifungal activity against phytopathogenic fungi. Their MIC and MFC varied from 312.5 to 2,500 ppm for the essential oil and from 1,250 to 5,000 ppm for terpinene-4-ol. Comparing the MIC values for the reference substances: benomyl and mancozeb and MIC values for the essential oil of fruit and terpinen-4-ol, it could be concluded that the reference substances demonstrated much lower MIC. However, the essential oil and terpinen-4-ol are usually less toxic than the synthetic compound: benomyl and mancozeb. The MIC and MFC values of the essential oil revealed more potent antifungal activity than terpinen-4-ol except for *C.cassiiicola* DOAC 1357. The maximum activity of essential oil was observed against *C. lunata* and *F. oxysporum* DOAC 1258 with MIC of 312.5 and 625 ppm and fungicidal activity with MFC of 312.5 and 625 ppm, respectively (**Figure 3.9**).

Tables 3.4 and **3.5** display that IC_{50} values of the essential oil and terpinen-4-ol could be related to MIC and MFC values. The inhibition and the killing of phytopathogenic fungi could be observed when using the mentioned essential oil even at low concentration. Additionally, from this study, terpinen-4-ol was disclosed to be a bioactive compound with antiphytopathogenic fungi activity. According to previous reports demonstrated that some oxygenated monoterpenes such as borneol, terpinen-4-ol, α-terpineol, menthol and essential oil containing oxygenated monoterpenes displayed board spectrum antifungal activity (Pattnaik *et al.*, 1997; Edris and Farrag, 2003; Kordali *et al.*, 2005; 2007a, b). Thus, the antifungal activity of the essential oil of fruit could be attributed to terpinen-4-ol.

3.4 Fractionation and Antibacterial and Anticandidal Activities of the Essential Oil, Fractions and its Major Components

The antibacterial and anticandidal activity screening of the essential oil of the fruits exhibited strong activity against Gram-positive bacteria (*B. subtilis* and *S. aureus*) and pathogenic yeast (*C. albicans*). Therefore, the bioactive compounds against bacteria and yeast of the fruits were further investigated.

3.4.1 Analysis of the Components in the Essential Oil of Fruit

The essential oil (100 mL) was first fractional distilled and the fractions were collected according to the temperature range. Three fractions (**I**, **II**, and **III**) were obtained at the temperature range of 55-90, 90-115 and above 115ºC, respectively. Percent yield of each fraction **I**, **II**, and **III** was shown in **Table 3.6**.

Fraction II gave the highest yield as 53.54% compared with the other two fractions. According to the GC analysis, fractions **I** and **II** composed of sabinene as the most abundant composition (53 and 41% for fractions **I** and **II**, respectively). Limonene was detected as the second most abundant component in these two fractions (13 and 18%, respectively). The analysis of fraction **III** revealed that the main composition was limonene (25%), terpinen-4-ol (19%) and sabinene (16%). It should be noted that the content of terpinen-4-ol was present in the highest amount in Fraction **III** (**Figure 3.10**).

Table 3.6 Fractions derived from the fractional distillation of the essential oil of *Z. limonella*

Fraction (Temperature range)	**Weight (g)**	**%Yield**
I (55-90ºC)	15.96	19.45
II (90-115ºC)	43.93	53.54
III (above 115ºC)	22.17	27.01

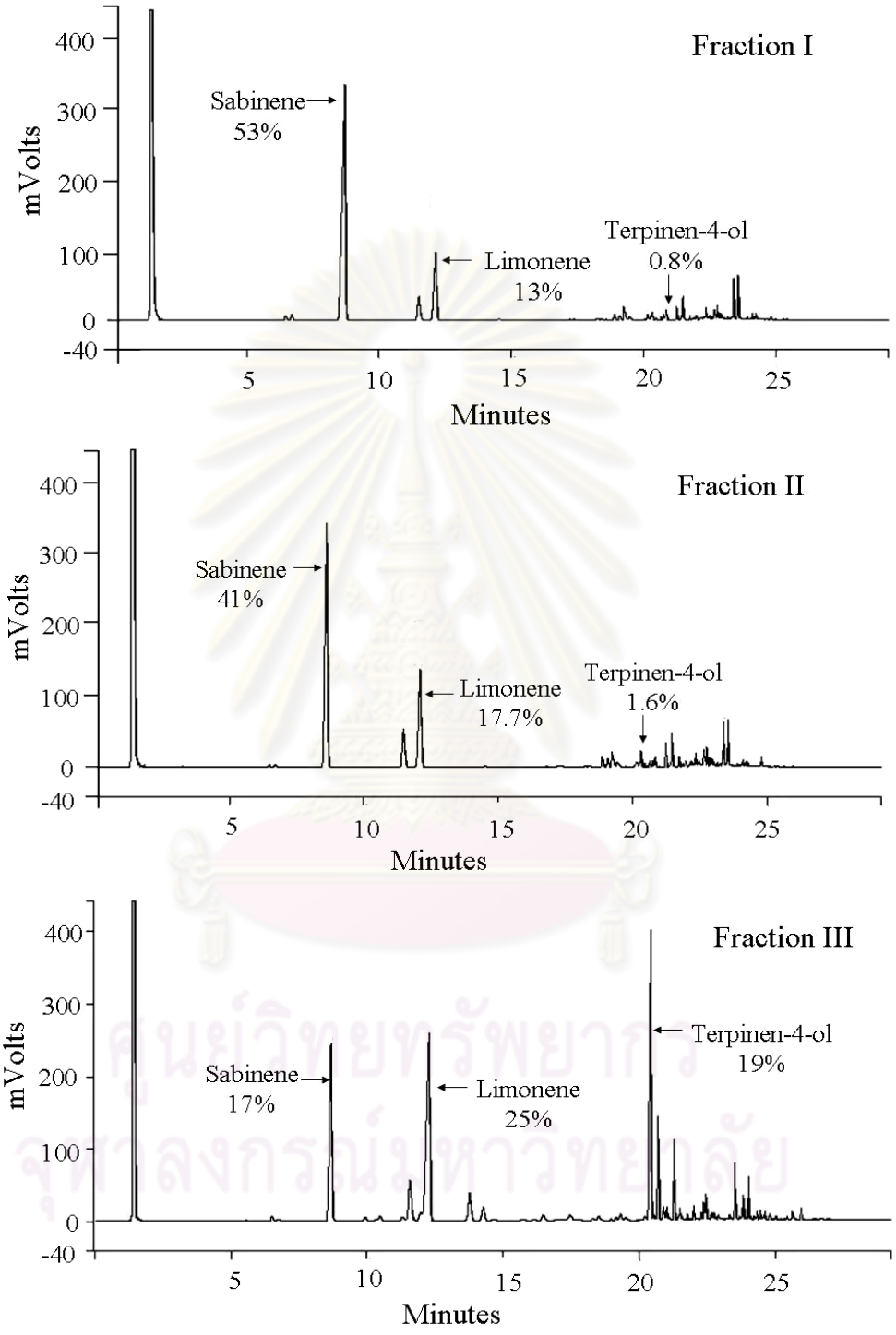


Figure 3.10 The GC-chromatogram of three fractions of essential oil from fruit of *Z. limonella*

A further examination on antibacterial and anticandidal activities of the native essential oil was conducted with three mentioned fractions and their major components such as sabinene, limonene and terpinen-4-ol at the final concentration of 5 mg per disc by paper disc diffusion method. Three antibiotics: Penicillin G, chloramphenicol and amphotericin B were used as a positive control. The inhibition zone was evaluated as shown in **Table 3.7**.

As the results presented in **Table 3.7**, fractions **I** and **II** exhibited significant susceptibility, with >10 mm inhibition diameter toward *S. aureus, B. subtilis, E. coli* and *C. albicans*. The effect on *P. aeruginosa* was weak (<10 mm inhibition diameter). The diameter of the inhibition zone of fractions **I** and **II** were close to the native essential oil, while fraction **III** had little activity against the test microorganisms. These data showed that the bioactive compounds should be present in fractions **I** and **II**. Considering the antibacterial activity result and the main composition of fractionated essential oil, sabinene was found as the most abundant component in fraction **I** (54%) and fraction **II** (41%) and should be responsible for the antibacterial activity. In addition, major constituents of the essential oil exhibited antibacterial and anticandidal activity property when tested separately. Sabinene was the most active, among the essential oil component tested. At the same concentration, terpinen-4-ol revealed moderately active in inhibiting the organisms, while limonene had slight activity. However, the result showed that the essential oil, fractions **I** and **II** displayed stronger antibacterial activity than the single compound. This is possible due to minor component that may be involved in some type of synergism with the other active compounds.

The essential oil, fractions **I** and **II**, and sabinene displayed good activity against Gram positive bacteria and only one of the Gram-negative bacteria namely *E. coli*. These observations suggest that this oil exhibit a broad spectrum antimicrobial agent. The essential oils containing phenolic compounds are also reported to possess antimicrobial activity (Dorman and Deans, 2000). The phenolic compounds have a broad spectrum of activity against a variety of both Gram-positive and Gram-negative bacteria (Kim *et al*., 1995; Helander *et al*., 1998; Dorman and Deans, 2000). The mechanism of action by which phenolic compounds aren thought to exert their

Table 3.7 Antibacterial and anticandidal activity of crude, fraction and major components of essential oil from fruit of *Z. limonella*

Microorganisms	Diameter of inhibition zone (mm)[a]									
	Crude oil	Fraction I	Fraction II	Fraction III	Sabinene	Limonene	Terpinen-4-ol	Pen G	Chloram	Ampho B
B. subtilis	18.9±0.5	21.3±0.5	20.0±0.4	14.3±0.3	14.8±0.5	NA	11.1±0.4	28.0±1.8	31.7±0.4	NA
S. aureus	29.5±0.7	24.4±0.5	24.1±0.6	13.7±0.4	18.0±0.5	NA	10.5±0.5	32.8±0.3	22.0±0.5	NA
E. coli	19.1±0.4	17.3±0.4	13.7±0.6	8.3±0.2	12.1±1.0	NA	6.7±0.4	NA	21.3±0.7	NA
P.aeruginosa	7.7±0.6	9.3±0.5	8.3±0.3	8.2±0.2	7.2±0.4	NA	NA	NA	15.2±0.5	NA
C. albicans	11.9±0.8	13.7±0.6	13.2±0.1	8.0±0.3	9.6±0.4	8.04±0.12	8.4±0.2	NA	NA	10.4±0.8

[a] Values, an average ± standard deviation of 3 replicates, of the mean inhibition zone of microorganisms

Pen G = Pencillin G, Chloram = Chloramphenicol, Ampho B = Amphotericin B, NA= Not active

antimicrobial activity is through the disturbance of the cytoplasmic membrane, disrupting the proton motive force, electron flow active transport, and coagulation of cell content (Burt, 2004). As known, the Gram-positive bacteria seem to be more susceptible to the inhibitory effects of the essential oil than the Gram-negative bacteria, a feature attributed to the difference in the respective cell wall structures. The greater susceptibility of Gram-positive bacteria may result from their sampler single layer cell wall structures, while the Gram-negative cell wall is a multi-layered structure and quite complex (Esen *et al*., 2007; Turker and Usta, 2008). However, it has been reported that the phenolic monoterpene, carvacrol is able to disintegrate the outer membrane of Gram-negative bacteria, releasing lipopolysaccharides (LPS) and increasing the permeability of the cytoplasmic membrane to adenosine triphosphate (ATP) (Dorman and Deans, 2000, Turker and Usta, 2008). This was shown to damage the cells irreversibly. It is hypothesized that the compounds containing in the essential oil with low molecular weight maybe enable to penetrate into the inner membrane of Gram-negative bacteria. Therefore, it could be concluded that the essential oil revealed its toxicity against Gram positive bacteria, some Gram-negative bacteria and yeast more than the isolated compounds. This data suggests that the complexity of the essential oil led to a complex activity that may act synergistically against the target organisms.

3.4.2 Antibacterial and Anticandidal Efficacy of Its Active Components of Essential Oil Combinations and Interaction with other Aroma Compounds

Essential oils contain numerous compounds, and one might wonder if their biological effects are the result of a synergism of all molecules or reflect only those of the main molecules present at the highest levels in volatile oils. In addition, the activity of essential oils can be expected to relate to the chemical configuration of the components, the proportions in which they are present and to interaction between them (Dorman and Dean, 2000). The complexity of the essential oil led to a various activities probably with synergistic and/or antagonistic phenomena. The present study was to investigate the effect of the bioactive compounds in this essential oil by examining as a single compound or the combination of two substances between

sabinene and terpinen-4-ol against microorganism tested. The results are displayed in **Table 3.8**.

The results show that the main inhibition zones of sabinene were more than those of the combined sabinene and terpinen-4-ol in certain different proportions. However, the inhibition zones observed from the mixture at higher concentrations of sabinene:terpinen-4-ol such as 9:1 and 3:1 (w/w) were more than those derived from the lower and equal proportions (1:1 and 1:3). This strongly indicated that none of these combinations displayed a synergistic activity against the bacteria and yeast used. In addition, this set of experiments also supported that sabinene played a major role for antimicrobial activity against microorganisms tested.

Many terpenes are known to be active against a wide variety of microorganisms including Gram-positive and Gram-negative bacteria and fungi. Several studies have also shown that monoterpenes exert microbial membrane damaging effects (Sikkama *et al*., 1995; Cox *et al*., 2000). The further study on the combination effect between sabinene and other aroma compounds, for instance α-terpinene, limonene, eugenol and safrole (**Figure 3.11**) has been investigated for antibacterial and anticandidal activity at the ratio of 9:1 (w/w). The results are shown in **Table 3.9**.

Table 3.9 shows that the overall diameter of the inhibition zone of Gram-positive bacteria was higher than that of Gram-negative bacteria. It was also found that using sabinene as a model, the Gram positive bacteria, *S. aureus* are more sensitive towards all combinations. The synergic effect could be observed in the combination of sabinene and α-terpinene against *S. aureus* and the mixture of sabinene and safrole against *B. subtilis*. These combinations created the inhibition zones against *S. aureus* and *B. subtilis* more than sabinene alone about 30 and 10%, respectively. In addition, sabinene and safrole diaplyed the inhibition zone about 11 mm against *E. coli*, while the combination of sabinene and aroma compounds showed antagonistic properties. The diameters of the inhibition zones of individual eugenol, sabinene and safrole exhibited the moderate activity against *C. albicans* (range zone

Table 3.8 Antibacterial and anticandidal activity of the mixture between sabinene and terpinen-4-ol by disc diffusion assay

Microorganisms	Diameter of inhibition zone (mm)[a]								
	Sa	T	Sa:T (9:1)	Sa:T (3:1)	Sa:T (1:1)	Sa:T (1:3)	Pen G	Chloram	Ampho B
B. subtilis	15.3±0.6	11.5±0.7	11.7±1.2	9.3±1.2	9.0±1.3	8.8±0.8	25.9±0.6	28.3±0.7	NA
S. aureus	18.8±0.4	10.6±0.8	18.5±0.6	12.8±0.4	11.3±1.5	8.0±0.4	30.1±0.6	22.4±1.0	NA
E. coli	12.1±1.0	6.7±0.4	8.2±0.3	7.1±0.5	7.2±0.4	7.1±0.4	NA	22.0±0.4	NA
P. aeruginosa	7.2±0.4	NA	7.0±0.26	8.0±0.5	7.0±0.5	7.0±0.4	NA	13.3±0.5	NA
C. albicans	9.0±0.4	8.1±0.3	8.3±0.5	8.5±0.5	8.0±0.2	8.8±0.3	NA	NA	9.9±0.4

[a] Values, an average ± standard deviation of 3 replicates, of the mean inhibition zone of microorganisms

Sa = Sabinene, T = Terpinen-4-ol, Pen G = Pencillin G, Chloram = Chloramphenicol, Ampho B = Amphotericin B

NA = Not active

Table 3.9 Antibacterial and anticandidal activity of the mixture between sabinene and α-terpinene, limonene, eugenol, safrole by disc diffusion assay

Microoraganisms	Diameter of inhibition zone (mm)[a]											
	Sa	α-T	Li	Eu	Saf	Sa:α-T (9:1)	Sa:Li (9:1)	Sa:Eu (9:1)	Sa:Saf (9:1)	Pen G	Chloram	Ampho B
B. subtilis	15.4±0.6	7.8±0.8	NA	7.1±0.6	20.8±1.7	13.4±1.0	15.3±1.2	11.3±1.2	17.1±2.1	23.4±1.2	25.6±0.2	NA
S. aureus	18.8±0.4	6.3±0.1	NA	10.0±0.6	10.4±0.8	24.2±0.3	18.7±0.6	17.2±0.8	17.8±0.8	30.5±1.0	20.7±0.4	NA
E. coli	10.8±0.3	NA	NA	8.7±0.3	11.0±0.5	NA	7.0±0.2	6.7±0.2	7.8±0.3	NA	20.3±0.9	NA
P.aeruginosa	7.2±0.4	NA	NA	NA	6.9±0.1	NA	NA	NA	7.0±0.3	NA	13.1±0.6	NA
C. albicans	9.0±0.4	6.5±0.1	7.6±0.2	13.2±0.8	10.0±0.4	8.6±0.5	8.0±0.2	9.4±0.4	9.2±0.1	NA	NA	9.6±0.3

[a] Values, an average ± standard deviation of 3 replicates, of the mean inhibition zone of microorganisms

Sa = Sabinene, α-T = α-Terpinene, Li = Limonene, Eu = Eugenol, Saf = Safrole, Pen G = Pencillin G, Chloram = Chloramphenicol, Ampho B = Amphotericin B, NA = Not active

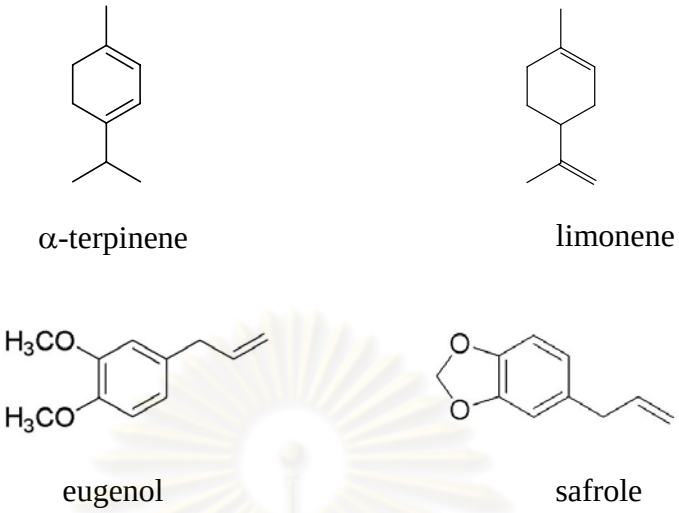


Figure 3.11 Structure of four monoterpenoid compounds.

size 10-13 mm). Nonetheless, when the combination of sabinene with other aromatic compounds, it did not display any benefit compared to sabinene alone.

Based on the above findings, the individual sabinene exhibited potential antimicrobial activity against the entire tested Gram-positive bacteria, one Gram-negative bacterium *E. coli* and yeast *C. albicans*. None of these combinations displayed a synergistic activity against the tested bacteria and yeast. However, the additive effect was found in the combination of sabinene and α-terpinene against *S. aureus* and the mixture of sabinene and safrole againt *B. subtilis*. *In vitro* data indicated that the essential oil and sabinene had antibacterial against *S. aureus*, *B. subtilis* and *E. coli*. They may exhibit the antibacterial efficacy against a clinical isolates, meticillin-resistant *S. aureus* (MRSA) and extended-spectrum β-lactamase-producing *E. coli* (ESBL). Therefore, for further investigation, the essential oil and sabinene were tested for quantitative antimicrobial evaluation against tested microorganisms and clinical isolates.

3.4.3 Minimum Inhibitory Concentrations (MIC), Minimum Bactericidal Concentration (MBC), and Minimum Fungicidal Concentration (MFC) of Essential Oil and Its Bioactive Compounds

According to the previous results, the essential oil from the fruit of *Z. limonella* and sabinene showed broad spectrum inhibitory against Gram-positive bacteria, some Gram-negative bacteria and yeast tested. However, the native essential oil was more effective than sabinene against almost tested microorganisms. Among Gram-positive and Gram-negative bacteria tested, *S. aureus* and *E. coli*, these two compounds gave large zone of inhibition. These results supported that the essential oil and sabinene may also be potential antibacterial against clinical isolates, MRSA and ESBL- producing *E. coli* strain. MRSA is a type of *S. aureus* that has become resistant to antibiotic, and can colonize the anterior nares, the area of broken skin, the groin and the axillae. MRSA is a major cause of nosocomial infection that has caused problems in hospital worldwide (Diekema *et al*., 2004). On the other hand, ESBL-producing *E. coli* are antibiotic resistant strains of *E. coli*. Normally, *E. coli* are very common bacterial that live harmlessly in the intestine. The ESBL-producing strains produce an enzyme called extend-spectrum β-lactamase (ESBL), which make them more resistant to cephalosporin antibiotics (Livermore, 1995). This makes in infection harder to treat. Both MRSA and ESBL have become a wide spread serious problem and several aspects of them a worrying. Therefore, in this study, the antibacterial and anticandidal effects of essential oil and sabinene were studied against tested microorganisms and clinical isolated of MRSA and ESBL using broth microdilution method to determine the MIC, MBC and MFC values as shown in **Table 3.10**.

Table 3.10 presents the MIC, MBC and MFC of the essential oil and sabinene tested against selected reference strains. The essential oil has variable levels of inhibition. The ranking of MIC values of tested microorganisms to the essential oil was as follows: *S. aureus* (MSSA) > MRSA = *E. coli* > ESBL = *B. subtilis* = *C. albicans*, while the MBC values of these sensitive strains have equivalent at 2 mg/mL. Only *P. aeruginosa* was resistant to the essential oil at the highest concentration tested. On the other hand, sabinene exhibited the same MIC and MBC values (4.22 and 16.88 mg/mL) against MSSA, MRSA, *E. coli* and ESBL, while sabinene

Table 3.10 Antibacterial and anticandidal activities of the essential oil from *Z. limonella* and Sabinene assayed by MIC, MBC and MFC values.

Microorganisms	Crude essential oil		Sabinene		Chloramphenicol		Amphotericin B	
	MIC (mg/mL)	MBC/MFC (mg/mL)	MIC (mg/mL)	MBC/MFC (mg/mL)	MIC (mg/mL)	MBC/MFC (mg/mL)	MIC (mg/mL)	MFC (mg/mL)
S. aureus ATCC 25923 (MSSA)	0.25	2.0	4.22	16.88	0.00625	0.0125	NA	NA
S. aureus ATCC 43300 (MRSA)	0.50	2.0	4.22	16.88	0.01250	0.0500	NA	NA
E. coli ATCC 25922	0.50	2.0	4.22	16.88	0.00313	0.0250	NA	NA
E. coli ESBL	1.0	2.0	4.22	16.88	0.00313	0.0500	NA	NA
P. aeruginosa ATCC 27853	NA	NA	NA	NA	0.10000	0.2000	NA	NA
P. aeruginosa (multidrug resistance)	NA	NA	NA	NA	0.10000	0.2000	NA	NA
B. subtilis ATCC 6633	1.0	2.0	8.45	40.0	0.00313	0.0500	NA	NA
C. albicans ATCC 10231	1.0	2.0	NA	NA	NA	NA	0.00250	0.0050

NA = not active

appeared to possess some activity against *B.subtilis* with a MIC of 8.45 mg/mL and MBC 40.0 mg/mL. In addition, the single sabinene did not show any activity against *P. aeruginosa* and *C. albicans* at the highest concentration tested. The results indicate that the essential oil showed stronger activity than individual sabinene. This also confirmed that the antimicrobial properties of the essential oil are mainly related to chemical components and structure of essential oil constituents. The modes of action of chemical compostions of the essential oil are different depending on microorganisms (Villar *et al*., 1986). In addition, when comparing the antimicrobial activity of the essential oil and sabinene with different methods used, the microdilution method showed better activity than disk diffusion method. This was particularly in the case of *E. coli* which was more sensitive in the microdilution method than disk diffusion method. It was noticed that the low water solubility of the essential oil and their components limited their diffusion through the agar medium. The miscibility of the essential oil may cause problems when assaying their activity. For the reasons, the disk diffusion method was appropriate to screen antimicrobial activity of the test substances. Therefore, broth method used in the microtitre trays, had the advantage of lower workload for large number of replicates and used small volumes of the test substances and growth medium. This method dilution of oil was better, there was no agar in the medium, and there was better diffusion through the liquid medium (Marina, 2006).

According to previous results, it could be concluded that the essential oil and individual sabinene showed broad spectrum inhibitory against *S. aureus* (MSSA and MRSA), *B. subtilis*, *E. coli*, ESBL-producing *E. coli* and *C. albicans*, whereas against *P. aeruginosa* it appeared to be completely inactive. MSSA, MRSA, *E. coli* and ESBL-producing *E.coli* strains were even more susceptible to essential oil and sabinene than other tested microorganisms. Further study was to examine the antibacterial potency of the essential oil and sabinene against MSSA, MRSA, *E. coli* and ESBL-producing *E. coli* by time-kill curve studies.

3.4.4 Time-Kill Curve Studies of the Essential Oil of Fruit of *Z. limonella* and Bioactive Compounds

In the previous study, the MICs and MBCs values were used as prediction tools for antimicrobial action of essential oil and individual sabinene. There are limitations to the use of data since it does not consider time-related antimicrobial effects, such as killing rate. The bactericidal potencies of the essential oil and sabinene in terms of the kinetics of bacterial death was determined by the number of remaining viable bacteria at varying time point after exposed to the tested substances. In this study, the kinetics of killing of essential oil and sabinene against two sensitive strains of bacteria (*S. aureus* ATCC 25923 (MSSA) and *E. coli* ATCC 25922) and two clinical strains (*S. aureus* ATCC 43300 (MRSA) and *E. coli* ESBL). The results of time-kill curve studies are presented in **Figure 3.12**.

The results show that the essential oil and sabinene were more active against MSSA and *E. coli* than against clinical isolate MRSA and ESBL-producing *E. coli*. At 4×MBC, essential oil (8 mg/mL) and sabinene (67.6 mg/mL) had a lethal effect against all tested bacteria. In case of *E. coli* and MSSA killed with in 3 min, whereas in the case of MRSA and ESBL total kill was attained with in 1 hour and 15 min, respectively. At 2×MBC, both essential oil (4 mg/mL) and sabinene (33.8 mg/mL) exhibited bactericidal effect on MSSA and *E. coli* with in 10 min, while there was complete eradication against MRSA and ESBL-producing *E. coli* with 90 min. However, at 1×MBC, MSSA and *E. coli* cells decrease to undectable levels within 15 and 30 min exposure to essential oil (2 mg/mL). In addition, at 1×MBC of essential oil exhibited bactericidal effect on MRSA and ESBL-producing *E. coli* for 3 hours, while for sabinene was able to kill the two organisms within 2 hours.

The doses at all MBC levels of the essential oil and sabinene were bactericidal to all the tested strains. The bactericidal activities of tested bacteria is also dose and time dependent. When the MBC values of essential oil and sabinene increased (2×MBC, 4×MBC), its antibacterial activity increased as shown by the shorter contact time required to inactive the tested bacteria. The rate of killing for essential oil and sabinene against MSSA and *E. coli* more rapid than MRSA and ESBL-producing *E. coli*. Comparison of the time killing of essential oil and sabinene showed closeness with the bactericidal activities of the same MBC values. However, the MBC values of

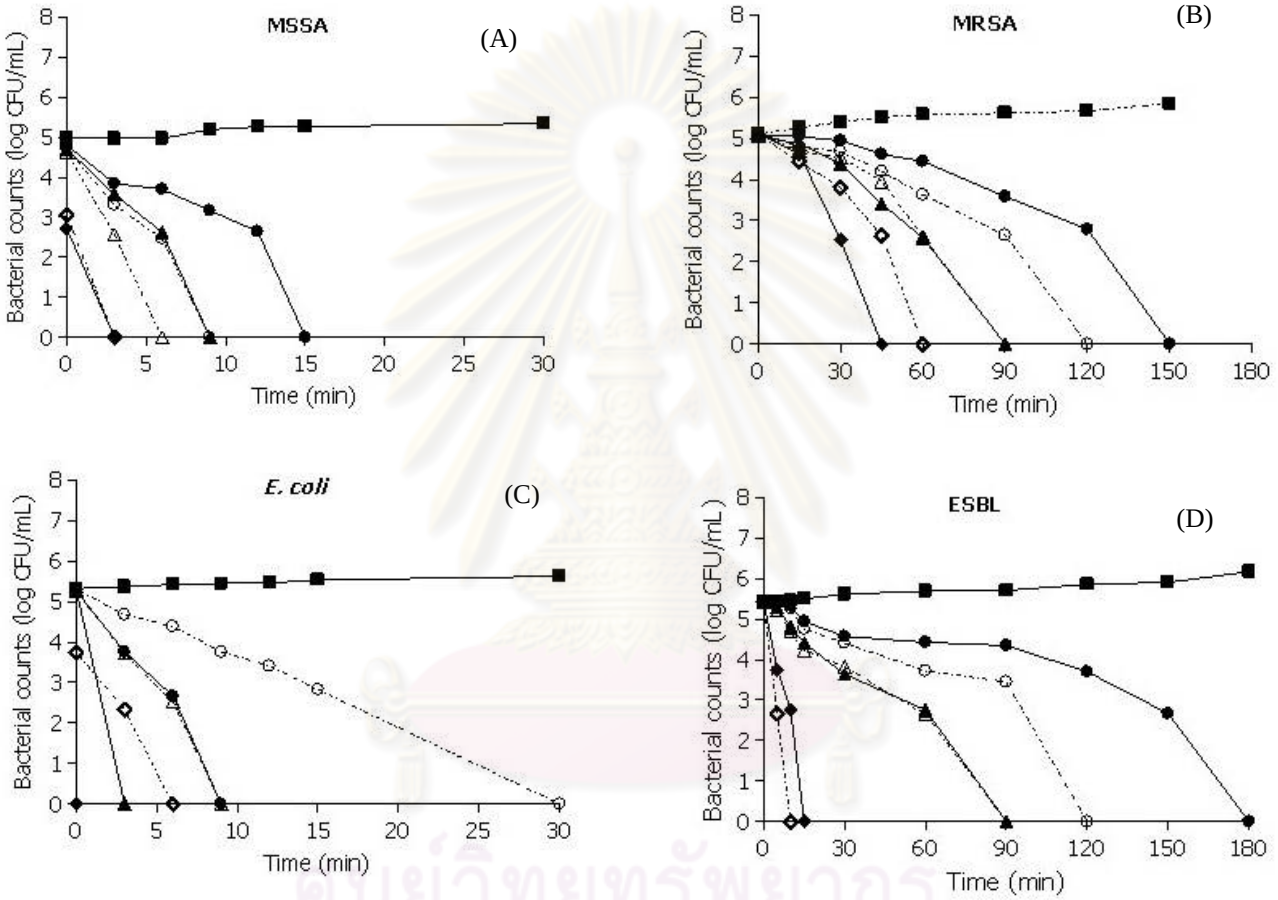


Figure 3.12 Time-kill curves of essential oil and sabinene against MSSA (A), MRSA (B)**,** *E. coli* (C) and ESBL (D). The concentrations used for the test: ●, MBC; ▲,2×MBC; and ◆, 4×MBC (= 2, 4 and 8 mg/mL for essential oil), ○ MBC; △, 2×MBC; and ◇,4×MBC (= 16.9, 33.8 and 67.6 mg/mL for sabinene) and ■, the control tube did not contain essential oil

the essential oil were eight times greater than that of the same MBC of sabinene. This data indicated that the essential oil was more active than sabinene against all the bacteria tested. On the other hand, time-kill curve showed that MSSA were more susceptible to sabinene than *E. coli*. Therefore, the Gram positive-bacteria were sensitive to sabinene than Gram-negative bacteria. These results indicated that Gram-positive bacteria more susceptible to sabinene compared with Gram-negative bacteria. Thus, the sabinene might be able to penetrate through cellular membranes or interact with intracellular components of Gram-positive bacteria more rapid than Gram-negative bacteria. It is notable, in particular, that the essential oil exhibited high efficacy against clinical isolate strains, MRSA and ESBL-producing *E. coli* more time-killing rate than sabinene. This finding suggested that the antimicrobial activity of essential oils be due to the presence of synergy between the major components and other constituents of the essential oil leading to various degree of antimicrobial activity. These results indicate possibility that the hydrophobicity of the essential oils is the main properties responsible for the distribution of bacterial structures increasing their permeability due to their inability to partition the lipids from bacterial cell membrane (Poolman *et al*., 1987). This causes expansion of the membrane, increase fluidity or disordering of the membrane structure and inhibition of membrane-embedded enzyme (Sikkema *et al*., 1995).

In conclusion, these findings suggest that the disc diffusion methods be suitable for screening purposes only and the qualitative data should sought using a broth dilution method and time-kill assay. However, limitation of the measurement of MICs and MBCs is the inability to the method to determine how quickly an antimicrobial agent acts on the microorganisms. Whereas, time-kill assays were expressed as the rate of killing by a fixed concentration of an antimicrobial agent. Therefore, time-kill assay allows determination of the speed and efficacy of bactericidal activity of substance at each time interval. In this study, the essential oil and sabinene had a broad spectrum antibacterial and anticandidal activity against Gram-positive, some Gram-negative bacteria and yeast. The rate of killing of the essential oil and sabinene appeared to be time and concentration dependent. At lower concentration of essential oil, more bacteria were completely killed. However, the bioactive compound from this essential oil, sabinene was responsible for antibacterial

activity. In addition, the essential oil shows potential to be considered as anti-MRSA and anti-ESBL producing *E. coli* agents commonly found in hospitals. Therefore, these data suggest that the essential oil could be blended into soaps, handwashes and shampoos, which could be used in hospital hygiene regimens to prevent the spread of such infections.

3.5 Experimental Section

3.5.1 Preparation of Essential Oil Fraction

Fractional distillation of the native essential oil (100 g) was carried out in batch mode under vacuum using a custom built, proprietary column. Many fractions were obtained during the fractional distillation process; samples of similar composition were pooled based on data derived from gas chromatographic analysis.

3.5.2 Chemical Composition of the Essential Oil

Analytical GC-MS of a distillated essential oil was performed on a GC-MS Thermo Electron. The 70%Cyanopropyl Polysilphenylene-siloxane TR-FAME column (60 m x 0.25 mm ID x 0.5 μm film thickness) was directly coupled to the mass spectrometer. The carrier gas was H_2 (1.1 mL/min) and the program used was 2 min isothermal at 50^{o}C, followed by 50-180^{o}C for 15 min; the injection port temperature was 250^{o}C. Ionization of the sample compounds was performed in E.I. mode (70eV). The constituents were identified by comparison of their mass spectra with those in the computer library and with authentic compounds. The identifications were confirmed by comparison of their retention time indices with those of authentic compounds or with literature data.

Fractionated essential oils were analyzed by GC-FID using CP-sil5 (30 m x 0.25 mm) with an oven temperature programmed as follows: from 50^{o}C (isothermal for 10 min) to 65^{o}C at 15 min^{-1}, and from 65^{o}C to 160^{o}C at 15 min^{-1}. Injector and detector temperatures were 220 and 250^{o}C respectively. Identification of oil components were compared their retention indices with those of authentic compounds.

3.5.3 Antifungal Assay

The native essential oil, fractionated essential oils and tested compounds were examined for antifungal activity against five phytopathogenic fungi by agar incorporation assay as described in the experimental section of Chapter 2 (Toudon, 1990 and Dwivedy and Dubey, 1993) and were determined MICs and MFCs by macrodilution method. Three independent experiments were performed and each experiment was run in triplicate.

A broth macrodilution method is used to determine the minimum inhibitory concentration (MIC) modified by the method described by Schmourlo *et al.*, 2005. Antifungal tests were performed in Potato dextrose broth (PDB). Bioactive compound(s) and essential oil were dissolved 1%tween 80 and then, 500 μL from each of serial two-fold dilution of the samples was added to 4.5 ml of culture broth containing 100 μL of 10^6 spore/mL for fungal strains. The PDB tube was treated with 1%tween 80 without essential oil and bioactive compound were used as a negative control, whereas benomyl was used as a positive control. The tubes were incubated on incubator shaker at room temperature about 5-7 days. The highest dilution (lowest concentration) showing no visible growth, is regarded as MIC. Cells from the tubes showing no growth were subcultured on PDB to determine if the inhibition was reversible of permanent. MFC was determined as the highest dilution (lowest concentration) at which no growth occurred in test tube.

3.5.4 Antibacterial and Anticandidal Assay

The native essential oil, fractionaed essential oils and test compounds were examined for antibacterial and anticandidal activity against four bacteria and one pathogenic yeast by disc diffusion method as describe in experimental section of Chapter 2 (CLSI, 2005) and were determined MICs, MBCs, and MFCs by a microdilution method.

MIC, MBC and MFC determination for essential oil and bioactive components were performed by the broth microdilution method, according to the Clinical and Laboratory Standards Institued protocol (CLSI, 2007). Antibacterial activity was tested in Mueller Hinton broth (MHB), whereas yeast was performed in Sabouraud dextrose broth (SDB). The essential oil and bioactive compounds were individually

dissolved in 1%tween 80 to obtain the highest concentrations. Serial two fold dilutions of essential oil or bioactive compounds were prepared in a 96-well microtiter plate at 100 µL final volume per well. Each well contained 20 µL of tested compound, 20 µL of cell suspension of the tested microorganism at 10^6 CFU/mL and 60 µL of culture broth. The microtiter plates then were incubated at 37ºC for 24 h, except for *C. albicans* which was incubated at 25ºC for 24 h. After incubation, the wells were examined for growth of microorganisms and the MICs were determined. The MIC was defined as the lowest concentration of essential oil or the active compound at which the microorganism did not demonstrate visible growth. The MBC and MFC were confirmed by re-inoculating onto fresh culture agar plate with 10 µL of each supernatant fluid from the MIC tested the wells with no microbial growth. The MBC was defined as the lowest concentration of the essential oil or the pure compound at which incubated microorganisms were completely killed. Chloramphenicol and amphotericin B were used as positive controls in parallel experiments. Three independent experiments were performed and each experiment was repeated three times.

3.5.5 Time-kill Studies

The bactericidal activity was determined according to the CLSI guideline (CLSI, 1999). Time-kill kinetics were determined by the number of remaining viable bacteria at varying time points after exposed to the essential oil and sabinene. The essential oil or sabinene was pre-mixed with 1%tween 80 and added into tubes containing MHB at various concentrations (1×, 2× and 4×MBC) and to achieve an initial inoculum of approximate 10^6 CFU/mL. Tubes were contained MHB, initial inoculum and 1%tween 80 were served as controls. All tubes were incubated at 37ºC. The samples were taken at 0, 15, 30, 45, 60, 90, 120, 150 and 180 min. When rapid killing occurred, samples were also taken at 3, 6, 9, 12, 15, and, 30 min. The survival bacterial counts were performed by making appropriate dilutions in 0.85%NaCl, then 100 µL of each dilution was spread onto the surface of MHA and incubated at 37ºC for 24 h, then the number of bacterial colonies in CFU/mL was counted. Time-kill curves were constructed by plotting $\log_{10}$CFU/mL against time (min). Experiments were carried out in triplicates.

CHAPTER IV

CHEMICAL COMPOSITIONS AND BIOACTIVE COMPOUNDS FROM THE STEMS OF *Z. limonella* Alston.

Based on biological screening tests, the CH_2Cl_2 extract of the stems exhibited good antimicrobial activity and the highest level of antioxidant activity. Therefore, it is rationalized to search for chemical composition and biological active constituents from this particular fraction.

4.1 Fractionation and Antimicrobial Activity Determination of the CH_2Cl_2 Extract from the Stems of *Z. limonella*

4.1.1 Fractionation

The dried stems of *Z. limonella* (12 kg) were extracted with CH_2Cl_2 by soxhlet apparatus. The CH_2Cl_2 crude extract, 100 g as a black-brown sticky was subjected to silica gel column using gradient solvent starting from hexane and increased polarity by mixing with EtOAc and MeOH. Each fraction was collected and combined according to the TLC results to obtain five fractions, Fr **1** - Fr **5**. The results of fractionation are presented in **Table 4.1**.

Table 4.1 The results of fractionation the CH_2Cl_2 extract from the stems of *Z. limonella* by silica gel column

Fraction No.	Solvent system	Remarks	Weight (g)
Fr **1**	100%hexane	white wax	13.35
Fr **2**	20-30%EtOAc in hexane	yellow oil	12.85
Fr **3**	30-50%EtOAc in hexane	orange oil	4.97
Fr **4**	50-100%EtOAc in hexane	brown syrup	30.21
Fr **5**	5%MeOH in EtOAc	dark brown syrup	34.41

Five fractions were obtained by silica gel column of the CH_2Cl_2 extract of stems. Fractions **4** and **5** gave the highest yield as 30.21 and 34.41 g, respectively. Each fraction was subjected to antimicrobial assay.

4.1.2 Antifungal, Antibacterial, and Anticandidal Activity Determination

Five fractions from the CH_2Cl_2 crude extract were tested at 5,000 ppm dose against five phytopathogenic fungi (*F. oxysporum, C. cassiicola, E. turcicum, F. oxysporum f.* sp. *vasinfectum* and *C. lunata*) by agar incorporation method. The percent growth inhibition is displayed in **Table 4.2**.

The data from **Table 4.2** indicated that Frs **2**, **3** and **5** displayed moderate antifungal activities, while Fr **1** had little activity against phytopathogenic fungi. In addition, Fr **4** displayed more potent activity than the other fractions and percentage inhibition was nearly to that of the original CH_2Cl_2 crude extract. The growth inhibition of tested fungi were observed in descending order as follows: *C. lunata* = *E. turcicum*>*F. oxysporum f. sp. vasinfectum*>*C. cassiicola*>*F. oxysporum*. These data pointed out that *C. lunata* and *E. turcicum* were the most susceptible while *F. oxysporum* was the least susceptible to the stem crude extract and Fr **4**. The CH_2Cl_2 crude extract and Fr **4** showed completely inhibition against *C. lunata* and *E. turcicum* at 5,000 ppm dose.

In addition, the antibacterial and anticandidal activities of the CH_2Cl_2 crude extract and five fractions were investigated towards *B. subtilis, S. aureus, E. coli, P. aeruginosa*, and *C. albicans*, at the final concentration of 5 mg per disc by paper disc diffusion method. The antibacterial spectra showing zone of inhibition in mm are shown in **Table 4.3**.

Table 4.3 shows that the CH_2Cl_2 extract and Fr **4** were more effective against tested organisms than other fractions. Frs **2**, **3** and **5** possessed moderate antibacterial against Gram-positive bacteria including *B. subtilis* and *S. aureus* and did not show inhibitory activity against Gram-negative bacteria and yeast *C. albicans*. In contrast, Fr **1** did not show any inhibitory activity against all bacteria and yeast tested. The CH_2Cl_2 crude extract and Fr **4** showed similar antibacterial activities against Gram-positive bacteria and yeast tested. Regarding, Fr **4** created larger inhibition zones against Gram-positive bacteria (*B. subtilis* (12.2 mm),), *S. aureus* (13.5 mm), and *C.*

Table 4.2 Growth inhibition of fungal species in agar incorporation method of the CH_2Cl_2 extract and each fraction from the CH_2Cl_2 extract

Extract/ Fraction No.	*F. oxysporum f.* sp. *vasinfectum*		*F. oxyspolum*		*C. lunata*		*C. cassiicola*		*E. turcicum*	
	Growth[a] (mm)	Inh (%)	Growth[a] (mm)	Inh (%)	Growth[a] (mm)	Inh (%)	Growth[a] (mm)	Inh (%)	Growth[a] (mm)	Inh (%)
CH_2Cl_2 crude extract of stems	10.7 ± 1.3	88.1 ± 1.4	29.0 ± 1.0	65.3 ± 1.2	0.0 ± 0.0	100.0 ± 0.0	24.7 ± 3.5	69.2 ± 4.4	0.0 ± 0.0	100.0 ± 0.0
Fr **1**	90.0 ± 0.0	0.0 ± 0.0	90.0 ± 0.0	0.0 ± 0.0	61.5 ± 1.8	31.7 ± 2.0	78.7 ± 1.2	17.0 ± 1.4	77.0 ± 0.0	14.4 ± 1.1
Fr **2**	72.0 ± 6.6	20.0 ± 7.3	90.0 ± 0.0	0.0 ± 0.0	40.0 ± 1.0	55.6 ± 1.1	76.3 ± 1.5	16.5 ± 1.9	63.2 ± 0.8	29.8 ± 0.8
Fr **3**	51.3 ± 1.5	43.0 ± 1.7	90.0 ± 0.0	0.0 ± 0.0	44.0 ± 1.0	51.1 ± 1.1	54.7 ± 2.5	31.7 ± 3.1	45.7 ± 1.5	49.3 ± 1.7
Fr **4**	30.3 ± 2.1	66.3 ± 2.3	30.0 ± 3.0	63.7 ± 3.8	0.0 ± 0.0	100.0 ± 0.0	18.7 ± 0.8	76.7 ± 1.0	0.0 ± 0.0	100.0 ± 0.0
Fr **5**	43.3 ± 2.1	51.9 ± 2.3	78.0 ± 2.0	7.0 ± 1.8	22.0 ± 1.0	75.6 ± 1.1	52.7 ± 1.5	34.2 ± 1.9	17.8 ± 1.0	80.2 ± 1.1
Benomyl (100 ppm)	0.0 ± 0.0	100.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	16.8 ± 1.4	69.1 ± 1.3	0.0 ± 0.0	100.0 ± 0.0	24.1 ± 1.4	73.2. ± 1.5

Inh.; Inhibition , [a] Values, an average ± standard deviation of 3 replicates, of the mean growth inhibition of fungi species

Table 4.3 Antibacterial and anticandidal activities of the CH_2Cl_2 extract and each fraction from the CH_2Cl_2 extract

Extract / Fraction No.	**Diameter of inhibition zone[a] (mm)**				
	B. subtilis	***S. aureus***	***E. coli***	***P. aeruginosa***	***C. albicans***
CH_2Cl_2 crude extract of stems	10.8 ± 0.3	10.7 ± 0.3	7.9 ± 0.2	7.2 ± 0.3	8.7 ± 0.3
Fr **1**	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Fr **2**	8.5 ± 0.5	9.2 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Fr **3**	8.2 ± 0.3	10.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Fr **4**	12.2 ± 1.0	13.5 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	10.5 ± 0.5
Fr **5**	10.3 ± 0.8	9.8 ± 1.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Pen G (10 unit/disc)	26.9 ± 0.6	31.1 ± 1.5	0.0 ± 0.0	0.0 ± 0.0	NT
Chloram (30 µg/disc)	31.2 ± 1.5	24.3 ± 0.8	19.4 ± 0.8	16.6 ± 0.9	NT
Ampho B (25 µg/disc)	NA	NA	NA	NA	10.7 ± 0.7

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

NA = Not active; NT = Not test

albicans (10.5 mm)) than that of the CH_2Cl_2 crude extract. However, the CH_2Cl_2 crude extract had a little active against Gram-negative bacteria (*E. coli* and *P. aeruginosa*), while Fr **4** did not show inhibitory activity against these bacteria. Thus, the order of the antibacterial activity of Fr **4** and the CH_2Cl_2 crude extract was *S. aureus* > *B. subtilis* > *C. albicans* > *E. coli* = *P. aeruginosa*, respectively.

These results indicated that Fr **4** possessed significant *in vitro* antimicrobial properties against Gram-positive bacteria (*B. subtilis* and *S. aureus*), yeast (*C. albicans*), and all phytopathogenic fungi tested. It could be concluded that Fr **4** may contain bioactive components as natural antimicrobial agents. However, all fractions of crude extract were further purified to search for chemical compositions and antimicrobial compounds since there was no report on the constituents of the stems of this plant.

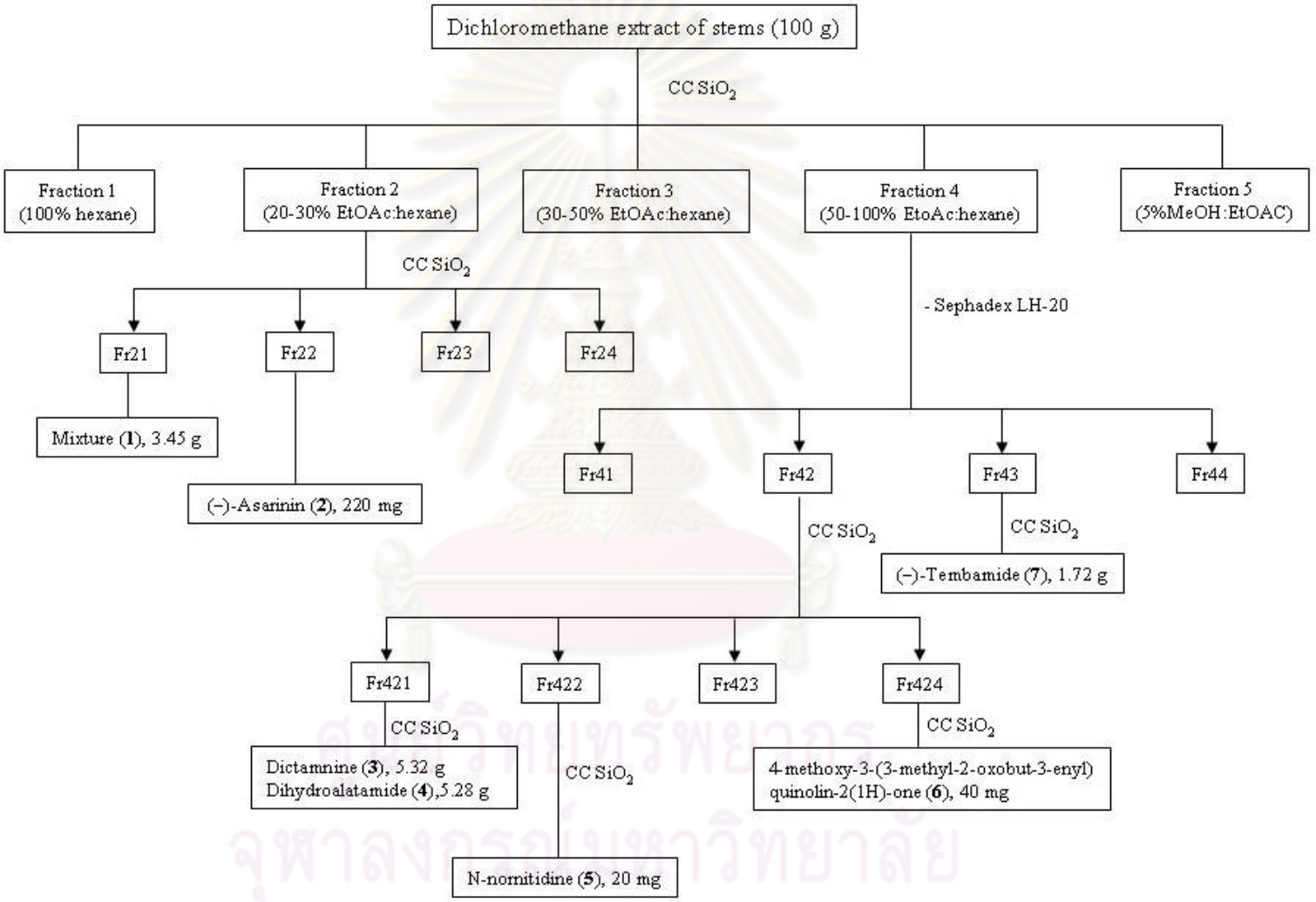
4.2 Separation and Structure Elucidation of Isolated Compounds

Each fraction, Frs **1**-**5**, was re-subjected to silica gel column using mixtures of hexane-EtOAc and EtOAc-MeOH of increasing polarity as eluents. Six compounds and a mixture of steroids were obtained after further separation by combination of silica gel chromatograph and Sephadex LH-20. Mixture **1** (3.45 g) were isolated from Fr **2**, compound **2** (0.22 g) was obtained from Fr **3**, compounds **3** (4.61 g), **4** (4.50 g), **5** (0.02 g), **6** (0.04 g) and **7** (1.72 g) were obtained from Fr 4. Among these isolated compounds, compound **6** has been characterized as a new naturally occurring compound. Compounds **3** and **4** were determined as a major compound of this extract. The known compounds **1-6** were identified in each case by comparisons of their ^{1}H, ^{13}C NMR, TLC, and/or mixed melting points with the corresponding authentic samples or literature data. The isolation procedures are summarized in **Scheme 4.1**.

4.2.1 Mixture 1

Mixture **1** was isolated as white paste solid (3.45 g) from silica gel column. Its melting point was 132-141ºC and R_f value was 0.55 (50% EtOAc/hexane). It could not be detected on TLC under UV, but could be detected with 10% H_2SO_4 in MeOH.

The GC chromatogram showed three peaks at Rt 15.98, 16.23, and 17.10 min, respectively. These peaks were compared with a standard mixture of phytosterols.



Sheme 4.1 The isolation procedure of dichloromethane extract of stems

The analysis of these peaks revealed that β-sitosterol (59%) was identified as a major component, followed by campesterol (32%), and stigmasterol (9%). The structures are shown in **Figure 4.1**. Generally, the steroid in nature was present as a mixture. Therefore, this mixture was analyzed by GC as presented in **Figure 4.2**.

According to previous studies, steroids have been detected in various parts of some *Zanthoxylum* species, such as four steroids: β-sitosterol, stigmasterol, campesterol and β-sitosteryl-3-*O*-β-D-glucoside were isolated from the leaves of *Z. schinifolium* and two steroids: β-sitosterol and stigmasterol were isolated from *Z. scandens* and *Z. integrifoliolum* (Chen *et al*., 2002; Cheng *et al*., 2007; Cheng *et al*., 2008).

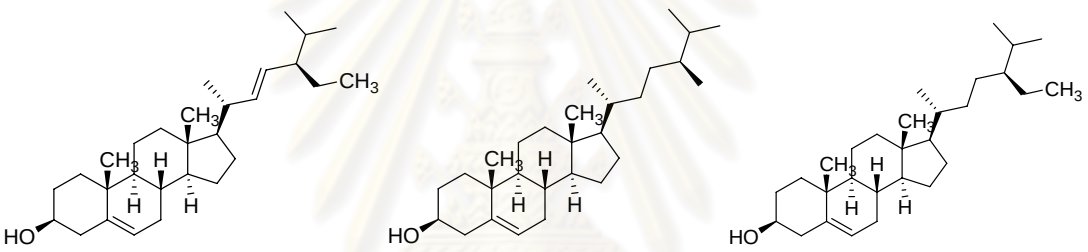


Figure 4.1 The structure of Mixture **1**

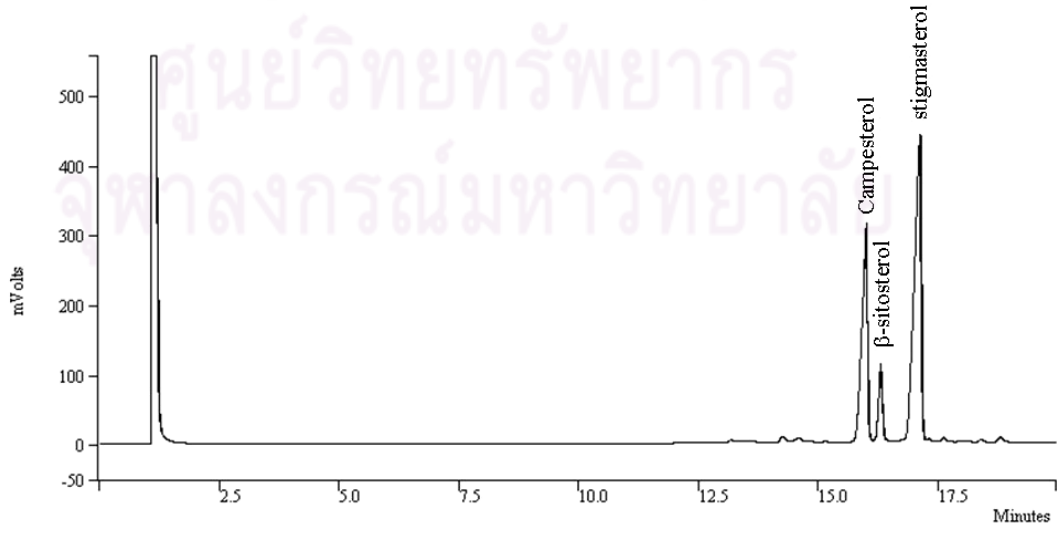


Figure 4.2 The GC Chromatogram of Mixture **1**

4.2.2 Compound 2

Compound **2** was isolated as a colorless needles, m.p. 120-122ºC, and $[\alpha]^{20}_{D}$ −111° (*c* 0.1, $CHCl_3$). This compound showed a single spot on TLC with R_f value of 0.55 (SiO_2; hexane: CH_2Cl_2 = 1:1). Its molecular formula was established as $C_{20}H_{18}O_6$ by ESIMS ($[M+Na]^+$ = at *m/z* 377).

The ^1H NMR ($CDCl_3$) spectrum is presented in Appendix B. Its structure was verified by comparing with report values (Wimalasina and Karunawansha, 1994). Its spectrum displayed six aromatic protons at δ_H 6.83-6.72 (m, 4H) and 6.86 (s, 2H), six protons connecting to oxygen atom at δ_H 3.94-3.80 (m, 1H), 3.32-3.27 (m, 1H), 3.94-3.80 (m, 1H), 4.09 (d, 1H, *J* = 9.5 Hz), 4.82 (d, 1H, *J* = 5.3 Hz), 4.38 (d, 1H, *J* = 7.2 Hz) and two protons observed at δ_H 2.86 (q, 1H, *J* = 13.9, 7.0 Hz), 3.22-3.27 (m, 1H). Four methylene protons being between two oxygen atoms at δ_H 5.95 (s, 4H) were also detected.

The ^{13}C NMR ($CDCl_3$) spectrum (Appendix B) showed twenty carbon signals. Twelve signals of aromatic carbons at δ_C 147.9, 147.6, 147.2, 146.5, 135.0, 132.2, 119.6, 118.6, 108.1, 106.5, 106.4 and 106.4, two methylene carbons between two oxygen atoms at δ_C 101.0 and 101.0, four sp^2 carbons connecting to one oxygen atom at δ_C 87.6, 82.0, 70.9 and 69.7, two sp^3 carbons at δ_C 54.6 and 50.1. By comparison of spectroscopic data of Compound **2** with the published in the literature (Wimalasena and Karunawansha, 1994), this compound was designated as (−)-asarinin (**Figure 4.3**). The ^1H and ^{13}C NMR spectral assignment are tabulated in **Table 4.4**.

The lignan, (−)-asarinin (**2**) has been detected in the barks of some Zanthoxylum species, such as *Z. clave-herculis* and *Zanthoxylum* sp. Sevenet 1183 (Rao and Devies, 1986; Vaquette and Waterman, 1979).

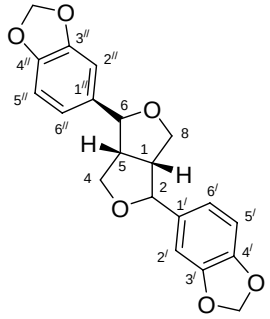


Figure 4.3 The structure of Compound **2**

Table 4.4 The ^{1}H and ^{13}C NMR spectral of Compound **2**

Position	HMQC		HMBC	
	δ_H (*J* in Hz)		δ_C (ppm)	
	Compound 2	Ref*	Compound 2	Ref*
1	3.22-3.27 (m, 1H)	3.31-3.27 (m, 1H)	50.1	50.1
2	4.82 (d, 1H, *J* = 5.3 Hz)	4.81 (d, 1H, *J* = 5.0 Hz)	82.0	82.0
4	3.94-3.80 (m, 1H)	3.83-3.77 (m, 1H)	70.9	70.9
	4.09 (d, 1H, *J* = 9.5 Hz)	4.06 (d, 1H, *J* = 9.0 Hz)		
5	2.86 (q, 1H, *J* = 13.9, 7.0 Hz)	2.95-2.85 (m, 1H)	54.6	54.6
6	4.38 (d, 1H, *J* = 7.2 Hz)	4.38 (d, 1H, *J* = 7.0 Hz)	87.6	87.6
8	3.94-3.80 (m, 1H)	3.83-3.77 (m, 1H)	69.7	69.7
	3.32-3.27 (m, 1H)	3.31-3.27 (m, 1H)		
1′	-	-	132.2	132.2
2′	6.86 (s, 2H)	6.99-6.77 (m, 6H)	106.4	106.4
3′	-	-	146.5	146.5
4′	-	-	147.2	147.2
5′	6.83-6.72 (m, 4H)	6.99-6.77 (m, 6H)	108.1	108.1
6′	6.83-6.72 (m, 4H)	6.99-6.77 (m, 6H)	118.6	116.7
1″	-	-	135.0	135.0
2″	6.83-6.72 (m, 4H)	6.99-6.77 (m, 6H)	106.5	106.5
3″	-	-	147.6	147.6
4″	-	-	147.9	147.9
5″	6.86 (s, 2H)	6.99-6.77 (m, 6H)	106.4	106.4
6″	6.83-6.72 (m, 4H)	6.99-6.77 (m, 6H)	119.6	119.6
OCH_2O	5.95 (s, 4H)	5.95 (s, 4H)	101.0	101.0
OCH_2O	5.95 (s, 4H)	5.95 (s, 4H)	101.0	101.0

*solvent = $CDCl_3$

4.2.3 Compound 3

Compound **3** (5.32 g) was obtained as a major compound from the CH_2Cl_2 extract. This compound was attained after being crystallized as colorless needles, m.p. 131-132ºC; $[\alpha]^{20}_D$ 0 (*c* 0.1, $CHCl_3$); R_f values 0.6 (silica gel, 100% CH_2Cl_2). Its molecular formula was proposed to be $C_{12}H_{11}NO_2$ by ESIMS ($[M+Na]^+$ at = *m/z* 224).

Compound **3** was confirmed its identity by ^1H and ^{13}C NMR and compared the spectroscopic data with those reported values (Bhoga *et al*., 2004) as collected in **Table 4.5**.

The ^1H NMR ($CDCl_3$) spectrum of Compound **3** (Appendix B) revealed four aromatic protons at δ_H 8.22 (d, 1H, *J* = 8.4 Hz), 7.99 (d, 1H, *J* = 8.8 Hz), 7.67 (ddd, 1H, *J* = 8.4, 6.8, 1.6 Hz) and 7.42 (ddd, 1H, *J* = 8.4, 6.8, 1.2 Hz), two doublet olefinic protons at δ_H 7.03 (d, 1H, *J* = 2.8 Hz) and 7.59 (d, 1H, *J* = 2.8 Hz), and three protons at δ_H 4.40 (s, 3H).

The ^{13}C NMR ($CDCl_3$) spectra (Appendix B) appeared twelve carbons signals. Six carbon signals of aromatic carbons were observed at δ_C 146.0, 129.5, 127.5, 124.0, 122.0 and 104.0. Four sp^2 carbons of furan ring at δ_C 164.0, 143.5, 119.0 and 106.0, one sp^2 carbon connecting to methoxy group at δ_C 158.0, and one methoxy carbon at δ_C 59.5 were also detected.

The above data led to establish the structure of Compound **3** as 4-methoxy-2,3-dihydrofuro[2,3-*b*]quinoline alkaloid. It should be noted that it is the first time for the report of the isolation of this compound from the stems of *Z. limonella*. Compound **3** was designated as dictamnine. The furoquinoline alkaloid, Compound **3** was previously isolated from *Z. cuspidatum*, *Z. decaryi*, *Z. belizense*, *Z. schinifolium*, *Z. nitidum*, *Z. scandeus* (Ishii *et al*., 1973; Vaquette *et al*., 1974; Najjar *et al*., 1975; Chen *et al*., 1997; Hu *et al*., 2007; Cheng *et al*., 2008). The ^1H and ^{13}C NMR spectral assignment are tabulated in **Table 4.5**.

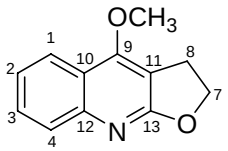


Figure 4.4 The structure of Compound **3**

Table 4.5 The ^{1}H and ^{13}C NMR spectral of Compound **3**

Position	HMQC δ_H (*J* in Hz)		HMBC δ_C (ppm)	
	Compound 3	**Ref***	**Compound 3**	**Ref***
1	8.22 (d, 1H, *J* =8.4 Hz)	8.19 (dd, 1H, *J* = 8.3, 1.7 Hz)	122.0	122.3
2	7.42 (ddd, 1H, *J* = 8.4, 6.8, 1.2 Hz)	7.39 (ddd, 1H, *J* = 8.3, 6.8, 1.5 Hz)	124.0	123.6
3	7.67 (ddd, 1H, *J* = 8.4, 6.8, 1.6 Hz)	7.64 (ddd, 1H, *J* = 8.3, 6.8, 1.7 Hz)	129.5	129.5
4	7.99 (d, 1H, *J* = 8.8)	8.0 (dd, 1H, *J* = 8.6, 1.5 Hz)	127.5	127.5
7	7.59 (d, 1H, *J* = 2.8 Hz)	7.53 (d, 1H, J = 2.7 Hz)	143.5	143.4
8	7.03 (d, 1H, *J* = 2.8 Hz)	7.53 (d, 1H, *J* = 2.7 Hz)	106.0	104.6
9	-	-	158.0	-
10	-	-	104.0	103.3
11	-	-	119.0	118.6
12	-	-	146.0	145.4
13	-	-	164.0	163.6
13–OCH_3	4.40 (s, 3H)	4.28 (s, 3H)	59.5	58.9

*solvent = $CDCl_3$

4.2.4 Compound 4

White needles (5.28 g) of Compound **4** were obtained from silica gel column, with m.p. 124-125 ºC; $[\alpha]^{20}_{D}$ 0 (*c* 0.1, $CHCl_3$) and R_f value 0.5 (SiO_2, CH_2Cl_2-MeOH, 99:1). The molecular formula of Compound **4** was determined as $C_{16}H_{17}NO_2$ on the basis of ESIMS *m/z* 278 $[M+Na]^+$.

The ^{1}H NMR ($CDCl_3$) spectrum is presented in Appendix B. Its spectrum displayed nine aromatic protons at δ_H 7.45 (s, 1H), 7.13 (d, 2H, *J* = 8.3 Hz), 7.39 (t, 2H, *J* = 7.4 Hz), 7.70 (d, 2H, *J* = 7.5 Hz) and 6.84 (d, 2H, *J* = 8.3 Hz), one singlet proton signal of amide group at δ_H 6.36 (s, 1H), three methyl protons at δ_H 3.78 (s, 3H), two protons connecting to amide group at δ_H 3.67 (q, 2H, *J* = 6.6, 13.0 Hz), and two methylene proton signals at δ_H 2.86 (t, 2H, *J* = 6.9 Hz).

The ^{13}C NMR ($CDCl_3$) spectrum (**Figure 7** in Appendix B) showed sixteen carbon signals. Twelve signals belonged to aromatic carbons could be detected at δ_C 158.3, 134.6, 131.4, 130.9, 129.7, 129.7, 128.5, 128.5, 126.8, 126.8, 114.1 and 114.1. Carbonyl carbon signal of amide at δ_C 167.5, a signal of methoxy carbon at δ_C 55.3, a carbon signals connecting to amide group at δ_C 41.3, a methylene carbon signal at δ_C 34.8 were observed. The ^{1}H and ^{13}C NMR spectral assignment are tabulated in **Table 4.6**.

The ^{13}C NMR spectrum of Compound **4** was further compared with published data of dihydroalatamide (Hsiao and Chiang, 1995) and could be concluded its structure as *N*-(4-methoxyphenethyl)benzamide. In previous reports, the aromatic amides, dihydroalatamide was isolated from the stem woods of *Z. alanthoides* (Sheen *et al*., 1994). However, Compound **4** was reported for the first time in *Z. limonella*.

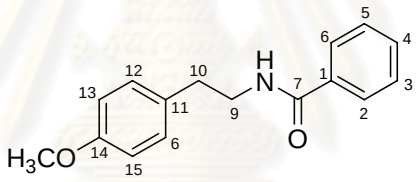


Figure 4.5 The structure of Compound **4**

Table 4.6 The ^{1}H and ^{13}C NMR Spectral of Compound **4**

Position	HMQC δ_H (J in Hz)	HMBC δ_C (ppm)	
	Compound 4	Compound 4	Ref*
1	-	134.6	135.3
2	7.7 (d, 2H, J = 7.5 Hz)	126.8	127.4
3	7.39 (t, 2H, J = 7.4 Hz)	128.5	129.2
4	7.45 (*s*, 1H)	131.4	132.0
5	7.39 (t, 2H, J = 7.4 Hz)	128.5	129.2
6	7.7 (d, 2H, J = 7.5 Hz)	126.8	127.4

Table 4.6 (continued)

Position	HMQC δ_H (*J* in Hz)	HMBC δ_C (ppm)	
	Compound 4	Compound 4	Ref*
7	-	167.5	168.1
8-NH	6.36 (s, 1H)	-	-
9	3.67 (q, 2H, *J* = 6.6, 13.0 Hz)	41.3	41.9
10	2.86 (t, 2H, *J* = 6.9 Hz)	34.8	35.4
11	7.45 (s, 1H)	131.4	132.0
12	7.13 (d, 2H, *J* = 8.3 Hz)	129.7	130.4
13	6.84 (d, 2H, *J* = 8.3 Hz)	114.1	114.8
14	-	158.3	159.0
14-OCH_3	3.78 (s, 3H)	55.3	55.9
15	6.84 (d, 2H, *J* = 8.3 Hz)	114.1	114.8
16	7.13 (d, 2H, *J* = 8.3 Hz)	129.7	130.4

*solvent = $CDCl_3$

4.2.5 Compound 5

Compound **5** was purified by silica gel column of the CH_2Cl_2 extract, red needles (20 mg) with m.p. 279-280 ºC; $[\alpha]^{20}_D$ 0 (*c* 0.1, $CHCl_3$); R_f value 0.4 (SiO_2, CH_2Cl_2-MeOH, 99:1). Molecular formula was determined to be $C_{20}H_{15}NO_4$ on the basis of ^1H, ^{13}C NMR and ESIMS. The ESIMS of this compound showed the molecular ion peak at m/z 356 $[M+Na]^+$.

The ^1H-NMR ($CDCl_3$) spectrum is presented in Appendix B. Its structure was verified by comparing with report values (Martin *et al*., 2005). Its spectrum displayed seven aromatic protons at δ_H 9.24 (s, 1H), 8.70 (s, 1H), 8.28 (d, 1H, *J* = 8.8 Hz), 7.89 (s, 1H), 7.83 (d, 1H, *J* = 8.8 Hz), 7.40 (s, 1H), 7.26 (s, 1H), two methylene protons connecting to oxygen atom at δ_H 6.13 (s, 2H) and six methyl protons at δ_H 4.16 (s, 3H) and 4.09 (s, 3H).

The ^{13}C NMR ($CDCl_3$) spectrum (**Figure 9** in Appendix B) showed twenty carbon signals. Sixteen carbon signals of aromatic carbons were observed at δ_C 153, 150, 148.2, 148.0, 140.4, 129.6, 129.1, 128.8, 126.6, 122.4, 120.2, 118.1, 107.3,

104.4, 102.2, 101.6, two methoxy carbon at δ_C 56.2 and 56.1, a methylene carbon between two oxygen atoms at δ_C 101.3, an imine carbon connecting to nitrogen atom at δ_C 149.8 were detected. The complete assignment protons and carbons for Compound **5** were presented in **Table 4.7**.

On the basis of all the spectral data, the structure of Compound **5** was established as *N*-nornitidine (**Figure 4.6**). The benzophenanthridine alkaloid, *N*-nornitidine (**5**) was isolated for the first time in *Z. limonella*. This alkaloid was previously obtained from the woods and fruits of *Z. microcarpum* and *Z. integrifoliotum*, respectively (Boulware and Stermitz, 1981; Chen *et al*., 1999).

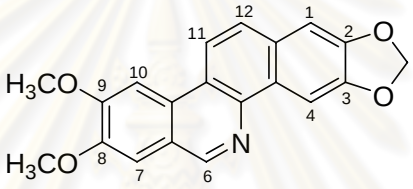


Figure 4.6 The structure of Compound **5**

Table 4.7 ^{1}H NMR and ^{13}C NMR spectral of Compound **5**

Position	HMQC δ_H (*J* in Hz)		HMBC δ_C (*J* in Hz)	
	Compound 5	**Ref***	**Compound 5**	**Ref***
1	7.26 (s, 1H)	7.46 (s,1H)	104.4	104.6
2	-	-	148.0	147.1
3	-	-	148.2	147.7
4	7.89 (s, 1H)	8.14 (s, 1H)	102.2	100.6
4a	-	-	129.1	126.2
4b	-	-	140.4	142.6
6	9.24 (s, 1H)	9.29 (s, 1H)	149.8	148.6
6a	-	-	122.4	126.1
7	7.40 (s, 1H)	7.68 (s, 1H)	107.3	146.0

Table 4.7 (continued)

Position	HMQC δ_H (*J* in Hz)		HMBC δ_C (*J* in Hz)	
	Compound 5	**Ref***	**Compound 5**	**Ref***
8	-	-	150	152.2
8-OCH_3	4.09 (s, 3H)	4.03 (s, 3H)	56.1	55.7
9	-	-	153	152.2
9-OCH_3	4.16 (s, 3H)	4.10 (s, 3H)	56.2	-
10	8.70 (s, 1H)	8.57 (s, 1H)	101.6	118.6
10a	-	-	128.8	126.2
10b	-	-	129.6	123.7
11	8.28 (d, 1H, *J* = 8.8 Hz)	8.24 (d, 1H, *J* = 9.9 Hz)	118.1	120.0
12	7.83 (d, 1H, *J* = 8.8 Hz)	7.93 (d, 1H, *J* = 9.9 Hz)	126.6	123.6
12a	-	-	120.2	130.8
OCH_2O	6.13 (s, 2H)	6.06 (s, 2H)	101.3	100.9

*solvent = $CDCl_3$

4.2.6 Compound 6

Compound **6** have been characterized as a new naturally occurring compound. It was isolated as white needles (40 mg) with m.p. 138-139ºC; $[\alpha]^{20}_{D}$ 0 (*c* 0.1, MeOH) and R_f value 0.61 (SiO_2, CH_2Cl_2-MeOH, 95:5). Molecular formula was determined to be $C_{15}H_{15}NO_2$ based on the evidence from the ^1H and ^{13}C NMR and HRESIMS data. The HRESIMS of this compound showed the molecular ion peak $[M+Na]^+$ at *m*/*z* 280.0943 (calcd for $C_{15}H_{15}NO_2Na$, 280.0950) in the positive HRESIMS.

The ^1H NMR spectrum is presented in Appendix B exhibited a characteristic signal for NH proton of amide moiety at δ_H 11.31. Two sets of doublet at δ_H 7.76 (*J* = 7.9 Hz, H-5) and 7.29 (*J* = 8.1 Hz, H-8) and two sets of triplet at 7.49 (*J* = 7.1 Hz, H-7) and 7.23 (*J* = 7.9 Hz, H-6) were assigned for five aromatic protons. The presence of two singlet signals at δ 6.21 and 5.88 was ascribed for H-4′ proton. The three singlets at δ_C 4.12 (H-1′), 3.91(3-OCH_3) and 1.96 (H-5′) were observed from the presence of two methylene, three methoxy and three methyl protons, respectively.

The ^{13}C NMR spectrum (Appendix B) showed all 15 resonances. A characteristic carbonyl peak of ketone and amide moieties at δ_C 198.8 (C-2′) and 165.0 (C-2) was detected. The peaks at δ_C 137.8 (C-8a), 123.0 (C-5), 122.4 (C-6), 130.5 (C-7), 116.0 (C-8), 117.7 (C-3), 116.9 (C-4a) were observed, indicating from the presence of 2-quinolone nucleus. Finally, the peaks at δ 144.3 (C-3′), 125.0 (C-4′), 33.8 (C-1′) and 17.8 (C-5′) were resonated.

The attachment of substituents was based on HMBC correlations (**Table 4.8** and **Figure 4.7**). It showed the correlations between the methoxy proton (3-OCH_3) and C-4 and C-1′ established the attachment of the methoxy group at C-4. The correlations between two methylene protons (H-1′) and C-2, C-3, C-4, C-1′ and C-2′ indicated that a methylene carbon C-1′ linked between C-3 of 2-quinolone core and C-1′ carbonyl group of methyl acryloyl motiety. A methyl group (H-5′) was substituted on C-3′ carbon due to the correlations between methyl protons and C-2′, C-3′ and C-4′. Thus, Compound **6** was determined as 4-methoxy-3-(3-methyl-2-oxobut-3-enyl)quinolin-2(1H)-one.

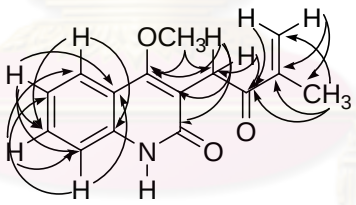


Figure 4.7 HMBC correlations of Compound **6**

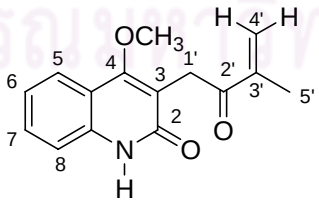


Figure 4.8 The structure of Compound **6**

Table 4.8 The ^{1}H and ^{13}C NMR spectral of Compound **6**

Position	HMQC δ_H (*J* in Hz)	HMBC δ_C (*J* in Hz)	HMBC correlations
1-NH	-	11.31 (s, 1H)	-
2	165.0	-	-
3	117.7	-	-
4	163.7	-	-
4-OCH_3	62.0	3.91 (s, 3H)	C-4, C-1′
4a	116.9	-	-
5	123.0	7.76 (d, 1H, *J* = 7.9 Hz)	C-8a, C-7
6	122.4	7.23 (t, 1H, *J* = 7.9 Hz)	C-4a, C-7, C-6, C-8
7	130.5	7.49 (t, 2H, *J* = 7.1 Hz)	C-5, C-8a,
8	116.0	7.29 (d, 2H, *J* = 8.1 Hz)	C-4a, C-6
8a	137.8	-	-
1′	33.8	4.12 (s, 2H)	C-2, C-3, C-4, C-2′, C-1′
2′	198.8	-	-
3′	144.3	-	-
4′	125.0	5.88 (s, 1H) 6.21 (s, 1H)	C-2′, C-3′, C-5′
5′	17.8	1.96 (s, 3H)	C-2′, C-3′, C-4′

4.2.7 Compound 7

Compound **7** (1.72 g) was isolated, from the CH_2Cl_2 extract. This compound was recrystallized as colorless solid, m.p. 152-153 ºC, $[\alpha]^{20}_{D}$ −12 (*c* 0.1, MeOH) and R_f value 0.55 (silica gel, CH_2Cl_2-MeOH: 95:5). The mass spectrum dosplayed the molecular ion at m/z 294 $[M+Na]^+$. The molecular formula of Compound **7** was confirmed as $C_{16}H_{17}NO_3$ from the NMR and ESIMS data (**Figure 4.9**).

The ^{1}H NMR (DMSO-d_6) spectrum of Compound **7** (Appendix B) exhibited 9 aromatic protons at δ_H 7.48 (s, 1H), 7.43 (t, 2H, *J* = 7.6 Hz), 7.26 (d, 2H, *J* = 8.8 Hz), 7.80 (d, 2H, *J* = 7.2 Hz) and 6.87 (d, 2H, *J* = 8.8 Hz). The appearance of a proton

connecting to hydroxyl group at δ_H 5.43 (d, 1H, J = 4.3 Hz), two protons connecting to amide group at δ_H 3.36-3.24 (m, 2H) and three protons of methoxy group were observed at δ_H 3.70 (s, 3H), a singlet proton signal of amide group at δ_H 8.46 (t, 1H, J =5.3 Hz) was also detected.

Seventeen carbons signals were detected in the ^{13}C NMR (DMSO-d_6) spectrum. The ^{13}C NMR spectrum (Appendix B) displayed twelve signals of aromatic carbons at δ_C 158.8, 136.2, 134.9, 131.5, 128.7, 128.7, 127.6, 127.6, 127.6, 127.6, 113.8 and 113.8. Two carbon signals of amide group and methoxy group were observed at δ_C 166.8 and 55.4, respectively. Two carbons connecting to hydroxyl group and amide group were observed at δ_C 71.1 and 48.1, respectively. The ^{1}H and ^{13}C NMR spectra are presented in **Table 4.9**.

Comparison of spectroscopic data of Compound **7** with the published in the literature (Lee *et al*., 2007) indicated that its structure was elucidateded as (–)-tembamide. The aromatic amides, (–)-tembamide was isolated for the first time in *Z. limonela*. This compound was previously isolated from the stem woods of *Z. alanthoides* and the stem bark of *Z. hyemale* (de Moura *et al*., 2002).

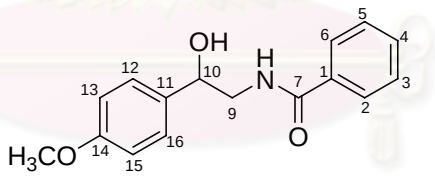


Figure 4.9 The structure of Compound **7**

Table 4.9 The ^{1}H and ^{13}C NMR spectral assignment of Compound **7**

Position	HMQC δ_H (*J* in Hz)		HMBC δ_C (ppm)	
	Compound 7	**Ref***	**Compound 7**	**Ref***
1	-	-	134.9	133.8
2	7.80 (d, 2H, *J* = 7.2 Hz)	7.76 (d, 2H, *J* = 6.9 Hz)	127.6	126.9
3	7.43 (t, 2H, *J* = 7.6 Hz)	7.43 (t, 2H, *J* = 7.5 Hz)	128.7	128.6
4	7.48 (s, 1H)	6.58 (s, 1H)	131.5	131.6
5	7.43 (t, 2H, *J* = 7.6 Hz)	7.43 (t, 2H, *J* = 7.5 Hz)	128.7	128.6
6	7.80 (d, 2H, *J* = 7.2 Hz)	7.76 (d, 2H, *J* = 6.9 Hz)	127.6	126.9
7	-	-	166.8	168.0
8-NH	8.46 (t, 1H, *J* = 7.3 Hz)	7.51 (t, 1H, *J* = 7.3 Hz)	-	-
9	3.36-3.24 (m, 2H)	3.56-3.48 (m, 2H)	48.1	47.7
9-OH	5.43 (d, 1H, *J* = 4.3 Hz)	3.09 (d, 1H, *J* = 3.5 Hz)	-	-
10	4.71 (pent, 1H)	4.94-4.89 (m,1H)	71.1	73.3
11	-	-	136.2	134.1
12	7.26 (d, 2H, *J* = 8.8 Hz)	7.33 (d, 2H, *J* = 8.7 Hz)	127.6	127.0
13	6.87 (d, 2H, *J* = 8.8 Hz)	6.90 (d ,2H, *J* = 8.7 Hz)	113.8	113.9
14	-	-	158.8	159.3
14–OCH_3	3.70 (s, 3H)	3.81 (s, 3H)	55.4	55.2
15	6.87 (d, 2H, *J* = 8.8 Hz)	6.90 (d ,2H, *J* = 8.7 Hz)	113.8	113.9
16	7.26 (d, 2H, *J* = 8.8 Hz)	7.33 (d, 2H, *J* = 8.7 Hz)	127.6	127.0

*solvent = $CDCl_3$

In conclusion, the chemical constituents of the CH_2Cl_2 extract led to the isolation of a novel quinolone alkaloid, limonelone (**6**), along with five known compounds. The known compounds included a lignan; (−)-asarinin (**2**), two aromatic amides: dihydroalatamide (**4**) and (−)-tembamide (**7**), a furoquinoline alkaloid: dictamnine (**3**), and a benzophenanthridine alkaloid: *N*-nornitidine (**5**). These known compounds were identified in each case by comparison their physical and spectroscopic data found in the literature. These isolated compounds are nevertheless reported for the first time in *Z. limonella*.

4.3 Antimicrobial Activities of Isolated Compounds of the CH_2Cl_2 Extract

The four isolated substances: Mixture **1**, Compounds **3**, **4** and **7** were subjected to antimicrobial activity, whereas Compounds **2**, **5** and **6** were not tested due to lack of material.

4.3.1 Antiphytopathogenic Fungi Activities of the CH_2Cl_2 Extract of Stems and Isolated Compounds

The five phytopathogenic fungi were tested at 1,000 ppm dose of each isolated compound and determined by agar incorporation method. The percent inhibition results of the CH_2Cl_2 extract of stems and its isolated compounds are shown in **Table 4.10** and **Figure 4.10**.

The data from **Table 4.10** and **Figure 4.10** revealed that Compound **3** gave more percent inhibition than those of other isolated compounds and the CH_2Cl_2 extract of stems. The aromatic amide Compound **4** and Compound **7** exhibited lower acitivity against fungi tested, while the Mixture **1** did not display growth inhibition for fungi. These results were correlated with part of screening test of antifungal activities from five fractions separated from the CH_2Cl_2 extracts of stems. It was observed that Fr **4** showed the most effective antifungal activity of five fractions and Compound **3** was isolated from this active fraction express higher potent activity against all fungi than the CH_2Cl_2 extract of stems. In addition, *C. lunata, C. cassiicola* and *E. turcicum* were completely inhibited at dose 1,000 ppm of Compound **3**.

Table 4.10 Growth inhibition of fungal species in agar incorporation method of the CH_2Cl_2 extract of stems and its major omponents of *Z. limonella*

Extract/ Compounds	*F.oxysporum f.* sp. *vasinfectum*		*F.oxyspolum*		*C. lunata*		*C. cassiicola*		*E. turcicum*	
	Growth[a] (mm)	Inh (%)	Growth[a] (mm)	Inh (%)	Growth[a] (mm)	Inh (%)	Growth[a] (mm)	Inh (%)	Growth[a] (mm)	Inh (%)
CH_2Cl_2 crude extract of stems	17.1 ± 0.8	51.2 ± 2.2	9.4 ± 0.8	73.1 ± 2.3	7.2 ± 0.3	79.5 ± 0.7	7.2 ± 0.4	79.3 ± 1.1	0.0 ± 0.0	100.0 ± 0.0
Mixture **1**	35.0 ± 0.0	0.0 ± 0.0	35.0 ± 1.0	0.0 ± 0.0	35.0 ± 0.0	0.0 ± 0.0	35.0 ± 0.0	0.0 ± 0.0	35.0 ± 0.0	0.0 ± 0.0
Compound **3**	11.4 ± 0.9	67.5 ± 2.5	5.9 ± 0.2	83.2 ± 0.6	0.0 ± 0.0	100.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0
Compound **4**	20.2 ± 1.1	42.4 ± 3.2	8.2 ± 0.3	76.7 ± 0.9	4.5 ± 0.3	87.2 ± 0.9	5.3 ± 0.3	84.8 ± 0.7	0.0 ± 0.0	100.0 ± 0.0
Compound **7**	26.6 ± 0.6	24.1 ± 1.7	25.7 ± 0.5	26.5 ± 1.5	35.0 ± 0.0	0.0 ± 0.0	35.0 ± 0.0	0.0 ± 0.0	20.9 ± 0.7	40.2 ± 1.9
Benomyl (100ppm)	0.0 ± 0.0	100.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	7.4 ± 0.5	78.9 ± 1.3	0.0 ± 0.0	100.0 ± 0.0	28.5 ± 0.8	18.6 ± 2.2

Inh.; Inhibition, [a] Values, an average ± standard deviation of 3 replicates, of the mean growth inhibition of fungi species

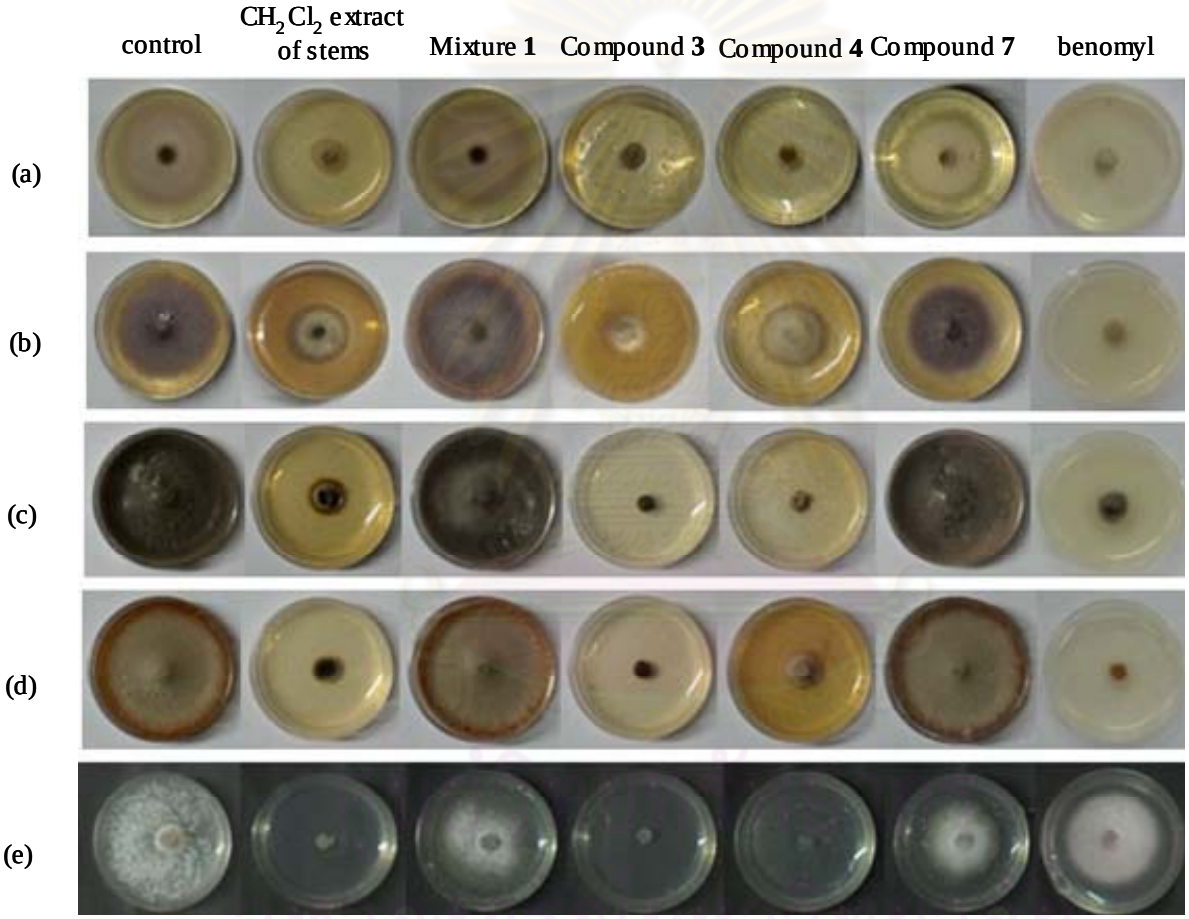


Figure 4.10 The mycelial growth inhibition zone of the CH_2Cl_2 extract of stems from *Z. limonella*, Mixture **1**, Compound **3**, Compound **4**, Compound **7** and standard fungicide; benomyl at 1,000 ppm against (a) *F.oxysporum f.* sp. *vasinfectum*, (b) *F.oxysporum*, (c) *C. lunata*, (d) *C.cassiicola* and (e) *E. turcicum*

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From this study indicated that Compound **3** was major component in the CH_2Cl_2 extract of stems, possessed the strongest antifungal activities compared with the other components, thus chosen Compound **3** for further investigation.

Based on previous result, five fungal strains were sensitive to the Compound **3**. Therefore, these phytopathogenic fungi were tested various concentration of Compound **3** and CH_2Cl_2 extract of stems to evaluated and compared IC_{50} value of each fungi. The growth inhibition were calculated and IC_{50} value (ppm) as shown in **Table 4.11** and **Figure 4.11**.

The data from **Table 4.11** and **Figure 4.11** displayed that the IC_{50} values of Compound **3** lower than the IC_{50} of CH_2Cl_2 extract of stems. Comparing with IC_{50} values showed that almost fungi such as *E. turcicum*, *C. lunata*, *C. cassiicola* and *F. oxysorum* were sensitive to Compound **3** with IC_{50} were 51.4, 67.3, 73.4 and 78.4 ppm, respectively, while *F. Oxysporum f.* sp. *vasinfectum* lower sensitivity with IC_{50} was 1,285.3 ppm. These data was observed that Compound **3** was major component of stems crude extract and showed the strongest antifungal activity with percentage inhibition and low IC_{50} values.

Table 4.11 IC_{50} values of Compound **3** and the CH_2Cl_2 extract of stems

Fungi	**IC_{50} (ppm)**	
	CH_2Cl_2 extract of stems	**Compound 3**
F.oxyspolum	429.5	78.4
F.oxysporum f. sp. *vasinfectum*	1,333.4	1,285.3
C. cassiicola	398.4	73.4
C. lunata	301.1	67.3
E. turcicum	199.1	51.4

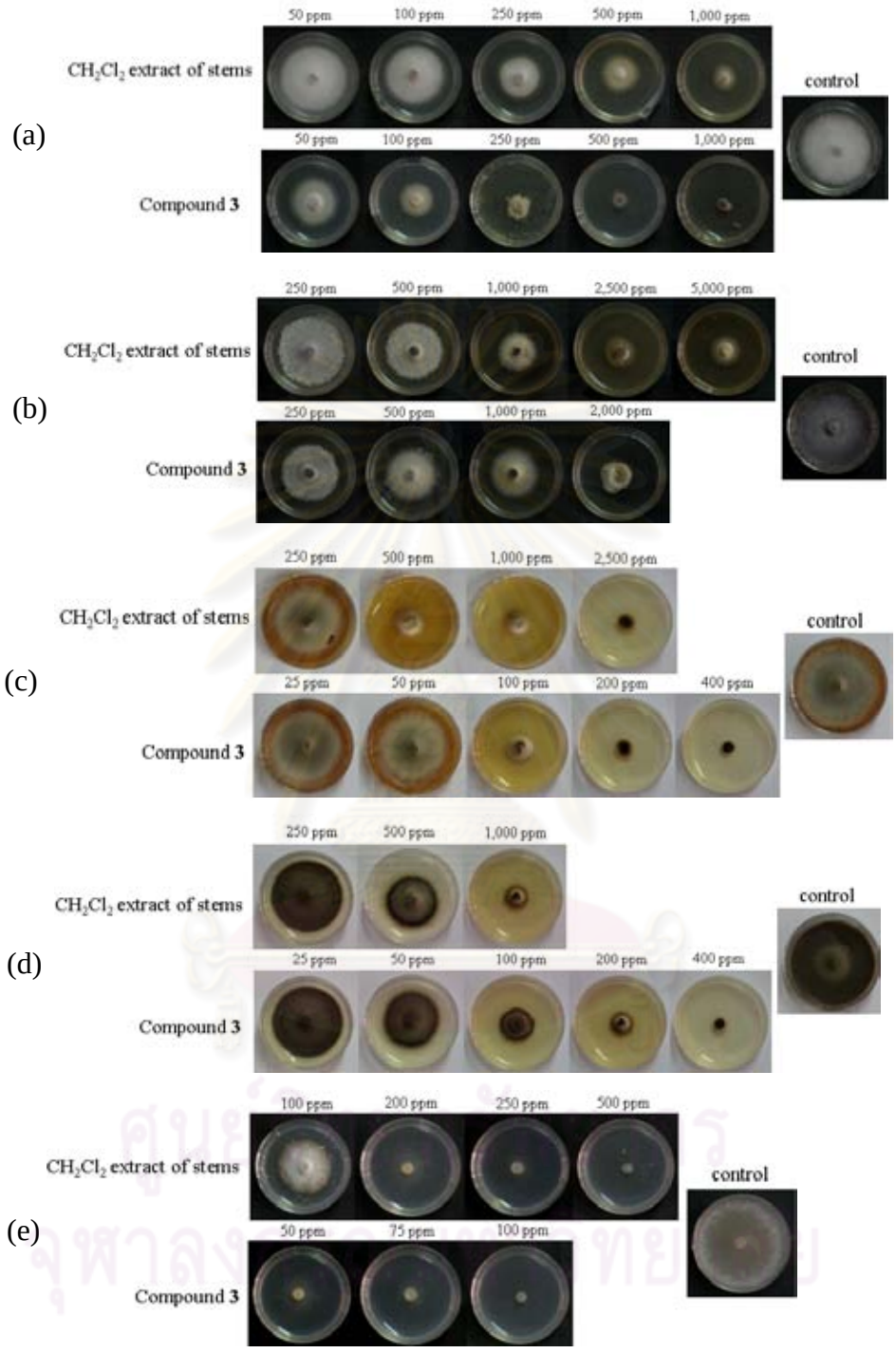


Figure 4.11 The mycelial growth inhibition zone of the CH_2Cl_2 extract of stems from *Z. limonella* and Compound **3** at various concentrations against (a) *F.oxysporum*,(b) *F. oxysporum f.* sp. *vasinfectum*, (c) *C. cassiicola*, (d) *C. lunata* and (e) *E. turcicum*

Thus, to evaluate the antifungal activity of compound **3** compared with the CH_2Cl_2 crude extract by broth macrodilution method. The antifungal activity was evaluated as MIC and MFC values. Two fungicides: mancozeb and benomyl were used as a positive control. The results are demonstrated in **Table 4.12**.

Table 4.12 displays that MIC values against five phytopathogenic fungi ranged from 0.312 to >5.00 mg/mL for the CH_2Cl_2 extract and from 0.156 to >5.00 mg/mL for compound **3**. The MIC and MFC values of compound **3** exhibited more potent than the CH_2Cl_2 extract in all fungi tested. Compound **3** displayed antifungal activity against *E. turcicum, C. lunata, C. cassiicola* and *F. oxysporum* with the MIC values of 0.156, 0.156, 0.156 and 0.625 respectively, which was much higher activity than the CH_2Cl_2 extract. In addition, compound **3** exhibited lower MIC against *F. oxysporum* sp. *vasinfectum* (MIC = 1.25 mg/mL).

These results showed that dictamnine exhibited a broad spectrum of fungitoxic behavior against phytopathogenic fungi tested. Compound **3** was previously reported to be an antimicrobial substance against fungi (Wolter and Eilert, 1981). Grayer and Harborn reported that furoquinoline alkaloids may also play a role in the defence of plants against potentially pathogenic fungi. Compound **3**, a common compound in Rutaceae, showed potent antifungal activity against the plant pathogenic fungi *Cladosporium cucumerinum*. It was also reported to be a phototoxic and photomutagenic compound (Zhao *et al*., 1998; Pfyffer *et al*., 1982; Schimmer and Kuhne, 1991). These activities have been attributed to the reactive furan ring (Pfyffer *et al*., 1982). From the above results, it seemed that the lignan and amide group in these compounds were not critical for the mycelial growth inhibitory effect, while the presence of furan ring might contribute to the inhibitory activity against phytopathogenic fungi (Zhao *et al*., 1998). Therefore, compound **3** was the main component of CH_2Cl_2 extract which possessed potent antifungal activity.

Table 4.12 Antifungal activities of the CH_2Cl_2 extract and compound **3** expressed by MIC and MFC values.

Fungi	CH_2Cl_2 extract of stems (mg/mL)		Compound 3 (mg/mL)		Benomyl (mg/mL)		Mancozeb (mg/mL)	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
F. oxysporum	1.25	0.625	3.90	1.25	0.0039	0.0039	0.0039	0.0039
F. oxysporum f. sp. *vasinfectum*	2.50	1.25	3.90	2.50	0.0039	0.0039	0.0039	0.0039
C. cassiicola	0.625	0.156	0.39	0.625	0.00039	0.00078	0.0312	0.0625
C. lunata	0.625	0.156	125	0.625	0.125	0.250	0.0078	0.0078
E. turcicum	0.312	0.156	65.5	0.312	0.0625	0.250	0.0156	0.0156

4.3.2 Antibacterial and Anticandidal Activities of the CH_2Cl_2 Extract and Isolated Substances

The CH_2Cl_2 crude extract and isolated compounds including mixture **1**, compounds **3**, **4** and **7** at 1,000 µg/disc were tested against 4 bacteria (*B. subtilis*, *S. aureus*, *E. coli* and *P. aeruginosa*) and 1 yeast (*C. albicans*) by disc diffusion method. The antibacterial and anticandidal activities were assessed as an inhibition zone around the paper disc containing the CH_2Cl_2 extract and isolated substances. Penicillin G, chloramphenicol, and amphotericin B were used as a positive control for growth inhibition. The results of the zone of inhibition tests are revealed in **Table 4.13**.

The CH_2Cl_2 crude extract exhibited moderate antibacterial and anticandidal activities against the growth of *B. subtilis* (7 mm), *S. aureus* (8 mm) and *C. albicans* (7 mm), while no inhibition effect was observed from this extract against Gram-negative bacteria such as *E. coli* and *P. aeruginosa*. Compound **3** showed the best zone values compared with other isolated substances. This compound was active against Gram-positive bacteria and yeast. It displayed much more potent activity against *B. subtilis* (9 mm), *S. aureus* (9 mm) and *C. albicans* (7 mm). Compound **4** demonstrated little activity, while compound **7** did not show any inhibitory activity against all tested microorganisms. In addition, the result showed that isolated substances and the CH_2Cl_2 extract did not produce any inhibition zone on Gram-negative bacteria (*E. coli* and *P. aeruginosa*). This can explained because the outer membrane of Gram-negative bacteria is known to have a barrier to the periplasmic space containing enzymes which are able to break down foreign molecule introduced from outside (Schaechter *et al*.,1999; Duffy and Power, 2001; Sortori *et al*., 2003). Many Gram-negative organisms also exhibit intrinsic high-level resistance to a range of antimicrobial agent and support a role for the outer membrane and active efflux as a barrier to antibiotics (Nikaido, 1989; Köhler, 1999; Van Bambeke *et al*., 2003).

Table 4.13 Antibacterial and anticandidal activities of the CH_2Cl_2 extract and isolated substances.

Extract / Fraction No.	Diameter of inhibition zone[a] (mm)				
	B. subtilis	***S. aureus***	***E. coli***	***P. aeruginosa***	***C. albicans***
CH_2Cl_2 crude extract of stems	6.9 ± 0.4	7.7 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	7.1 ± 0.4
Mixture **1**	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Compound **3**	9.2 ± 0.7	9.2 ± 0.6	0.0 ± 0.0	0.0 ± 0.0	7.2 ± 0.2
Compound **4**	6.4 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	6.4 ± 0.2
Compound **7**	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Pen G (10 Unit/disc)	24.6 ± 0.9	30.5 ± 1.1	0.0 ± 0.0	0.0 ± 0.0	NA
Chloram (30 µg/disc)	28.3 ± 0.7	21.7 ± 0.4	20.3 ± 0.9	13.1 ± 0.6	NA
Ampho B (25 µg/disc)	NA	NA	NA	NA	9.5 ± 0.9

[a]values, an average ± standard deviation of 3 replicaes, of the mean diameter of inhibition zone

Pen G = Penicillin G, Chloram = Chloramphenicol, Ampho B = Amphotericin B, NA = Not active

Previous results showed that compound **3** gave the highest antibacterial and anticandidal activities compared with other compounds. Compound **3**, a bioactive compound from the stems of *Z. limonella* is a potent against *B. subtilis*, *S. aureus* and *C. albicans*. Therefore, compound **3** and the CH_2Cl_2 crude extract were thoroughly investigated for their antibacterial and anticandidal activities by broth microdilution assay. The MIC values for compound **3** and the CH_2Cl_2 crude extract were examined and the results are shown in **Table 4.14**.

The MIC for compound **3** and the CH_2Cl_2 crude extract ranged from 1 - >5 mg/mL against microorganism tested. Chloramphenicol and amphotericin B were used as a positive control for growth inhibition and showed MIC value less than 0.1 mg/mL for bacteria and 0.0025 mg/ml for *C.albicans*. Compound **3** was also noticed to be more susceptible to *S. aureus* and *C. albicans* and their MIC values of 1 mg/mL for *S. aureus* and *C. albicans*. Moreover, the CH_2Cl_2 extract exhibited moderate antibacterial and anticandidal activity against *B. subtilis* and *S. aureus* with MIC value 2 mg/mL and the yeast *C. albicans* with MIC 1 mg/mL. Both compound **3** and the CH_2Cl_2 extract showed no activity against *E. coli* and *P. aeruginosa*. In addition, MCC values ranged from 2 - >5 mg/ml against bacterial and yeast tested. The MBC values of dictamnine and the CH_2Cl_2 crude extract against *S. aureus* were 2 and 4 mg/mL and *C. albicans* were 2 and 2 mg/mL, respectively. These results indicated that the MIC values of CH_2Cl_2 extract and compound **3** were lower than their MBC values. This suggests that they were bacteriostatic at lower concentration but bactericidal at higher concentrations against *S. aureus* and *C. albicans.*

In view of the results obtained using both disc diffusion and micro-well dilution assays, compound **3** was found to be active only against Gram-positive bacteria and yeast *C. albicans*. The furoquinoline alkaloid, compound **3** was previously reported to be mutagenic and phototoxic in Gram-positive bacteria, yeasts, and filamentous fungi in near ultraviolet light (Pfyffer *et al*., 1982). It showed interesting MIC values against the microorganisms *S. sanguinis* (0.4 mg/mL), *S. mutans* (0.4 mg/mL), and *L. casei* (0.1 mg/mL) (Severino *et al*., 2009). It participates in the severe skin phototoxicity of the plant, and this photobiological activity has been shown to be connected with the reactive furan double bond (Schempp *et al*., 1996).

Table 4.14 Antibacterial and anticandidal activities of the CH_2Cl_2 crude extract and compound **3** assayed by MIC, MBC, and MFC values.

Microorganisms	CH_2Cl_2 extract of stems (mg/mL)		Compound 3 (mg/mL)		Chloram (mg/mL)		Ampho B (mg/mL)	
	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MFC
S. aureus	2	4	1	2	0.00625	0.0125	NA	NA
E. coli	>5	>5	>5	>5	0.00313	0.0250	NA	NA
P. aeruginosa	>5	>5	>5	>5	0.10000	0.2000	NA	NA
B. subtilis	2	4	>5	>5	0.00313	0.0500	NA	NA
C. albicans	1	2	1	2	NA	NA	0.00250	0.00500

Chloram = Chloramphenicol, Ampho B = Amphotericin B, NA = Notactive

Therefore, the presence of furan ring in compound **3** may also contribute to the observed inhibitory activity against the microorganisms.

In summary, the present study has shown the growth inhibition or killing of the CH_2Cl_2 extract and active isolated compound **3** from *Z. limonella* against tested microorganisms. Compound **3** was determined as a major compound and possessed the antifungal and antibacterial activities. This compound could be useful for natural antimicrobials that could be used to reduce or kill human and plant pathogen. These extract and active compound **3** of *Z. limonella* may be useful as an alternative antimicrobial agent as natural medicine for the treatment of many infectious diseases because of its potency.

4.4 Experimental section

4.4.1 Equipments

Melting points were determined on Fishers-Johns melting point apparatus and are uncorrected. Optical rotations were measured using a Perkin Elmer instrument Model 341 polarimeter. NMR spectra, including COSY, HMBC, and HSQC experiments, were recorded on Varian model Mercury+400 which operated at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR. Chemical shifts were internally referenced to the solvent signals in $CDCl_3$ and methanol-d_6 with TMS as the internal standard. High-resolution EIMS spectra were recorded on micrOTOR Bruker mass spectrometer. Adsorbent such as silica gel 60 Merck cat. No. 7734 was used for column chromatography. Thin-layer chromatography (TLC) was performed on pre coated Merck silica gel 60 F254 plates (0.25 mm thick layer). Most solvents used in this research were commercial grade and were distilled prior to use.

4.4.2 Extraction and Isolation

Dried stems (12 kg) were crushed and extracted with CH_2Cl_2. The solvent was evaporated under vacuum to give 110 g of CH_2Cl_2 extract. The CH_2Cl_2 extract (100 g) was subjected to silica gel column, eluting with *n*-hexane and gradually increasing the polarity with EtOAc and MeOH to afford five fractions as follows: fractions Fr **1** (6 L, n-hexane); Fr **2** (10 L, n-hexane/EtOAC, 4:1); Fr **3** (12 L, n-hexane/EtOAC, 1:1); Fr **4**

(8 L, EtOAC); and Fr **5** (8 L, EtOAC/MeOH, 19:1). Fraction Fr **2** (17.85 g) was rechromatographed on silica gel using proportion of *n*-hexane and EtOAc to furnish four fractions Fr **21** to Fr **24**. Fractions Fr **21** and Fr **22** was chromatographed over silica gel, eluting with *n*-hexane and gradually increasing the polarity with EtOAc to obtain mixture **1** (3.45 g) and compound **2** (22 mg), respectively. Fraction Fr **4** (35.21 g) was rechromatrographed over Sephadex LH-20 (500g) eluting with *n*-hexane:CH_2Cl_2:MeOH (2.5:1.5:1) to yield four fractions (Fr **41** to Fr **44**). Fraction Fr **42** (14.8 g) was washed with CH_2Cl_2-MeOH to give compounds **3** (5.32g) and **4** (5.28 g). The concentrated filtrate (13.47 g) was rechromatographed on silica gel eluting with *n*-hexane-EtOAc, gradually increased the polarity with EtOAc, and 4 fractions (Fr **421** to Fr **424**) were collected. Fraction Fr **422** was applied to a silica gel column using varying proportion of *n*-hexane and EtOAc to afford compound **5** (20 mg). Fraction Fr **424** was further rechromatographed on silica gel eluting with EtOAc and gradually increasing the polarity with MeOH to obtain compound **6** (40 mg). Fraction Fr **43** (5.04 g) chromatographed on silica gel using varying proportion of EtOAc and MeOH to afford compound **7** (172 mg).

4.4.3 Antifungal Assay

The CH_2Cl_2 extract, five fractions, and isolated compounds were examined for antifungal activity against five phytopathogenic fungi by agar incorporation assay and were determined for MICs and MFCs by macrodilution method as described in the experimental section of Chapter 2 (Toudon, 1990 and Dwivedy and Dubey, 1993) and Chapter 3 (Schmourlo *et al.*, 2005), respectively. Each test was carried out in triplicate.

4.4.4 Antibacterial and Anticandidal Assay

The CH_2Cl_2 extract, five fractions, and isolated compounds were tested for antibacterial and anticandidal activity against four bacteria and one pathogenic yeast by disc diffusion method and were determined for MICs, MBCs, and MFCs by a microdilution method as described in experimental section of Chapter 2 (CLSI, 2005) and Chapter 3 (CLSI, 2007).

(−)-asarinin (2): colorless neddles; m.p. 120-122 ºC; $[\alpha]^{20}_{D}$ −111 (*c* 0.1, $CHCl_3$); R_f 0.55 (silica gel, hexane-CH_2Cl_2 (1:1)); ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H 2.86 (1H, q, *J* = 13.9, 7.0 Hz), 3.22-3.27 (1H, m), 3.32-3.27 (1H, m), 3.94-3.80 (1H, m), 4.09 (1H, d, *J* = 9.5 Hz), 4.38 (1H, d, *J* = 7.2 Hz), 4.82 (1H, d, *J* = 5.3 Hz), 5.95 (4H, s), 6.83-6.72 (4H, m), 6.86 (2H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C 50.1, 54.6, 69.7, 70.9, 82.0, 87.6, 101.0, 101.0, 106.4, 106.5, 108.1, 118.6, 119.6, 132.2, 135.0, 146.5, 147.2, 147.6, 147.9, ESIMS *m/z* 377 $[M + Na]^+$

Dictamnine (3): White needles; m.p. 131-132 ºC; $[\alpha]^{20}_{D}$ 0 (*c* 0.1, $CHCl_3$); R_f = 0.6 (CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H 4.40 (3H, s), 7.03 (1H, d, *J* = 2.8 Hz), 7.42 (1H, ddd, *J* = 8.4, 6.8, 1.2 Hz), 7.59 (1H, d, *J* = 2.8 Hz), 7.67 (1H, ddd, *J* = 8.4, 6.8, 1.6 Hz), 7.99 (1H, d, *J* = 8.8 Hz), 8.22 (1H, d, J = 2.8 Hz), ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C 58.9, 103.3, 104.9, 118.6, 122.3, 123.6,127.5, 129.5, 143.4, 145.4, 163.6; ESIMS *m/z* 224 $[M+Na]^+$

Dihydroalatamide (4): White needles; m.p. 124-125 ºC; $[\alpha]^{20}_{D}$ 0 (*c* 0.1, $CHCl_3$); R_f = 0.5 (CH_2Cl_2-MeOH, 99:1) ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H 2.86 (2H, t, *J* = 6.9 Hz), 3.67 (2H, q, *J* = 6.6, 13.0 Hz), 3.78 (3H, s), 6.36 (1H, s), 6.84 (2H, d, *J* = 8.3 Hz), 7.13 (2H, d, *J* = 8.3 Hz), 7.39 (2H, t, *J* = 7.4 Hz), 7.45 (1H, s), 7.7 (2H, d, *J* = 7.5 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C 34.8, 41.3, 55.3, 55.3, 114.1, 126.8, 128.5, 129.7, 130.9, 131.4, 134.6, 158.3, 167.5; ESIMS *m/z* 278 $[M+Na]^+$

***N*-nornitidine (5):** Red needles; m.p. 279-280 ºC; $[\alpha]^{20}_{D}$ 0 (*c* 0.1, $CHCl_3$); R_f = 0.4 (CH_2Cl_2-MeOH, 99:1); ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H 4.09 (3H, s), 4.16 (3H, s), 6.13 (2H, s), 7.26 (1H, s), 7.40 (1H, s), 7.83 (1H, d, *J* = 8.8 Hz), 7.89 (1H, s), 8.28 (1H, d, *J* = 8.8 Hz), 8.70 (1H, s), 9.24 (1H, s); ^{13}C NMR ($CDCl_3$, 100 MHz δ_C 56.1, 56.2, 101.3, 101.6, 102.2, 104.4, 107.3, 118.1, 120.2, 122.4, 126.6, 128.8, 129.6, 140.4, 148.0, 148.2, 149.8, 150, 153; ESIMS *m/z* 356 $[M+Na]^+$

4-methoxy-3-(3-methyl-2-oxobut-3-enyl)quinolin-2(1H)-one (6): Yellow needles; m.p. 138-139ºC; $[\alpha]^{20}_{D}$ 0 (*c* 0.1, MeOH); R_f = 0.61 (CH_2Cl_2-MeOH, 95:5); ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H 1.96 (3H, s), 3.91 (3H, s), 4.19 (2H, s), 5.88 (1H, s), 6.21 (1H,

s), 7.23 (1H, t, *J* = 7.9 Hz), 7.29 (2H, d, *J* = 8.1 Hz), 7.49 (1H, t, *J* = 7.1 Hz), 7.76 (1H, d, *J* = 7.9 Hz), 11.31 (1H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C 17.8, 33.8, 62.0, 116.0, 116.9, 117.7, 122.4, 123.0, 125.0, 130.5, 137.8, 144.3, 163.7, 165.0, 198.8; HRESIMS *m/z* 280.0943 $[M+Na]^+$ (calcd for $C_{15}H_{15}NO_3Na$, 280.0950)

(−)-tembamide (7): White crystalline solid; m.p. 152-153 ºC; $[\alpha]^{20}_D$ −12 (*c* 0.1, MeOH); R_f = 0.55 (CH_2Cl_2-MeOH, 95:5)^{1}H NMR ($CDCl_3$, 400 MHz) δ_H 3.36-3.24 (2H, m), 3.70 (3H, s), 4.7 (1H, pent), 5.43 (1H, d, *J* = 4.3 Hz), 6.87 (2H, d, *J* = 8.8 Hz), 7.26 (2H, d, *J* = 8.8 Hz), 7.43 (2H, t, *J* = 7.6 Hz), 7.48 (1H, s), 7.80 (2H, d, *J* = 7.2 Hz), 8.46 (1H, t, *J* = 7.3 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) 48.1, 55.4, 71.1, 113.8, 127.6, 127.6, 128.7, 131.5, 134.9, 136.2, 158.8, 166.8; ESIMS *m/z* 294 $[M+Na]^+$

CHAPTER V

Antioxidant Activity of *Zanthoxylum limonella* Alston.

The preliminary screening of antioxidant activity of CH_2Cl_2, MeOH crude extracts from various parts and the essential oil of fruits were examined for antioxidant activity by using TLC autographic method with DPPH. The CH_2Cl_2 extract from stems and the essential oil from fruits were selected for investigation its primary antioxidative potential in cell-free system and further extend to prostate cancer cell lines.

5.1 Primary Antioxidative Potential in Cell-Free System

The CH_2Cl_2 extract of stems, essential oil, three isolated compounds from CH_2Cl_2 of stems (compound **3**, compound **5**, and compound **7**) and three major components of essential oil (sabinene, limonene, and terpinen-4-ol) were tested for their antioxidant activity using DPPH scavenging assay and trolox equivalent antioxidant capacity (TEAC) assay. The principle of quantitative analysis of DPPH antioxidant assay is based on a reaction in which a blue solution of stable free radical DPPH accepts an electron from a free radical scavenger, antioxidant compounds, and decolorizes. The amplitude of decolorization can be quantitatively determined by reading the absorbance. A large antioxidant capacity is revealed by low IC_{50} value. On the other hand, TEAC assay showed percentage inhibition of $ABTS^{\bullet+}$ absorbance as a function of the concentration. Each extract and compound was determined at 1-min time point. The results are shown in **Table 5.1** and **5.2**.

Table 5.1 Antioxidative capacities of the CH_2Cl_2 extract of stems and essential oil from the fruits of *Z. limonella*

Plant extracts	50% DPPH scavenging activity (IC_{50}, μg/mL)	Trolox equivalent antioxidant capacity (μM Trolox/g crude extract)
CH_2Cl_2 extract of stems	117.5 ± 4.7	14.3 ± 0.3
Essential oil of fruits	5,764.7 ± 6.5	7.1 ± 0.3
Butylated hydroxytoluene (BHT)	19.7 ± 0.2	–

– = Not determined

Table 5.2 Antioxidant activity of isolated compounds from CH_2Cl_2 crude extract of stems and essential oil from the fruit of *Z. limonella*

Compounds	50% DPPH scavenging activity (IC_{50}, mM)	Trolox equivalent antioxidant capacity (μM Trolox)
Compound **3**	4.3 ± 0.2	42.8 ± 5.3
Compound **4**	5.6 ± 0.9	22.1 ± 0.5
Compound **7**	4.9 ± 0.4	36.6 ± 3.5
Sabinene	ND	ND
Limonene	ND	ND
Terpinen-4ol	6.6 ± 0.3	19.7 ± 0.8
BHT	0.087 ± 0.002	–

ND = Not detectable, – = Not determined

The data from **Table 5.1** showed that IC_{50} values of CH_2Cl_2 extract of stems and essential oil of fruits were 117.5 ± 4.7 and 5,764.7 ± 6.5 μg/mL. The IC_{50} values in **Table 5.2** of four isolated compounds such as Compound **3**, Compound **4**, Compound **7**, and terpinen-4-ol were 4.3 ± 0.2, 5.6 ± 0.9, 4.9 ± 0.4, and 6.6 ± 0.3 mM, respectively. In contrast, sabinene and limonene were not determined to the unability to decolorize the methanolic DPPH solution.

TEAC values of CH_2Cl_2 extract of stems and essential oil were 14.3 ± 0.3 and 7.05 ± 0.3, respectively. In addition, the isolated compounds presented the following order obtained by the $ABTS^{\bullet+}$ decolorization assay: Compound **3** > Compound **7** > Compound **4** > terpinen-4-ol, where as the TEAC values of sabinene and limonene were not detectable.

The TEAC and IC_{50} values in the same ranking order implied to the information regarding the quality and reactivity, indicating to the amount of radical scavenger compounds in each sample. The results showed higher antioxidant capacity of CH_2Cl_2 extract from stems and high potential antioxidative substance such as Compound **3**, Compound **4**, and Compound **7** in comparison with essential oil of fruits. However, considering the antioxidant capacities result and the main compounds of essential oil, terpinen-4-ol should be responsible for the antioxidant activity.

The results for these isolated compound confirmed that hydroxyl and methoxy group in chemical structures are the main requirement for radical scavenging capacity. The Compound **3**, Compound **4**, and Compound **7** from CH_2Cl_2 extract of stem showed methoxy and hydroxyl group in chemical structures. Thus, these compounds showed an activity to scavenge $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ at different values. However, the position of hydroxyl group and methoxylation influence the antioxidant potential. In addition, the chemical structures of major compounds in essential oil of fruits indicated that oxygenated monoterpene, terpinen-4-ol showed the hydroxyl group and radical scavenging activity, whereas sabinene and limonene are monoterpene and showed inactive antioxidant activity.

The data from DPPH and TEAC assays of extract, essential oil, and isolated compounds revealed a wide range of antioxidant activity. These antioxidant properties were proven to be correlated with literature report. This previous report on the antioxidant activity of *Z. limonella* crude extract from seed was found to moderate degree of activity (Plalsuwan *et al*., 2005). Furthermore, the isolated compounds have been no report regarding antioxidant activity in *Z. limonella* and other plants. In further study, we extended the study to test the antioxidant capability via cell-base system with prostate cancer cell line, PC-3 and DU-145.

5.2 Antioxidative Activity of Extract in Prostate Cancer Cell Lines

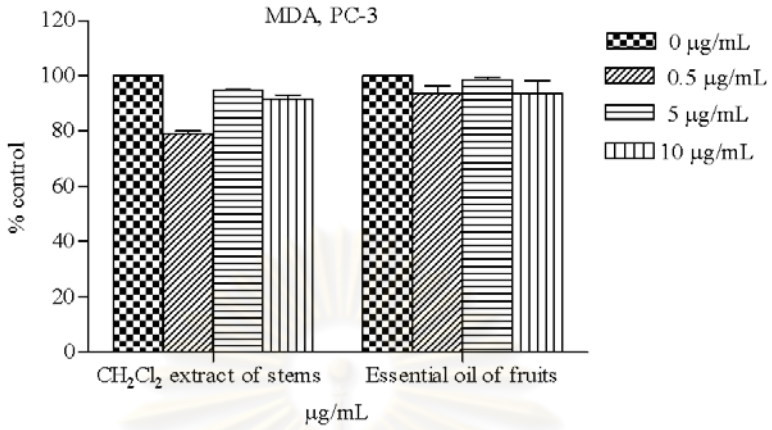
Prostate cancer is the most prevalent type of internal malignancy found in men over the age of fifty. Extensive research has established a strong relationship between ROS generation and carcinogenesis including cancer progression. ROS can induce mutations and alter gene function resulting in carcinogenesis *via* oxidation processes (Halliwell, 1994). In the erythrocytes of patients with prostate cancer, malondialdehyde (MDA) levels have been found to be significantly higher (Aydin *et al.*, 2006). It was previously mentioned that there is a higher oxidative stress in benign epithelium of prostate cancer patients than normal men. Many reports mentions that some natural compounds and dietary agents such as selenium, vitamin E and D, lycopene, soy, and isoflavone, etc. reduced the possibility of prostate cancer both growth and progression (Liao, *et al*., 1995).

In this study, the ability of CH_2Cl_2 extract of stems and essential oil to change on MDA levels in untreated cell lysates of PC-3 and DU-145 were shown in **Figure 5.1**. **Figure 5.2**, **5.3**, and **5.4** show the evaluation of MDA levels and intracellular antioxidant system, glutathione (GSH) and catalase (CAT), was done in PC-3 and DU-145 24 h-pretreated with extract, essential oil, and isolated compounds at the various concentrations.

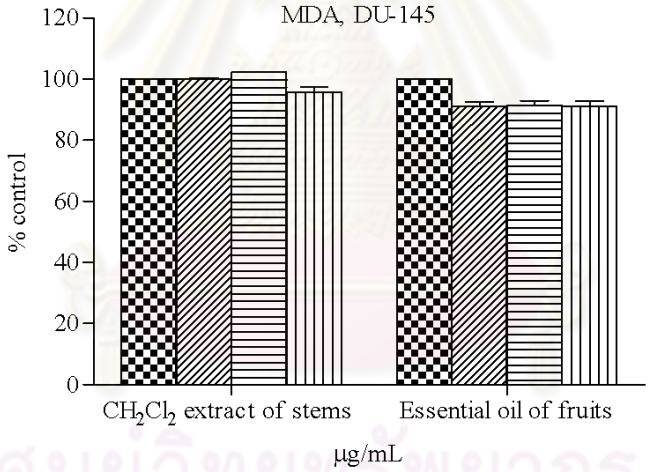
5.2.1 Lipid Peroxidation Determination

In the present study, lipid peroxidation product, i.e. MDA, were analyzed using TBAR assay, with the spectrophotometrically measurement the color intensity of MDA-TBA complex. The data from **Figure 5.1** generally show the slightly decrease in MDA level. Moderately reduction of MDA, approximately 20% of control, was found by CH_2Cl_2 extract of stems at 0.5 μg/mL. Both extracts at 5 and 10 μg/mL seemed to exert no effect. In addition, CH_2Cl_2 extract of stems and essential oil at 0.5, 5 and 10 μg/mL exhibited no effect to MDA levels in DU-145 untreated cell lysate.

The information in **Figure 5.1** showed quite low ability of CH_2Cl_2 extract of stems and essential oil of fruit to change the MDA concentration in untreated cell lysate of PC-3 and DU-145. These results showed that the extract and essential oil had weak activity against MDA level in both cell lysates and can not directly protect cell



(a)



(b)

Figure 5.1 Change on MDA levels with CH_2Cl_2 extract of stems and essential of fruits in PC-3 (a) and DU-145 (b) cell lysates at 0 (control), 0.5 µg/ml, 5 µg/ml, and 10 µg/ml

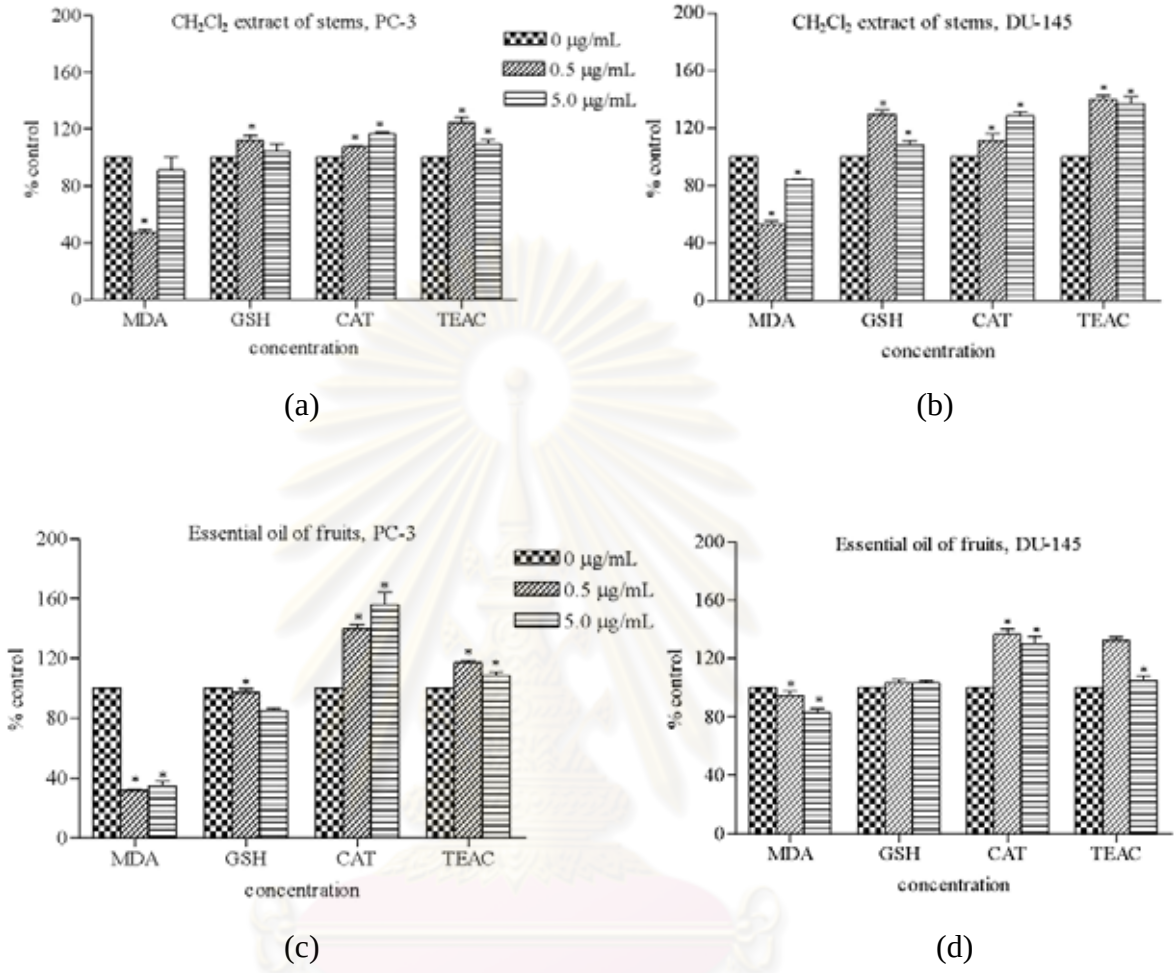


Figure 5.2 Change on MDA levels (ng μg^{-1} protein) , GSH levels (mg mg^{-1} protein) and CAT (μmoles mg^{-1} protein min^{-1}), TEAC activities (μM Trolox mg^{-1} protein) with CH_2Cl_2 of stems and essential oil of fruits in PC-3 and DU-145 prostate cancer cells (a – d) at 0 (control), 0.5µg/ml , 5 µg/ml for 24 h.* $p<0.05$ versus untreated control as analyzed by one way ANOVA and Turkey's multiple-comparison test

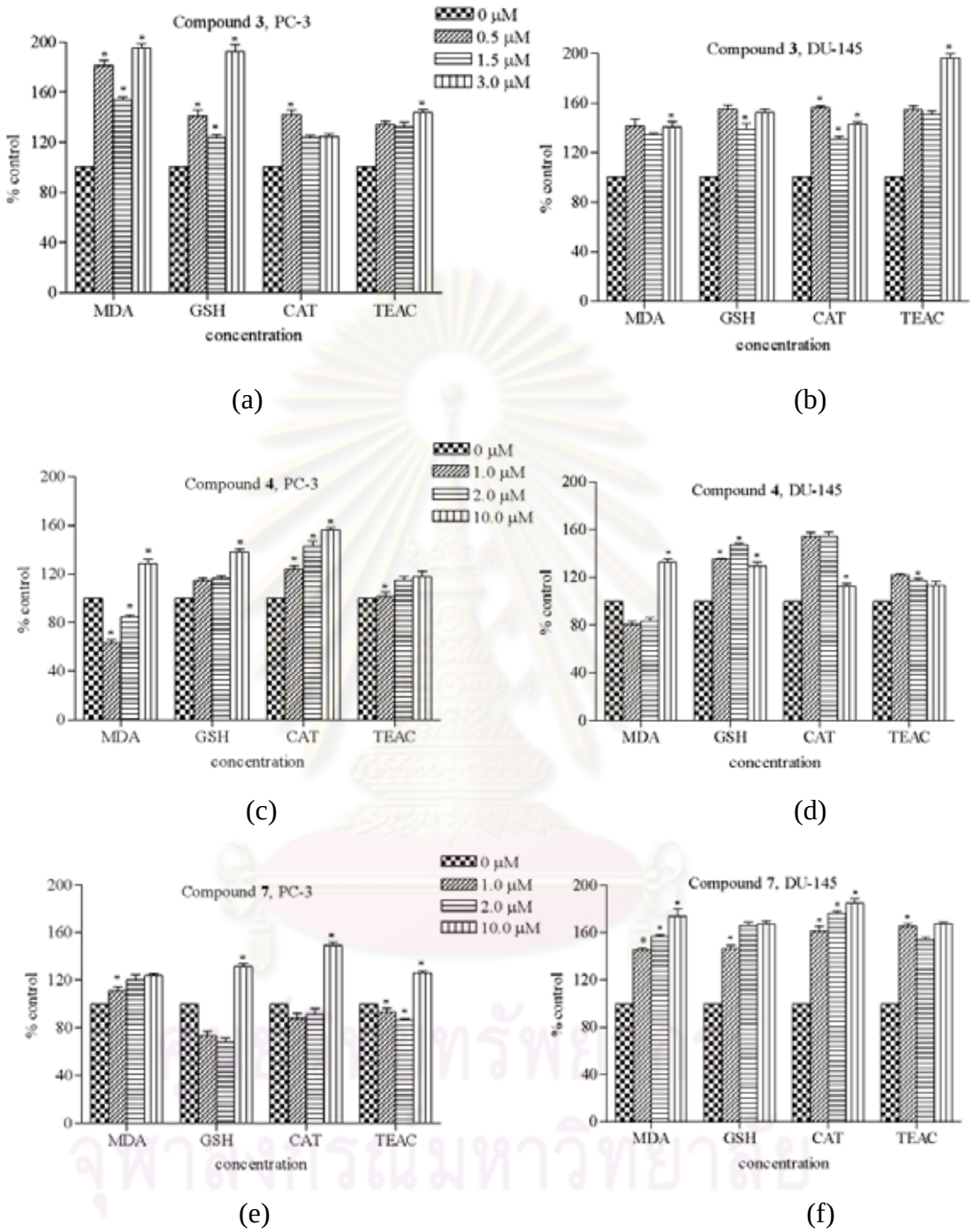


Figure 5.3 Change on MDA levels (ng μg^{-1} protein) , GSH levels (mg mg^{-1} protein) and CAT (μmoles mg^{-1} protein min^{-1}), TEAC activities (μM Trolox mg^{-1} protein) with dictamnine, benzamide and tembamide in PC-3 and DU-145 prostate cancer cells (a – f) at various concentrations for 24 h.* $p<0.05$ versus untreated control as analyzed by one way ANOVA and Turkey's multiple-comparison test

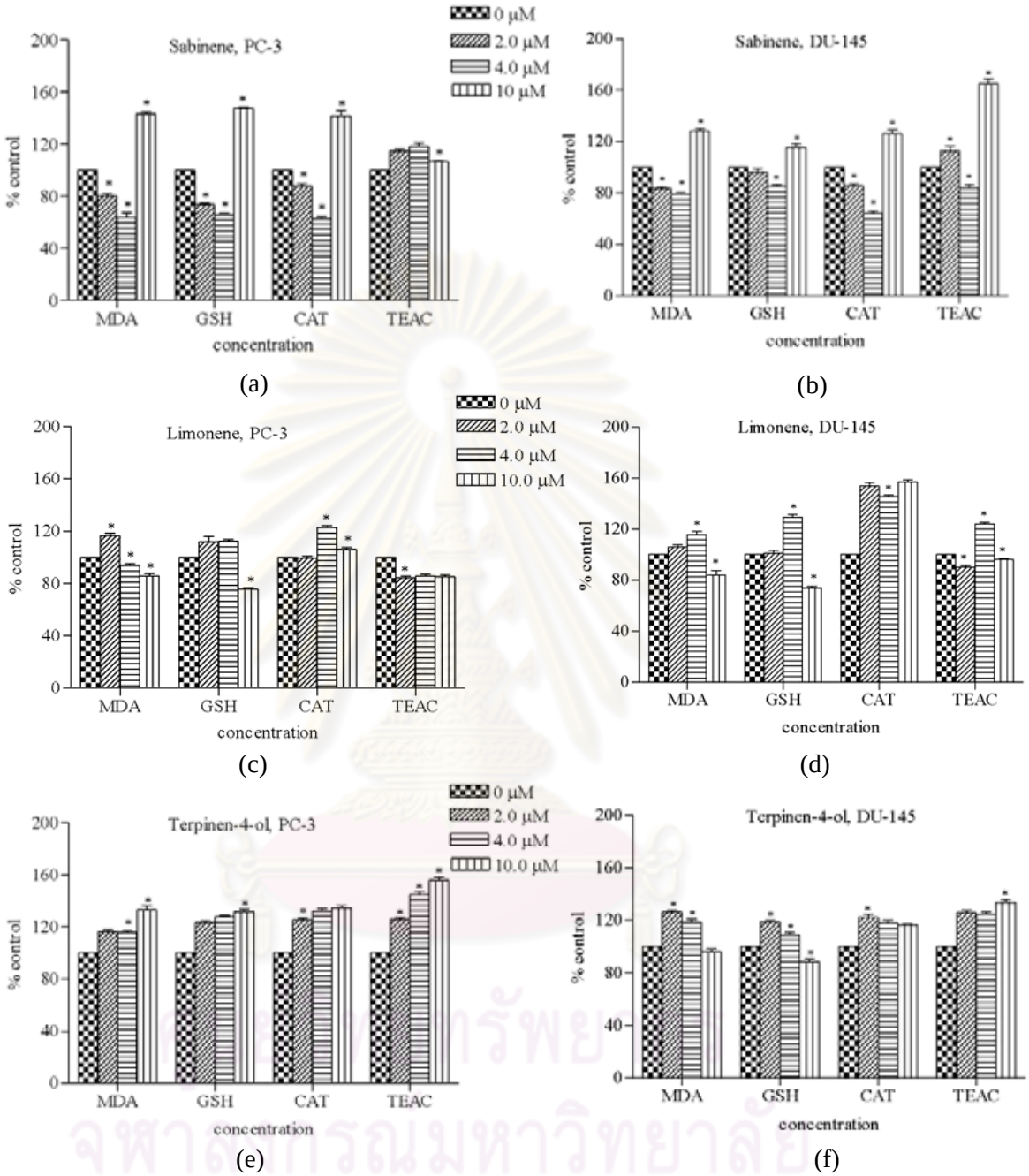


Figure 5.4 Change on MDA levels (ng μg^{-1} protein) , GSH levels (mg mg^{-1} protein) and CAT (μmoles mg^{-1} protein min^{-1}), TEAC activities (μM Trolox mg^{-1} protein) with sabinene, limonene and terpinen-4-ol in PC-3 and DU-145 prostate cancer cells (a–f) at various concentrations for 24 h.* $p<0.05$ versus untreated control as analyzed by one way ANOVA and Turkey's multiple-comparison test

membrane from the free radical caused lipid peroxidation. However, the indirect effect *via* intracellular antioxidant system might be possible.

According to **Figure 5.2** and **5.4**, the intracellular antioxidant systems were investigated in pretreated PC-3 and DU-145 with different concentrations of each sample. The CH_2Cl_2 extract of stems and essential oil pretreated both cell lines at the concentration of 0, 0.5, and 5 μg/mL, whereas the isolated compounds, Compund **3** pretreated cell lines at dose of 0.5, 1.5 and 3 μM, Compound **4** and Compound **7** pretreated cell line at 1, 2, and 10 μM, sabinene, limonene and terpinen-4-ol pretreated cell lines at 2, 4, and 10 μM. The concentration of the extracts and isolated compounds were previously proved not to influence cell growth *via* the proliferation assay.

The lipid peroxidation of CH_2Cl_2 extract of stems was found in DU-145 and PC-3, approximately 50% of control at 0.5 μg/mL**,** whereas the concentration at 5 μg/mL exhibited no effect to MDA levels in PC-3 and DU-145 pretreated cells. In addition, isolated compounds of CH_2Cl_2 extract of stems in all dose of Compound **3** and Compound **7** were increased the MDA levels when compared to the control group in both pretreated cells. However, the MDA level of both cell lysates pretreated with Compound **4** were decreased with concentration at 1 and 2 μM while an increased level was observed at a dose of 10 μM. On the other hand, the major compounds of essential oil were investigated lipid peroxidation in pretreated prostate cancer cells, the MDA levels of PC-3 and DU-145 pretreated with sabinene at dose of 2 and 4 μM seem to significantly decreased compared to the control group, approximately 20-30% of control and significantly increase in MDA level was observed at a dose of 10 μM. MDA levels in PC-3 and DU-145 pretreated with limonene at all dose seemed to exert no effect. In addition, the concentration of 2 and 4 μM of Compound **7** displayed slightly increase on the MDA level in both pretreated cells while Compound **7** at dose of 10 μM exhibited no effect in pretreated DU-145.

5.2.2 Catalase activity

CAT activity from cell lysates pretreated with CH_2Cl_2 extract of stems in both PC-3 and DU-145 significantly increased in a dose dependent manner. This was also

found in cell lysates pretreated with essential oil in PC-3. The same magnitude of CAT elevation was observed in cell lysates pretreated with essential oil in DU-145 (**Figure 5.1**).

As the results presented in **Figure 5.3**, the same pattern of higher increase in CAT activity could be observed in DU-145 pretreated with all concentration of Compound **3**, Compound **4**, and Compound **7**. In addition, Compound **3**, 0.5 and 1.5 μM, and tembamide, 1 and 2 μM, exhibited no effect to CAT activity in pretreated PC-3 cell lysates, while Compound **3** 3 μM, Compound **7** 10 μM and all dose of Compound **4** increased significantly compared to control group.

The data from **Figure 5.4** displayed that CAT activity of major compounds changed in essential oil of fruits. The activity of CAT was increased significantly when pretreated PC-3 and DU-145 cells with terpinen-4-ol at concentration of 0.5 and 1.5 μM. Limonene at all doses were increased in pretreated PC-3 cells compared to the control. The both cells pretreated with sabinene at concentration of 2 and 4 μM significantly decreased CAT activity compare with the control cells. On the other hand, the both cells were pretreated with sabinene at concentration of 10 μM, a marked increase in the CAT activity.

5.2.3 Glutathione Level

The result from **Figure 5.1** showed that reduced GSH levels of the cell lysate from PC-3 24 h-pretreated with the CH_2Cl_2 extract of stems at concentration of 0.5 μg/mL seemed to significantly increase compared to the control group 111.97 ± 3.77 and 112.48 ± 3.23%, respectively. The increase percentage was lower when the concentration of CH_2Cl_2 extract of stems was increased 10 times, 104.83 ± 4.85 and 104.20 ± 3.11%, respectively. The same pattern of higher increase in reduced GSH could be observed in DU-145 pretreated cell lysates. GSH level in PC-3 cell lysates pretreated with essential oil at 0.5 μg/mL was slightly decreased but significantly decreased with essential oil at 5 μg/mL.

Cell lysates from PC-3 and DU-145 pretreated with Compound **3** and Compound **4** at all concentrations, GSH levels were found to increase with difference magnitude compared to the control (**Figure 5.3**). However, GSH in PC-3 pretreated

cell lysates was decreased with Compound **7** at 1 and 2 μM whereas treatment of PC-3 with Compound **7** at dose of 10 μM was found to increase the level of GSH compared to control group. In contrast, Compound **7** in all doses were increased the GSH levels in pretreated DU-145 cell lysates.

Reduced GSH levels in PC-3 and DU-145 cell lysates pretreated with sabinene at 2 and 4 μM were increased (approximately 20-30% of control) and magnitude of GSH levels elevation was observed in sabinene at dose of 10 μM. In addition, GSH levels in both pretreated cell lysates with limonene at 2 and 4 μM was slightly increased and significantly decrease at 10 μM. For PC-3 pretreated cell lysates, GSH levels were increased by terpinen-4-ol at all doses. The GSH levels of DU-145 pretreated cell lysates was increased, approximately 20% of control, by terpinen-4-ol at 2 and 4 μM and was slightly decreased at 10 μM.

5.2.4 Trolox Equivalent Antioxidant Capacity (TEAC) Assay in Cell Lysates

The results from **Figure 5.2** and **5.4**, cell lysates from PC-3 and DU-145 at 24 h pretreated with CH_2Cl_2 extract of stems, essential oil of fruits and isolated compounds from stems and essential oil were also tested by TEAC assay. In all treatment, TEAC values were found to increase with difference magnitude compare to control.

In the previous study, the several isolated compounds were isolated from *Z. limonella* Alston. Somanabandhu *et al*. (1991) isolated five compounds from the bark of *Z. limonella*: ubiquitous lupeol, alkaloid rutaecarpine and three coumarins, xanthoxyletin, osthol and scopoletin. In addition, there has been no report regarding antioxidant compound in *Z. limonella* Alston. However, some of these compounds previously isolated from other plants were studied to possess antioxidant potential. Kim *et al.* (1997) reported that one of the components of *Artemisia iwayomogi*, scopoletin was unable to scavenge the $DPPH^{\bullet}$. In contrast, scopoletin was found to inhibit lipid peroxide and generation of superoxide and hydroxyl radicals. Kang *et al.* (1998) reported that the isolation of scopoletin from *Solanum lyratum* and protects hepatocyte from CCl_4-induced toxicity by maintaining the GSH content, the activity of SOD, and inhibiting the production of MDA as a result of its antioxidation and free

radical-scavenging effect. Lupeol, a triterpene found in many fruits and vegetables was found to contain antioxidant, antimutagenic, antiimflammatory effect in *in vitro* and *in vivo* system (Saleem *et al*. 2001; Geetha and Varalakshmi, 2001). Lupeol and its ester lupeol linoate effectively scavenge free radicals and reduce oxidative stress indices by enhancing the antioxidant capacity of the liver of cadmium treated rats (Sunitha *et al*., 2001).

These results showed that the intracellular antioxidant system, GSH and CAT were modified in PC-3 and DU-145 pretreated with extract, essential oil and isolated compounds at the various concentrations. The CH_2Cl_2 extract of stems and its isolated compounds, Compound **3**, Compound **4**, and Compound **7** seemed to act through GSH level and CAT activity, leading to the control of intracellular antioxidant power *via* an increasing TEAC value. These observations may support that the CH_2Cl_2 extract of stems and its isolated compounds might have antioxidant capacity *via* the thiol-regulated cellular activity. GSH, the major low-molecular-mass thiol in the cytoplasm, acts as a free radical scavenger, trapping ROS that would otherwise interact with cellular thiol through an enzyme-catalyzed reaction. Glutathione peroxidase (GPx) is used as an electron donor in the reduction of peroxidation, including lipid peroxide (Ghezzi *et al*., 2005).

On the other hand, the essential oil of fruit has high possibility to regulate CAT activity more than GSH level in both cell lines. CAT prevents the hydrogen peroxide from harming the cell itself. CAT is frequently used by cells to rapidly catalyze the decomposition of hydrogen peroxide into less reaction gaseous oxygen and water molecules (Gaetani, 1996). In addition, major components of essential oil, sabinene, limonene, and terpinen-4-ol seemed to function *via* both GSH and CAT activity. Data obtained by GSH level and CAT activity assay indicated that limonene and sabinene at all concentration exhibited slightly increase in both pretreated cell lysates. In contrast, GSH level and CAT activity decrease in both pretreated cell lysates with sabinene at 2 and 4 μM. It is proposed that the decreased in GSH level and CAT activity in cell lysates may partially be due to control MDA levels in pretreated cells. The levels of glutathione and CAT activity were increased significantly in the PC-3 and DU-145 pretreated with sabinene at 10 μM. This

concentration of sabinene might make a cytotoxic in both cells. The increased GSH level and CAT in cells were returned MDA to normal levels as in control group.

In summary, the antioxidant capability of the CH_2Cl_2 extract of stems, essential oil of fruits, and isolated compounds from *Z. limonella* was found in a wide range depended on various parts of plant. The all treatments expressed quite and interesting indirect action *via* intracellular antioxidant content, GSH and CAT activity, to monitor the lipid peroxidation process. The gene expression of catalase and enzymes involved in glutathione metabolism should be further studied for the well understanding of these contents from *Z limonella*.

5.3 Experimental Section

5.3.1 DPPH Assay

The $DPPH^{\bullet}$ test is the conventional $DPPH^{\bullet}$ capacity assay widely use for plant, food on natural product to screen and evaluate the free radical-scavenging effect. The radical form $DPPH^{\bullet}$ with dark-blue color can be protonated by the antioxidant compounds and reduced to the more stable radical $DPPH^{\bullet}$ with the yellow colored diphenylpicrylhydrazine and terminate radical chain reaction.

Dimethylsulphoxide (DMSO) was used to dissolve the crude extracts. Serial dilutions of extracts were carried out to obtain a suitable concentration. A 250 μL of BHT or diluted extract and compound were added to 250 μL DPPH ethanolic solution (2.4 mg in 100 mL ethanol). After incubation at ambient temperature for 20 min, the absorbance was monitored against ethanol as a blank at 520 nm by UV-spectrophotometer (Shimadzu, UV-160A). The percentage of scavenged DPPH was calculated as the percentage of inhibition using the following formula.

$$\% \text{ inhibition} = [(A_{blank} - A_{extract/compound}) / A_{blank}] \times 100$$

where A_{blank} and $A_{extract/compound}$ are the absorbance of blank and extract/compound, respectively. Ethanolic solution of butylated hydroxyltoluene (BHT) was used as positive control. DPPH, BHT, and reconstituted solution were freshly prepared. All extracts were performed in triplicate and concentration of extracts/compound

exhibited 50 % inhibition (IC_{50}) obtained from dose response curve was averaged and used to compare the scavenging activity of each extract.

5.3.2 Trolox Equivalent Antioxidant Capacity (TEAC) Assay

2,2/-azinodi-3-ethylbenzthiazoline sulphonate (ABTS) and potassium persulphate solution were dissolved in deionized water to a concentration of 14 and 5 mM, respectively. The reaction mixture was left at ambient temperature for 12 to 16 h in a dark place to get the blue-green coloured $ABTS^{\bullet+}$ radical cation. The radical cation solution was diluted with deionized water to obtain an absorbance of 0.8 ± 0.05 at 734 nm. The water soluble vitamin E analogue, Trolox®, or various concentrations of extracts prepared by serial dilutions were added to the dilute radical cation solution. The decrease in absorbance after 1 min was spectrophotometically read at 734 nm. All of the assays were performed at least in triplicates. The $ABTS^{\bullet+}$ radical cation solution was freshly prepared daily. The decline in absorbance after 1 min caused by Trolox or extracts was plotted against concentration. The TEAC value obtained from the ratio between slopes of the linear plot of the slope of Trolox.

5.3.3 Cell Cultures

Human prostate adenocarcinoma cell lines DU-145 and PC-3 were a generous gift from Professor Thompson EW, Department of Surgery, St. Vincent's Institute, the University of Melbourne, Australia. The cancer cells were grown in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin as monolayer in 55 cm^2 tissue culture dishes. Both cell cultures were maintained at 37°C in CO_2 incubator at a humidified atmosphere containing 5% CO_2 until 80% confluency and then subcultered twice a week

5.3.4 Lipid Peroxidation Determination

Whole tumor cell lysates were obtained by repeated freeze-thaw procedures according to the protocol previously described by Ohkawa *et al*., (1979). Briefly, cancer cells were trypsinized, harvested, washed with phosphate buffered saline (PBS), subjected to repeated freeze-thaw procedures in ice-cold bath, and centrifuged.

The supernatant was collected and gently vortexed to get a uniform suspension. Homogenous lysates from cancer cells pretreated with various concentrations of crude extracts were used for lipid peroxidation measurement using thiobarbituric acid reactive substance (TBARS). For untreated cancer cells, lysates were combined with various concentrations of crude extract followed by the lipid peroxidation assayed using TBARS.

Thiobarbituric acids tested for malondialdehyde (MDA) were used as a lipid peroxidation following the method previously described by Ohkawa *et al*. (1979). Briefly, a 500 µL aliquot of cell lysates was combined with a reaction mixture containing 75 µL of 8.1% SDS, 565 µL of 20% acetic acid, and 565 µL of 0.8% thiobarbituric acid (TBA). The resulting mixture was vigorously mixed, incubated in a water bath at 95 °C for 1 h, and cooled to room temperature with tap water. A 500 µL of *n*-butanol and pyridine (15:1, v/v) mixture was added to each sample, shaken vigorously, and centrifuged at 1200 g for 10 min. The supernatant fraction with the pink color of MDA-TBA complex was isolated and the absorbance at 532 nm was measured against mixture of *n*-butanol and pyridine as a blank. The same procedure was repeated with malonaldehyde bis(dimethyl acetal) as a positive control. The content of lipid peroxidation was expressed as nM MDA per mg protein by interpolation in a standard curve in water covering a concentration range of 0-200 mM. Protein concentration was estimated by Bradford's method. All samples were conducted independently in triplicate.

5.3.5 Catalase (CAT) Assay

CAT catalyzes the decomposition of H_2O_2 molecules into water and oxygen. The reaction kinetics of CAT activity was conducted at 25 °C using 50 mM phosphate buffer of pH 7.0 containing H_2O_2 as a substrate. After the 1-min incubation with cell lysates, the decrease of absorbance at 240 nm was monitored as the CAT activity to decompose hydrogen peroxide molecules. A molar extinction coefficient of 43.6 $M^{-1}cm^{-1}$ for H_2O_2 was used.

5.3.6 Glutathione (GSH) Assay

The total GSH contents of the cell lysates were estimated by colorimetric assay using the reaction between sulphydryl group of 5,5/-dithiobis-2-nitrobenzoic acid (DTNB) to produce a yellow-color of 5-thio-2-nitrobenzoic acid (TNB). TNB production is directly proportional to the concentration glutathione in cell lysates. All experiments were done in triplicate. Total glutathione was determined by measuring the absorbance 410 nm after 30 min incubation. The results were expressed in µmole per mg protein.

5.3.7 Statistical Analysis

The statistic analysis of the obtained data was performed by SPSS (version 11.5) software for window. Data were statistically analyzed using one-way ANOVA and Turkey's multiple comparison testes in which the significance level was defined as $p < 0.05$.

CHAPTER VI

CONCLUSION

Previous results of the various parts of *Z. limonella* were screened for their antimicrobial and antioxidant activities. Essential oil of fruits and the CH_2Cl_2 extract of stems were displayed the effective inhibiting the growth of tested microorganisms and showed the highest level of antioxidant activity. This work involves continuing studies on the chemical constituents and biological activity of essential oil of fruits and the CH_2Cl_2 extract of stems.

The essential oil from the fruits contain terpinoid group as a major components such as sabinene (42.73%), limonene (39.05%) and terpinen-4-ol (5.40%), respectively. The essential oil of fruits and major components were investigated for antifungal and antibacterial activities. The essential oil was exhibited strong activity against strains of phytopathogenic fungi and major component, terpinen-4-ol. In addition, the crude essential oil of fruits and bioactive component, sabinene, possessed a broad spectrum antibacterial activity. The rate of killing of the essential oil and sabinene appeared to be time and concentration dependent. The essential oil and sabinene were rapidly bactericidal at 2×MBC and 4×MBC achieving a complete elimination of MSSA and *E. coli* within 10 min, but MRSA and ESBL were killed within 90 min.

The chemical constituents of the CH_2Cl_2 extract of stems of *Z. limonella* led to the isolation of novel quinoline alkaloid, Compound **6**, along with five known compounds and one mixture of steroid. The known compounds included one lignan; (−)-asarinin (**2**), two aromatic amides; dihydroalatamide (**4**) and (−)-tembamide (**7**), one furoquinoline alkaloid, dictamnine (**3**), and one benzophenanthridine alkaloid; *N*-nornitidine (**5**), three components in mixture 1 such as β-sitosterol (59%), campesterol (32%), and stigmasterol (9%). All isolated compounds from the CH_2Cl_2 extract of stem from *Z. limonella* were summarized in **Table 6.1**.

Table 6.1 Structures of the isolated compounds from the CH_2Cl_2 extract of stems from *Z.limonella* and their physical properties

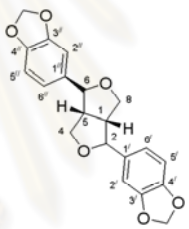
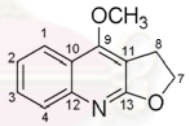
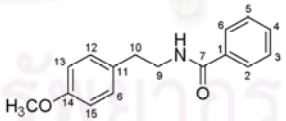
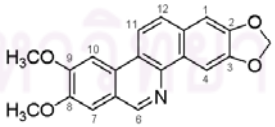
Compounds	Remarks	Molecular formula	Structure	Melting point (°C)	$[\alpha]^{20}_D$
(–)-Asarinin (**2**)	Colorless needles	$C_{20}H_{18}O_6$		120-122°C	-111 (c 0.1, $CHCl_3$)
Dictamnine (**3**)	Colorless needles	$C_{12}H_{11}NO_2$		131-132°C	-
Dihydroalatamide (**4**)	White needles	$C_{16}H_{17}NO_2$		124-125°C	-
N-nornitidine (**5**)	Red needles	$C_{20}H_{15}NO_4$		279-280°C	-

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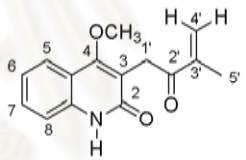
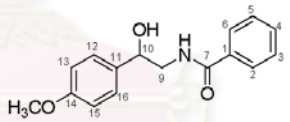
Table 6.1 (continued)

Compounds	**Remarks**	**Molecular formula**	**Structure**	**Melting point (°C)**	**$[\alpha]^{20}_D$**
Compound **6**, new compound	White needles	$C_{15}H_{15}NO_2$		138-139°C	-
(–)-tembamide (**7**)	Colorless solid	$C_{16}H_{17}NO_3$		152-153°C	-12 (*c* 0.1, MeOH)

The CH_2Cl_2 extract of stem and major components (Compound **3**, **4** and **7**) were investigated antimicrobial activity. The IC_{50}, MIC, and MFC values against tested phytopathogenic fungi of Compound **3** were more potent than the CH_2Cl_2 extract of stem. Compound **3** showed the strongest antifungal activity. On the other hand, the MIC and MCC values of the CH_2Cl_2 extract of stems and Compound **3** were found only active against tested gram-positive bacteria and yeast, *C. albicans*. Compound **3** was determined as a major compound and possessed the antifungal and antibacterial activities.

In this study, the CH_2Cl_2 of stems, the essential oil from fruits and their major components were investigated for primary antioxidative potential in cell-free system and extend to prostate cancer cell lines (PC-3 and DU-145). The CH_2Cl_2 extract of stems and isolated compounds such as Compoud **3**, **4** and **7** showed higher antioxidant capacity than the essential oil of fruits and its major components. In addition, the evaluation of MDA levels and intracellular antioxidant system (CAT, GSH) were investigated in pretreated PC-3 and DU-145 with the CH_2Cl_2 extract of stem, essential oil, and isolated compounds. The CH_2Cl_2 extract of stem and Compound **3**, **4** and **7** seemed to act through GSH level and CAT activity, leading to the control of intracellular antioxidant power *via* an increasing TEAC value. On the other hand, the essential oil of fruit has high possibility to regulate CAT activity more than GSH level in both cell lines. The all treatements expressed quite and interesting indirect action *via* intracellular antioxidant content, GSH and CAT activity, to monitor the lipid peroxidation process.

REFERENCES

Agosta, W. C. 1996. *Bombardier Beetles and Fever Trees*. New York. Addison-Wesley.

Ahmad, A., Davies, J., Randall, S. and Skinner, G. R. B. 1996. Antiviral properties of extract of *Opuntia streptacantha*. *Antiviral. Res.* 30: 75–85.

Ahsan, M., Zaman, T. A., Hasan, C. M., Ito, C. and Islam, S. K. N. 2000. Constituents and cytotoxicity of *Zanthoxylum rhesta* stem bark. *Fitoterapia.* 71: 697-700.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. and Walter, P. 2002. *Molecular Biology of the Cell*, 4th ed., New York: Garland Science.

Almahy, H. A., and Elegami, A. A. 2007. Alkaloids and antimicrobial activity of *Plumeria autifolia* (Rubra). *Int. J. Mole. Med. Adv. Sci.* 3: 12-15.

Ames, B. N., Shigenaga, M. K. and Hagen, T. M. 1993. Oxidants, antioxidants, and the degenerative diseases of aging. *Proc. Natl. Acad. Sci.* 90: 7915-7922.

Armstrong, J. S. 2006. Mitochondrial membrane permeabilization: the sine qua non for cell death. *BioEssays.* 28: 253–260.

Avorn, J. 1996. The effect of cranberry juice on the presence of bacteria and white blood cells in the urine of elderly women. What is the role of bacterial adhesion? *J. Adv. Exp. Med. Biol.* 408: 185-186.

Aydin, A., and others. 2006. Oxidative stress and antioxidant status in non-metastatic prostate cancer and benign prostatic hyperplasia. *Clin. Biochem.* 36: 176-179.

Babu, B. and Wu, J. T. 2008. Production of natural butylated hydroxytoluene as an antioxidant by freshwater phytoplankton. *J. Phycol.* 44: 1447-1454.

Bagchi, K. and Puri, S. 1998. Free radicals and antioxidants in health and disease. *East. Mediter. Health. J.* 4(2): 350-360.

Bakkali, F., Averbeck, S., Averbeck, D. and Idaomar, M. 2008. Biological effects of essential oils ─ A review. *Food Chem. Toxicol.* 46: 446-475.

Bandow, J. E., Brotz, H., Leichert, L. I. O., Labischinski, H. and Hecker, M. 2003. Proteomic approach to understanding antibiotic action. *Antimicro. Agents Chemotherap.* 47: 948-955.

Batista, O., Duarte, A., Nascimento, J. and Simones, M. F. 1994. Structure and antimicrobial activity of diterpenes from the roots of *Plectranthus hereroensis*. *J. Nat. Prod.* 57: 858-861.

Beuchat, L. R. 1983. Influence of water activity on growth, metabolic activities and survival of yeasts and molds. *J. Food Prot.* 46(2): 135-141.

Bhattacharya, S. and Zaman, M. K. 2009. Pharmocognostical the evaluation of *Zanthoxylum nitidum* bark. *Int. J. Pharm.Tech. Res.,* 1: 292-298.

Bhoga, U., Mali, R.S. and Adapa, S.R. 2004. New synthesis of linear furoquinoline alkaloids. *Tetrahedron Lett.* 45: 9483-9485.

Biesalski, H. K. and McGregor, G. P. 2007. Antioxidant therapy in critical care is the microcirculation the primary target? *Crit. Care. Med.* 35: 577-583.

Binns, S. E., Arnason, J. T. and Baum, B. R. 2002. Phytochemical variation within populations of *Echinacea angustifolia* (Asteraceae). *Biochem. Syst. Eco.* 30: 837-854.

Bonjean, K., and others. 1998. The DNA intercalating alkaloid cryptolepine interferes with topoisomerase II and inhibits the primary DNA synthesis in B 16 melanoma cells. *Biochem.* 37: 5136-5146.

Boon, E.M., Downs, A. and Marcey, D. Catalase: H_2O_2: H_2O_2 Oxidoreductase. Catalase Structural Tutorial Text [online]. Availble from: http://biology. kenyon.edu /BMB/Chime/catalase/frames/cattx.htm. [2007, Feb 11].

Borris, R. P. 1996. Natural products research: The perspectives from a major of the pharmaceutical company. *J. Ethnopharmacol*. 51: 29-38.

Boulware, R. T. and Stermitz, F. R. 1981. Some alkaloids and other constituents of *Z. microcarpum* and *Z. procerum*. *J. Nat. Prod.* 44: 200-205.

Brantner, A., Males, Z., Pepeljnjak, S. and Antolic, A. 1996. Antimicrobial activity of *Paliurus spina*-christi mill. *J. Ethnopharmacol.* 52: 119–122.

Buckingham, J. 2005. *Dictionary of Natural Products on CD-ROM*. Boca Raton, Florida: Chapman and Hall/CRC Press.

Buettner, G. R. 1998. *Antioxidant enzymes and functions*. Sunrise Free Radical School for oxygen Society, Electronic publication.

Burt, S. 2004. Essential oils: their antibacterial properties and potential applications in foods—a review. *Int. J. Food Microbiol.* 94: 223–253.

Carson, C. F., Mee, B. J. and Riley, T. V. 2002. Mechanism of action of *Melaleuca alternifolia* (tea tree) oil on *Staphylococcus aureus* determined by time-kill, lysis, leakage and salt tolerance assays and electron microscopy. *J. Antimicrob. Agents. Chemother.* 46: 1914–1920.

Chabot, S., Bel-Rhlid, R., Chenevert, R. and Piche, Y. 1992. Hyphal growth promotion in vitro of the VA mycorrhizal fungus, Gigaspora margarita Becker and Hall, by the activity of structurally specific flavonoid compounds under CO_2-enriched conditions. *New. Phytol.* 122: 461–467.

Cheij, R. 1988. *The McDonald Encyclopedia of Medical Plants*. pp. 54-59. London: McDonald & Co. Ltd.

Chen, I. S., Chen, T. L., Chang, Y. L., Teng, C. M. and Lin, W. Y. 1999. Chemical constituents and biological activities of the fruit of *Zanthoxylum integrifoliolum*. *J. Nat. Prod.* 62: 833-837.

Chen, I. S., and others. 1995. Coumarins and anti-platelet aggregation constituents from *Zanthoxylum schinifolium*. *Phytochem.* 39: 1091-1097.

Chen, M. J., Yang, C. H., Lin, W. Y., Tsai, I. L. and Chen, I. S. 2002. Chemical constituents from the leave of *Zanthoxylum schinifolium*. *J. Chin. chem. soc.* 49: 125-128.

Cheng, M. J., Lin, C. F., Cheng, H. S. and Chen, I. S. 2008. Chemical constituents from the stem bark of *Zanthoxylum scadens*. *J. Chil. Chem. Soc.* 53: 1631-1634.

Cheng, M. J., Lin, C. F., Wang, C. J., Tsai, I. L. and Chen, I. S. 2007. Chemical constituents from the root wood of *Zanthoxylum integrifoliolum*. *J. Chin. chem. soc.* 54: 779-783.

Chu, F. F., Doroshow, J. H. and Esworthy, R. S. 1993. Expression, characterization, and tissue distribution of a new cellular selenium-dependent glutathione peroxidase GSHPx-GI. *J. Biol. Chem.* 268: 2571–2576.

Colin, N. O. 1991. Differential effects of condensed and hydrolyzable tannin on polyphenol oxidase activity of attine symbiotic fungus. *J. Chem. Eco.* 17: 1811-1819.

Cowan, M. M. 1999. Plant products as antimicrobial agents. *Clin. Microbiol. Rev.* 12: 564-582.

Cox, S. D., and others. 2000. The mode of antimicrobial action of essential oil of *Melaleuca alternifolia* (tea tree oil). *J. Appl. Microbiol.* 88: 170–175.

Croteau, R., Kutchan, T. M. and Lewis, N. G. 2000. Natural products (secondary metabolites). In: *Biochemistry and Molecular Biology of Plants.* ed., American Society of Plant Physiologists.

Dassnneville, L., and others. 2000. Cytotoxicity and cell cycle effect of the plant alkaloids cryptolepine and neocryptolepine: relation to a drug-induced apoptosis. *Eur. J. Pharmacol.* 409: 9-18.

Davidson, P. M. 2001. Chemical preservatives and natural antimicrobial compounds. In M. P. Doyle, L. R. Beuchat, and T. J. Montville (ed.), *Food microbiology: Fundamental and frontiers*, pp. 593–627. Washington, DC: ASM Press.

de Moura, N. F., and others. 2002. Alkaloids, amides and antipasmodic activity of *Zanthoxylum hyemale*. *Planta Med.* 68: 534-538.

Dekkers, J., van Doornen, L. and Kemper, H. 1996. The Role of Antioxidant Vitamins and Enzymes in the Prevention of Exercise-Induced Muscle Damage. *Sports. Med.* 21: 213-238.

Dewick, P. M. 2002. *Medicinal Natural Products.* New York: John Wiley and Sons.

Di Pasqua, R., Hoskins, N., Betts, G. and Mauriello, G. 2006. Changes in membrane fatty acids composition of microbial cells induced by addiction of thymol, carvacrol, limonene, cinnamaldehyde, and eugenol in the growing media. *J. Agric. Food. Chem.* 54: 2745–2749.

Didry, N., Pinkas, M. and Dubreil, L. 1986. Activité antibacterienne de naphtho-quinones d'origine végetale. *Annal. Pharmaceu. Françaises.* 44: 73-78.

Diekema, D. J., Dodgson, K. J., Sigurdardottir, B. and Pfaller, M. A. 2004. Rapid detection of antimicrobial-resistant organism carriage: an unmet clinical need. *J. Clin. Microbiol.* 42: 2879–2883.

Dixon, R. A., Dey, P. M. and Lamb, C. J. 1983. Phytoalexins: enzymology and molecular biology. *Adv. Enzymol.* 55: 1–69.

Dogazaki, C., Shindo, T., Furuhata, K. and Furuyama, M. 2002. Identification of chemical antibacterial components against *Legionella pneumophila* in a coffee beverage. *J. Pharm. Soc. Jpn.* 122: 487-494.

Dorman, H. J. D. and Dean, S. G. 2000. Antimicrobial agents from plants: anti-bacterial activity of plant volatile oils. *J. Appl. Microbiol.* 88: 308–316.

Duffy, C. F. and Power, R. F. 2001. Antioxidant and antimicrobial properties of some Chinese plant extracts. *Int. J. Antimicrob. Agents.* 17: 527-529.

Duke, J. A. 1985. *Handbook of medicinal herbs*, ed., Florida: CRC Press

Eckert-Maksic, M., Kovacek, I., Maksic, Z. B., Osmak, M. and Paveli, K. 1991. Effect of ascorbic acid and its derivatives on different tumors in vivo and in vitro. In Z. B. Maksic and M. Eckert-Maksic, (eds.), *Molecules in Natural Science and Medicine*, pp. 509-524. New York: Ellis Horwood.

Edris, A. E. and Farrag, E. S. 2003. Antifungal activity of peppermint and sweet basil essential oils and their major aroma constituents on some plant pathogenic fungi from the vapour phase *Nahrung. Food.* 47: 117–121.

Esen, G., Azaz, A. D., Kurkcuoglu, B., Baser, K. H. C. and Tınmaz, A. 2007. Essential oil and antimicrobial activity of wild and cultivated *Origanum vulgare* L. subsp. hirtum (Link) letswaart from the Marmara region. *Turkey. Flav. Fragr. J.* 22: 371-376.

Fee, J. A. and Teitelbaum, H. D. 1972. Evidence that superoxide dismutase plays a role in protecting red blood cells against peroxidative hemolysis. *Biochem. Biophys. Res. Commun.* 49: 150- 158.

Fessenden, R. J. and Fessenden, J. S. 1982. *Organic chemistry*, 2nd ed., Boston, Mass: Willard Grant Press.

Fish, F., Gray, A. I. and Waterman, P. G. 1975. Coumarin, alkaloid and flavonoid constituents from the root and stem bark of *Zanthoxylum avicennae*. *Phytochem.* 14: 840-841.

Frei, B. 1999. On the role of vitamin C and other antioxidants in atherogenesis and vascular dysfunction. *Proc. Soc. Exp. Biol. Med.* 222: 196–204.

Fujii, T., Shimaya, C., Yano, A., Terado, K., Sugino, H. and Fukuda, H. 2002. Suppression of the growth of the basidiomycete yeast *Rhodotorula minuta*, by cinnamic acid. *Biotechnol. Lett.* 24: 151–154.

Funatogawa, K., and others. 2004. Antibacterial activity of hydrolyzable tannins derived from medicinal plants against *Helicobacter pylori*. *Microbiol. Immunol.* 48(4): 251-261.

Furuhata, K., Dogasaki, C., Hara, M. and Furuyama, M. 2002. Inactivation of *Legionella pneumophila* by phenol compounds contained in coffee. *J. Antibact. Antifung. Agents.* 30: 291-297.

Gaetani, G., Ferraris, A., Rolfo, M., Mangerini, R., Arena, S. and Kirkman, H. 1996. Predominant role of catalase in the disposal of hydrogen peroxide within human erythrocytes. *Blood.* 87: 1595–1599.

Garbor, M. 1986. Anti-inflammatory and anti-allergic properties of flavonoids. In *Biochemical Pharmacological and Structure Activity Relationships*, pp. 471-480. Alan: Liss Inc.

Geetha, T. and Varalakshmi, P. 2001. Anti-inflammatory activity of lupeol and lupeol linoleate in rats. *J. Ethnopharmacol.* 76: 77–80.

Ghezzi, P. 2005. Oxidoreduction of protein thiols in redox regulation. *Biochem. Soc.T.* 33: 1378-1381.

Giulia, D. C., Nicola, M., Angelo, A. and Francesco, C. 1999. Flavonoids: Old and new aspects of a class of natural therapeutic drugs. *Life. Sci.* 65: 337-353.

Goodsell, D. S. 2004. Catalase. Molecule of the Month. RCSB Protein Data Bank [on line]. Available from http://www.rcsb.org/pdb/static.do?p=education_ discussion/molecule_of_the_month/pdb57_1.html. [2004, Sep 1].

Guittat, L., and others. 2003. Interaction of cryptolepine and neocryptolepine with unusual DNA structures. *Biochem.* 85: 535-541.

Halliwell, B. 1994. Free radicals, antioxidants and human disease: curiosity, cause or consequence. *The Lancet.* 344: 721-724.

Halliwell, B. 1995. Oxidation of low-density lipoproteins: questions of initiation, propagation, and the effect of antioxidants. *Am. J. Clin. Nutr.* 61: 670-677.

Hara, Y. 1997. Influence of tea catechins on the digestive tract. *J Cell. Biochem.* 27: 52-58.

Hara, Y. and Ishigami, T. 1989. Antibacterial activities of tea polyphenols against foodborne pathogenic bacteria. *J. Jpn. Soc. Food Sci. Technol.* 36: 996-999.

Haslam, E. 1989. *Plant polyphenols: vegetable tannins revisited*. New York: Cambridge university press.

Haslam, E. 1996. Natural polyphenols (vegetable tannins) as drugs: possible modes of action. *J. Nat. Prod.* 59: 205-215.

Helander, I. M., and others. 1998. Characterization of the action of selected essential oil components on Gram-negative bacteria. *J. Agr. Food. Chem.* 46: 3590–3595.

Herrera, E. and Barbas, C. 2001. Vitamin E: action, metabolism and perspectives. *J. physio. biochem.* 57(2): 43-56.

Hostettmann, K., Terreaux, C., Marston, A. and Patterat, O. 1997. The role of planar chromatography in the rapid screening and isolation of bioactive compounds from medicinal plants. *J. Planar. Chromat.* 10: 251–257.

Hsiao, J. J. and Chiang, H. H. 1995. Lignans from the wood of *Aralia bipinnata*. *Phytochem.* 39: 899-902.

Hu, J., and others. 2007. Alkaloids from *Zanthoxylum nitidum* (Roxb.) DC. *Biochem. sys. eco.* 35: 114-117.

Ikigai, H., Nakae, T., Hara, Y. and Shimamura, T. 1993. Bactericidal catechins damage the lipid bilayer. *Biochimica. et. Biophysica. Acta.* 1147: 132–136.

Ishii, I., Ishikawa, T. and Chen, I. S. 1973. Alkaloids of Rutceous plants. XXII. Cuspidiol, a new monomeric phenyl propaneid. *Tetrahedron Lett.* 14: 4189-4192.

Islam, A., Sayeed, A., Bhuiyan, M. A. S. and Ashik, M. 2001. *In vitro* Antimicrobial effect of three terpenes, isolated from the bark of *Zanthoxylum budrunga*. *Pakist. J. Biol. Sci.* 6: 711-713.

Islam, A., Sayeed, A. Bhuiyan, M. S. A., Mosaddik, M. A. and Astaq Modal Khan, G. R. M. 2001. Antimicrobial activity and cytotoxicity of *Zanthoxylum budrunga*. *Fitoterapia.* 72: 428-430.

Ittipanichpong, C., Ruangrungsi, N. and Pattanaautsahakit, C. 2002. Chemical compositions and pharmacological effect of essential oil from the fruit of *Zanthoxylum limonella*. *J. Med. Assoc. Thai.* 85: 344-353.

Jirovetz, L., Buchbauer, G., Shafi, M. and Saidutty, A. 2006. Analysis of the aroma compounds of the essential oil of seeds of the spice plant *Zanthoxylum rhetsa* from southern India. *ZLebensm. Unters. Forsch.* 206: 228-229.

Joanny, F. 2005. Superoxide dismutase (SOD), a powerful antioxidant, is now available orally. *Phytothérapie.* 3: 1-4.

Juglal, S., Govinden, R. and Odhav, B. 2002. Spice oil for the control of co-occuring mycotoxin producing fungi. *J. Food. Protect.* 65: 683–687.

Kaczmarski, M., Wójcicki, J., Samochowiec, T., Dutkiewicz, T. and Sych, Z. 1999. The influence of exogenous antioxidants and physical exercise on some parameters associated with production and removal of free radical. *Pharmazie.* 54: 303-306.

Kallner, A., Hartmann, D. and Hornig, D. 1981. On the requirements of ascorbic acid in man: steady-state turnover and body pool in smokers. *Am. J. Clin. Nutr.* 34: 1347-1355.

Kang, S. Y., Sung, H., Park, J. H. and Kim, Y. C. 1998. Hepatoprotective activity of scopoletin, a constituent of *Solanum lyratum*. *Arch. Pharmacal. Res.* 21: 718–722.

Karlsson, J. 1997. Introduction to Nutraology and Radical Formation. In: *Antioxidants and Exercise*. pp. 1-143. Illinois: Human Kinetics Press.

Kazmi, M. H., Malik, A., Hameed, S., Akhtar, N. and Noor Ali, S. 1994. An anthraquinone derivative from *Cassia italica*. *Phytochem.,* 36: 761–763.

Kersten, W. 1971. Inhibition of RNA synthesis by quinone antibiotics. *Prog. Mol. Subcell. Biol.* 2: 48-57.

Kim, J. M., Marshall, M. R., Cornell, J. A., Preston, J. F. and Wei, C. I. 1995. Antibacterial activity of carvacrol, citral and geraniol against *Salmonella typhimurium* in culture medium and on fish cubes. *J. Food. Sci.* 60: 1364-1368.

Kim, S. S., Lee, C. K., Kang, S. S., Jung, H. A. and Choi, J. S. 1997. Chlorogenic acid, an antioxidant principle from the aerial parts of *Artemisia iwayomogi*. *Arch. Pharmacal. Res.* 20: 148-154.

Kinnula, V. L., Crapo, J. D. and Raivio, K. O. 1995. Generation and disposal of reactive oxygen metabolites in the lung. *Lab. Invest.* 73: 3-19.

Kocacaliskan, I., Talan, I. and Terzi, I. 2006. Antimicrobial activity of catechol and pyrogallol as allelochemicals. *Z. Naturforsch.* 61: 639-642.

Kordali, S., Cakir, A., Mavi, A., Kilic, H. and Yildirim, A. 2005. Screening of chemical composition and antifungal and antioxidant activities of the essential oils from three Turkish Artemisia species. *J. Agric. Food Chem.* 53: 1408–1416.

Kostova, I. 2005. Synthetic and natural coumarins as cytotoxic agents. *Curr. Med. Chem. Anti-Cancer. Agents.* 5: 29–46.

Kostova, I. 2007. Studying plant-derived coumarins for their pharmacological and therapeutic properties as potential anticancer drugs. *Expert. Opin.Drug. Discov.* 2: 1605-1618.

Köhler, T., Pechère, J. C. and Plèsiat, P. 1999. Bacterial antibiotic efflux systems of medical importance. *Cell. Mol. Life Sci.* 56: 771-778.

Lambert, R. J. W., Skandamis, P. N., Coote, P. and Nychas, G. J. E. 2001. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *J. Appl. Microbiol.* 91: 453–462.

Lee, D. M., Lee, J. C., Jeong, N. and Lee, K. I. 2007. Asymmetric transfer hydrogenation of 2-tosyloxy-1-(4-hydroxyphenyl)ethanone derivatives: synthesis of R-tembamide, (R)-aegeline, (R)-octopamine, and (R)-denopamine. *Tetrahedron Asymm.* 18: 2662-2667.

Lee, S. J. and Lim, K. T. 2007. Glycoprotein of *Zanthoxylum piperitum* DC. has a hepatoprotective effect via anti-oxidative character *in vivo* and *in vitro*. *Toxicol. In Vitro.* 22: 376–385.

Liao, S., Umekita, Y., Guo, J., Kokontis, J. M. and Hiipakka, R. A. 1995. Growth inhibition and regression of human prostate and breast tumors in athymic mice by tea epigallocatechin gallate. *Cancer Lett.* 96: 239-243.

Livermor, D. M. 1995. Beta-Lactamase in laboratory and clinical resistance. *Clin. Microbiol. Rev.* 8: 557-584.

Mahmoud, S. S. and Croteau, R. B. 2002. Strategies for transgenic manipulation of monoterpene biosynthesis in plants. *Trends. Plant. Sci.* 7: 366– 373.

Manosroi, A., Saraphanchotiwitthaya, A. and Manosroi, J. 2006. Effects of *Pouteria cambodiana* extracts on in vitro immunomodulatory activity of mouse immune system. *Fitoterapia.* 77: 189-193.

Marina, S. and Leo, J. L. D. 2006. Antimicrobial activity of essential oils and their components against the three major pathogens of the cultivated buttom mushroom, *Agaricus bisporus*. *Eur. J. Plant. Pathol.* 116: 211-224.

Marklund, S. L. 1985. Pyrogallol autoxidation. In R. A. Greenwald (ed.), *Handbook of Methods for Oxygen Radical Research*, pp. 243–247. Boca Raton, Florida: CRC Press.

Martin, M. T., Rasoanaivo, L. H. and Raharisololalao, A. 2005. Phenanthridine alkaloids from *Zanthoxylum madagascariense*. *Fitoterapia.* 76: 590-593.

Martin, Y. C., Bustard, T. M. and Lynn, K. R. 1973. Relationship between physical properties and antimalarial activities of 1, 4-naphthoquinones. *J. Med. Chem.* 16: 1089-1093.

Mason, T. L. and Wasserman, B. P. 1987. Inactivation of red beet betaglucan synthase by native and oxidized phenolic compounds. *Phytochem.* 26: 2197–2202.

Matern, U. and Lüer, P. 1999. Polyketides and Other Secondary Metabolites Including Fatty Acids and Their Derivatives. In U. Sankawa, D. H. R. Barton, K. Nakanishi, and O. Meth-Cohn (eds.), *Comprehensive Natural Products Chemistry*, pp. 623-637. UK: Elsevier Science Ltd.

Matsukawa, R., Dubinsky, Z., Kishimoto, E., Masak, K., Masuda, Y. and Takeuchi, T. 1997. A comparison of screening methods for antioxidant activity in seaweeds. *J. Applied. Phycol.* 9: 29–35.

May, J., Chan, C. H., King, A., Williams, L. and French, G. L. 2000. Time-kill studies of tea tree oils on clinical isolates. *J. Antimicrob. Chemoth.* 45: 639-643.

Menvielle-Bourg, F. J. 2005. Superoxide Dismutase (SOD), a Powerful Antioxidant, is now Available Orally. *Phytothérapie.* 3: 118-121.

Morton, R. A. 1985. *Biochemistry of Quinones*, ed., Academic Press, New York,

Moser, U. and Bendich, A. 1990. *Handbook of Vitamins*. New York: Marcel Dekker.

Muller, F. L., Lustgarten, M. S., Jang, Y., Richardson, A. and Van Remmen, H. 2007. Trends in oxidative aging theories. *Free. Rad. Biol. Med.* 43(4): 477-503.

Naidu, K. A. 2003. Vitamin C in human health and disease is still a mystery ? An overview. *Nutr. J.* 2: 1-10.

Najjar, S., Cordell, G. A. and Farnsworth, N. R. 1975. Alkaloids and coumarins from *Zanthoxylum belizense*. *Phytochem.* 14: 2309-2310.

Nakutsu, T., Lupo, A., Chinn, J. and Kang, R. 2000. Biological activity of essential oils and their constituents. Studies in Natural products chemistry. *Atta-ur-Rahman.* 21: 571-631.

Nayak, K. P. and Dutta, N. K. 1961. Role of essential oils and allied drugs in experimental cholera of the rabbit. *Indian. J. Med. Res.* 49: 51-54.

Naz, X., Ahmad, S., Ajaz, R. S., Asad, S. S. and Siddiqi, R. 2006. Antibacterial activity directed isolation of compounds from *Onosma hispidum*. *Microbiol. Res.* 161: 43-48.

Ngane, A. N., Biyiti, L., Zollo, P. H. A. and Bouchet, P. 2000. Evaluation of the anti-fungal activity of extracts of two Cameroonian rutaceae: *Zanthoxylum leprieurii* Guill., *Zanthoxylum xanthoxyloides* Waterm. *J. Ethnopharmacol.* 70: 335-342.

Ngassoum, M. B., Essia-Ngang, J. J., Tatsadjieu, L. N., Jirovetz, L., Buchbauer, G. and Adjoudji, O. 2003. Antimicrobial study of essential oils of *Ocimum gratissimum* leaves and *Zanthoxylum xanthoxyloides* fruits from Cameroon. *Fitoterapia.* 74: 284-287.

Nikaido, H. 1989. Outer membrane barrier as a mechanism of antimicrobial resistance. *Antimicrob. Agents Chemother.* 33: 1831-1836.

Nkerre, C. K. 2003. *Antibacterial properties of the stem bark extracts of Picralima nitida (Stapf) Th. and Hel Dur (Akumma plant).* Master's thesis, Michael Okpara University of Agriculture.

Nohl, H., Jordan, W. and Youngman, R. I. 1986. Quinones in Biology:Functions in electron transfer and oxygen activation. *Adv. Free. Rad. Biol. Med.* 2: 211-279.

Notka, F., Meier, G. and Wagner, R. 2004. Concerted inhibitory activities of *Phyllanthus amarus* on HIV replication in vitro and *ex vivo*. *Antiviral. Res.* 64: 93–102.

Novgorodov, S. A. and Gudz, T. I. 1996. Permeability transition pore of the inner mitochondrial membrane can operate in two open states with different selectivities. *J. Bioenerg. Biomembr.* 28: 139–146.

Nychas, G. J. E. 1995. Natural antimicrobials from plants. In: G. J. E. Nychas (ed.), *New Methods of Food Preservation*, pp. 58– 89. London: Blackie Academic and Professional.

O'Kennedy, R. and Thornes, R. D. 1997. *Coumarin: biology, applications, and mode of acion*. Chichester, UK: John Wiley&Sons.

Ohkawa, H., Ohishi, N. and Yagi, K. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Chem.* 95: 351-358.

Ojala, T., and others. 2000. Antimicrobial activity of some coumarin containing herbal plants growing in Finland. *J. Ethnopharmacol.* 73: 299–305.

Okibo, R. N. and Omodamiro, O. D. 2006. Antimicrobial effects of leaf extracts of Pigeon Pea (*Cajanus cajan* (L.) Millsp) on some human pathogen. *J. Herbs. Spices. Med. Plants.* 12: 117-127.

Olenick, C. G. and Hahn, F. E. 1974. Bactericidal action of a 2-hydroxy-3-alkyl-1, 4 naphthoquinone. *Ann. NY. Acad. Sci.* 235: 542-552.

Olson, J. A. 1999. Bioavailability of carotenoids. *Arch. Latinoamer. Nutr.* 49: 21-25.

Oussalah, M., Caillet, S. and Lacroix, M. 2006. Mechanism of action of Spanish oregano, Chinese cinnamon, and savory essential oils against cell membranes and walls of *Escherichia coli* O157:H7 and *Listeria monocytogenes*. *J. Food. Prot.* 69: 1046–1055.

Packer, L., Weber, S. U. and Rimbach, G. 2001. Molecular aspects of alpha-tocotrienol antioxidant action and cell signalling. *J. Nutr.* 131(2): 369-373.

Palasuwan, A., Soogarun, S., Lertlum, T., Wiwanitkit, V. and Pradniwat, P. 2005. Inhibition of Heinz body induction in vitro model and total antioxidant activity of medicinal Thai plants. *APJCP*. 6: 458-463.

Papageorgiou, V. P., Assimopoulou, A. N., Couladouros, E. A., Hepworth, D. and Nicolaou, K. C. 1999. The chemistry and biology of alkannin, shikonin, and related naphthazarin natural products. *Angewandte. Chemie. Int.* 38: 270-300.

Park, P. J., and others. 2005. Reactive oxygen scavenging effect of enzymatic extracts from *Sargassum thunbergii*. *J. Agric. Food. Chem.* 53: 6666-6672.

Pattnaik, S., Subramanyam, V. R., Bapaji, M. and Kole, C. R. 1997. Antibacterial and antifungal activity of aromatic constituents of essential oils. *Microbios.* 89: 39-46.

Pepeljnjak, S., Kalodera, Z. and Zovko, M. 2005. Antimicrobial activity of flavonoids from *Pelargonium radula* (Cav.) L´Hérit. *Acta. Pharm.* 55: 431- 435.

Perry, N. S., Bollen, C., Perry, E. K. and Ballard, C. 2003. Salvia for dementia therapy: review of pharmacological activity and pilot tolerability clinical trial. *Pharmacol. Biochem. Behav.* 75: 651-659.

Pfyffer, G. E., Panfil, I. and Tower, G. H. N. 1982. Monofunctional covalent photo-binding of dictamnine, a furoquinoline alkaloid, to DNA as a target in vitro. *Photochem. Photobiol. Sci.* 35: 63-68.

Pichersky, E., Noel, J. P. and Dudareva, N. 2006. Biosynthesis of plant volatiles: nature's diversity and ingenuity. *Science.* 311: 808-811.

Pina-Vaz, C., and others. 2004. Antifungal activity of Thymus oils and their major compounds. *J. Eur. Acad. Dermatol.* 18: 73–78.

Pitasawat, B., and others. 2007. Aromatic plant-derived essential oil: An alternative larvicide for mosquito control. *Fitoterapia.* 78: 359-364.

Pongboonrod, S. 1979. *Mai-tet-muang-thai*. Kasem Bannakich Press. Bangkok.

Poolman, B., Driessen, A. J. M. and Konings, W. N. 1987. Regulation of solute transport in Streptococci by external and internal pH values. *Microbiol. Rev.* 51: 498-508.

Pruthi, J. S. 1976. *Spices and Condiments*. New Delhi, India: National Book Trust, Green Park.

Punturee, K., Wild, C. P. and Vinitketkumneun, U. 2004. Thai medicinal plants modulate nitric oxide and tumor necrosis factor-α in J774.2 mouse macrophages. *J. Ethnopharmacol.* 95: 183–189.

Rahman, A. U., and others. 1997. Antifungal diterpinoid alkaloids from Delphinium denudatum. *J. Nut. Prod.* 60: 474-474.

Rahman, M. T., Alimuzzaman, M., Ahmad, S. and Chowdhyry, A. A. 2002. Antinociceptive and antidiarrhoeal activity of *Zanthoxylum rhetsa*. *Fitoterapia.* 73: 340-342.

Raman, M. S., Sultana, N. and Anwar, M. N. 2004. *In vitro* antimicrobial activity of Holarrifine24-ol isolated from the stem bark of *Holarrhena antidysenteriaca*. *Int. J. Agric. Biol.* 6: 698-700.

Randhir, R., Lin, Y. T. and Shetty, K. 2004. Phenolics, their antioxidant and antimicrobial activity in dark germinated fenugreek sprouts in response to peptide and phytochemical elicitors. *Asia. Pac. J. Clin. Nutr.* 13: 295–307.

Rang, H. P., Dale, M. M., Ritter, J. M. and Flower, R. J. 2007. *Rang and Dale's Pharmacology*. New York: Elsevier.

Rao, K. V. and Davies, R. 1986. The ichthyotoxic principles of *Zanthoxylum clava-herculis*. *J. Nat. Prod.* 49: 340-342.

Rauha, J. P., and others. 2000. Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. *Int. J. Food. Microbiol.* 56: 3-12.

Rimm, E. B., Stampfer, M. J., Ascherio, A., Giovannucci, E., Colditz, G. A. and Willett, W. C. 1993. Vitamin E consumption and the risk of coronary heart disease in men. *New. Eng. J. Med.* 328: 1450–1456.

Rosenthal, G. A. and Berenbaum, M. R. 1991. *Herbivores: Their Interaction with Secondary Plant Metabolites*. London: Academic Press.

Sakanaka, S., Kim, M., Taniguchi, M. and Yamamoto, T. 1989. Antibacterial substances in Japanese green tea extract against *Streptococcus mutans*, a carcinogenic bacterium. *Agric. Biol. Chem.* 53: 2307–2311.

Sakanaka, S., Shimura, N., Aizawa, M., Kim, M. and Yamamoto, T. 1992. Preventive effects of green tea polyphenols against dental carries in conventional rats. *Biosci. Biotech. Biochem.* 56: 592–594.

Saleem, M., Alam, A., Arifin, S., Shah, M. S., Ahmed, B. and Sultana, S. 2001. Lupeol, a triterpene, inhibits early responses of tumor promotion induced by benzoyl peroxide in murine skin. *Pharm. Res.* 43: 127–134.

Satori, M. R. K., and others. 2003. Antifungal activity of fractions and two pure compounds of flowers from *Wedelia paludosa* (*Acmela brasiliensis*) (Asteraceae). *Pharmazie.* 58: 567-569.

Sawer, I. K., Berry, M. I. and Ford, J. L. 2005. The killing effect of cryptolepine on *Staphylococcus aureus*. *Lett. Appl. Microbiol.* 40: 24-29.

Scalbert, A. 1991. Antimicrobial properties of tannins. *Phytochem.* 30: 3875–3883.

Schaechter, M., Engleberg, N. C., Eisenstein, B. I. and Medoff, G. 1999. *Mechanisms of Microbial Disease*, 3 rd ed., New York: Lippincott Williams&Wklins.

Schempp, C. M., Sonntag, M., Schöpf, E. and Simon, J. C. 1996. *Dermatitis bullosa* striata pratensis durch *Dictamnus albus* L. (Brennender Busch). *Hautarzt.* 47: 708-710.

Schimmer, O. and Kuhne, I. 1991. Furonoquinoline alkaloid as photosensitizer in *Chlamydomonas reinhardtii*. *Mutat. Res.* 249: 105-110.

Schmidt, H. 1988. Phenol oxidase (E.I.14.18.1), a marker enzyme for defense cells. In H. Schmidt (ed.), *Progress in histochemistry and cytochemistry,* pp. 47-66. New York: Gustav Fischer.

Setzer, W. N. and Volger, B. 2006. Bioassays for activity. In W. N. Setzer and B. volger., (eds.), *Natural Products from Plants,* pp. 389-414. Florida: Taylor & Francis Group.

Seung, S. A., Lee, J. Y., Lee, M. Y., Park, J. S. and Chung, J. H. 1998. The relative importance of oxidative stress versus arylation in the mechanism of quinone-induced cytotoxicity to platelets. *Chem. Biol. Interact.* 113: 133-144.

Shafi, P. M., Saidutty, A. and Clery, R. A. 2002. Volatile constituents of *Zanthoxylum rhesta* leaves and seeds. *J. Essent Oil. Res.* 12: 697-700.

Shahverdi, A. R., Iranshahi, M., Mirjani, R., Jamalifar, H., Amin, G. and Shafiee, A. 2005. Bioassy-guided isolation and idenfication of an antibacterial compound from *Ferula persica* var. Persica roots. *DARU.* 13: 17-19.

Shankaracharya, N. B., Puranik, J., Nagalakshmi, S. and Rao, L. J. M. 1994. Chemical composition and flavour quality of Triphal (*Zanthoxylum rhesta*). *Pafai. J.* 16: 15-21.

Sheen, W. S., Tsai, I. L. and Chen, I. S. 1994. Nor-neolignan and phenyl propanoid from *Zanthoxylum ailanthoides*. *Phytochem.* 36: 213-215.

Shelef, L. A. 1983. Antimicrobial effects of spices. *J. Food. Safety.* 6: 29– 44.

Shi, Z., Shen, S., Zhou, W., Wang, F. and Fan, Y. 2008. *Fusarium graminearum* growth inhibition due to glucose starvation caused by osthol. *Int. J. Mol. Sci.* 9: 371-382.

Shin, K. S., Lee, S. and Cha, B. 2007. Antifungal activity of Plumbagin purified from leaves of *Nepenthes ventricosa* x *maxima* against phytopathogenic fungi. *Plant. Pathol.* 23: 113-115.

Sies, H. 1991. *Oxidative Stress: Oxidants and Antioxidants*. London: Academic Press.

Sigaud-Kutner, T. C. S., Neto, A. M. P., Pinto, E. and Colepicolo, P. 2005. Diel activities of antioxidant enzymes, photosynthetic pigments and malondialdehyde content in stationary-phase cells of *Tetraselmis gracilis* (Prasinophyceae). *Aquat. Bot.* 82: 239–249.

Sikkema, J., DeBont, J. A. M. and Poolman, B. 1995. Mechanisms of membrane toxicity of hydrocarbons. *Microbiol. Rev.* 59: 201–222.

Sikkema, J., De Bont J. A. M. and Poolman, B. 1994. Interactions of cyclic hydrocarbons with biological membranes. *J. Biol. Chem.* 269: 8022–8028.

Singh, G., Upadhyay, R. K., Narayanan, C. S., Padmkumari, K. P. and Rao, G. P. 1993. Chemical and fungi toxic investigation on the essential oil of *Citrus sinensis* (L.) Pers. *Z. Deuts. Zeitsc. Pflanze*. 100: 69-74.

Singh, S. K., Sarma, B. K., Srivasta, J. S., Singh, U. P. and Ray, A. B. 1999. Antifungal activity of Δ^3-Alstovenine, a plant alkaloid isolated from *Alstonia venenata*. *Folia. Microbiol.* 44: 510-512.

Singh, U. P., Singh, K. P., Trpathi, V. K. and Pandey, V. B. 1994. Antifungal activity of some naturally occurring plant alkaloids. *Int .J. Trop. Plant. Dis.* 12: 209-212.

Sinha, R. P., Ambasht, N. K., Sinha, J. P. and Hapder, D. P. 2003. Wavelength dependent induction of a mycosporine-like amino acid in a rice-field cyanobacterium, Nostoc commune: role of inhibitors and salt stress. *Photochem. Photobiol. Sci.* 2: 171–176.

Smitinand, T. 1980. *Thai Plant Name*. Bangkok: Funny Publishing Ltd.

Somanabandhu, A., Ruangrungsi, N., Lange, G. L. and Organ, M. G. 1992. Constituents of the stem bark of *Zanthoxylum limenella*. *J. Sci. Soc. Thailand.* 18: 181-185.

Spencer, C. M., and others. 1988. Polyphenol complexation. Some thoughts and observations. *Phytochem.* 27: 2397-2409.

Stein, A. C., Álvarez, S., Avancini, C., Zacchino, S. and Poser, G. V. 2006. Antifungal activity of some coumarins obtained from species of Pterocauon (Asteraceae). *J. Ethnopharmacol.* 107: 95-98.

Suffredini, I. B., Paciencia, M. L. B., Varella, A. D. and Younes, R. N. 2006. Antibacterial activity of Brazilian Amazon plant extracts. *Braz. J. Infect. Dis.* 10: 401-402.

Sukenik, A. and Zmora, O. 1993. Biochemical quality of marine unicellular algae with special emphasis on lipid composition. II *Nannochloropsis* sp. *Aquacult.* 117: 313-326.

Sunitha, M., Nagaraj, M. and Varalakshmi, P. 2001. Hepatoprotective effect of lupeol and lupeol linoleate on tissue antioxidant defense system in cadmium-induced hepatotoxicity in rats. *Fitoterapia.* 72: 516-523.

Syed, M., Fenoglio-Preiser, C., Skau, K. A. and Weber, G. F. 2008. Acetylcholin-esterase supports anchorage independence in colon cancer. *Clin. Experimen. Metas.* 25: 787-798.

Taiz, L. and Zeiger, E. 2002. *Plant physiology*. Sunderland: Sinauer Associates.

Tanaka, J. C. A., Silva, C. C., Oliveira, A. J. B., Nakamura, C. V. and Dias Fiho, B. P. 2006. Antibacterial activity of indole alkaloids from *Aspidosperma ramiflorum*. *Braz. J. Med. Biol. Res.* 39: 387-391.

Tanvetyanon, T. and Bepler, G. 2008. Beta-carotene in multivitamins and the possible risk of lung cancer among smokers versus former smokers: a meta-analysis and evaluation of national brands. *Cancer.* 113(1): 150-157.

Taudon, A. 1990. *Activite antifongigue des Labiatae Donnees Bibliographiques. Etudes in vitro de treize huiles essentielles (interet dela microemulsion).* These de Doctorat d'Etates-Sciences pharmacentiques, Universite Paul Sabatier Toulouse.

Thappa, R. K., Dhar, K. L. and Atal, C. K. 1976. A new monoterpene triol from *Zanthoxylum budranga*. *Phytochem.* 15: 1568-1569.

Thara, S., Ingham, J., Nakahara, S., Mizutani, J. and Harborne, J. B. 1984. Antifungal isoflavones in Lupinus. *Phytochem.* 23: 1889-1900.

Thomson, W. A. R. 1978. *Medicines from the Earth*. New York: McGraw-Hill.

Toda, M., Okubo, S., Ikigai, S. and Shimamura, T. 1990. Antibacterial and antihemolysin activities of tea catechins and their structural relatives. *Jap. J. Bacteriol.* 45: 561-566.

Traber, M. G. and Atkinson, J. 2007. Vitamin E, antioxidant and nothing more. *Free. Rad. Bio. Med.* 43: 4-15.

Tsuchiya, H., Sato, M. and Miyazaki, T. 1996. Comparative study on antibacterial activity of phytochemical flavonones against methicillin-resistant *Staphylococcus aureus*. *J. Ethnopharmacol.* 50: 27-34.

Tsuchiya, M., Kagen, V. E., Freisleben, H. J., Manabe, M. and Packer, L. 1994. Antioxidant activity of α-tocopherol, β-carotene and ubiquinol in membranes: cis-Parinaric acid-incorporated liposomes. *Methodes Enzymol*. 234: 371–383.

Turina, A. V., Nolan, M. V., Zygadlo, J. A. and Perillo, M. A. 2006. Natural terpenes: self-assembly and membrane partitioning. *Biophys. Chem.* 122: 101–113.

Turker, A. U. and Usta, C. 2008. Biological screening of some Turkish medicinal plant extracts for antimicrobial and toxicity activities. *Nat. Prod. Res.* 22: 136-146.

Ultee, A., Kets, E. P. W., Alberda, M., Hoekstra, F. A. and Smid, E. J. 2000a. Adaptation of the food-borne pathogen *Bacillus cereus* to carvacrol. *Archives. Microbiol.* 174: 233– 238.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M. and Telser, J. 2007. Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell. Biol.* 39: 44–84.

Van Bambeke, F., Glupczynski, Y., Plèsiat, P., Pechère, J. C. and Tulkens, P. M. 2003. Antibiotic effulus pumps in prokaryotic cells: occurrence, impact on resistance and strategies for the future of antimicrobial therapy. *J. Antimicrob. Chemother*. 51: 1055-1065.

Vaquette, J., Cave, A. and Waterman, P. G. 1979. Alkaloids, triterpenes and lignans from *Zanthoxylum* sp. Sevenet 1183- a species indigenous to new Caledonia. *Planta. Med.* 35: 42-47.

Vaquette, J., Pousset, J. L., Cavé, A., Delaveau, P. and Paris, R. R. 1973. Study of the constituents of the trunk bark of *Zanthoxylum decaryi* H. Perrier (Rutaceae). I. Non-alkaloid constituents. *Annal. pharm. fran.* 31(1): 49-55

Vijaya, K., Ananthan, S. and Nalini, R. 1995. Antibacterial effect of theaflavin, polyphenon 60 (*Camellia sinensis*) and *Euphorbiahita* on Shigella spp.—a cell culture study. *J. Ethnopharmacol.* 49: 115-118.

Villar, A., Rios, J. L., Recio, M. C., Cortes, D. and Cave, A. 1986. Antimicrobial activity of Benzylisoquinoline alkaloids. II. Relation between chemical composition and antimicrobial activity. *Planta. Med.* 6: 556-557.

Vouldoukis, I., Conti, M. and Kolb, J. P. 2003. Induction of Th1-dependent immunity by an orally effective melon superoxide dismutase extract. *Curr. Trends. Immunol.* 5: 141-145.

Vouldoukis, I., Lacan, D. and Kamate, C. 2004. Antioxidant and antiimflammatory properties of a *Cucumis melo* LC. Extract rich in superoxide dismutase activity. *J. Ethnopharmacol.* 94(1): 67-75.

Wang, Y., Corr, J. G. and Thaler, H. T. 1995. Decreased growth of established human prostate LNCaP tumors in nude mice fed a low- fat diet. *J. Nat.Cancer. Inst.* 87: 1456-1462.

Wang, Y., Xu, X., van Lieshout, M., West, C. E. and Lugtenburg, J. 2000. A liquid chromatography-mass spectrometry method for the quantification of bioavailability and bioconversion of beta-carotene to retinol in humans. *J. Anal. Chem.* 72: 4999–5003.

Wattad, C., Dinoor, A. and Prusky, D. 1994. Purification of pectate lyase produced by *Colletotrichum gloeosporioides* and its inhibition by epicatechin: A possible factor involved in the resistance of unripe avocado fruits to anthracnose. *Mol. Plant-Microbe. Interact.* 7: 293-297.

Westh, H., Zinn, C. S. and Rosdahl, V. T. 2004. An international multicenter study of antimicrobial consumption and resistance in *S. aureus* isolates from 15 hospitals in 14 countries. *Microbe. Drug. Resist.* 10: 169 -176.

Wild, R. 1994. *The complete book of natural and medicinal cures*. Pennsylvania: Rodale Press.

Wimalasina, S. and Karunawansha, E. 1994. Characterization of a new aryl alkanone and other compounds present in *Horsfieldia irya* seeds. *J. Nation Sci. Counc. Sri Lanka,* 22: 301-304.

Wolters, B. and Eilert, U. 1981. Antimicrobial substances in callus cultures of *Ruta graveolens*. *Planta Med.* 43: 166.

Yamazaki, E., Inagaki, M., Kurita, O. and Inoue, T. 2007. Antioxidant activity of Japanese pepper (*Zanthoxylum piperitum* DC.) fruit. *Food. Chem. Toxicol.* 100: 171-177.

Yoon, H. S., Moon, S. C., Kim, N.D., Park, B. S., Jeong, M. H. and Yoo, Y. H. 2000. Genistein induces apoptosis of RPE-J cells by opening mitochondrial PTP. *Biochem. Biophys. Res. Commun*. 276: 151-156.

Yoshimura, S., Suemizu, H., Taniguchi, Y., Arimori, K., Kawabe, N. and Moriuchi, T. 1994. The human plasma glutathione peroxidase-encoding a gene: organization, sequence and localization to chromosome 5q32. *Gene.* 145: 293-297.

Zhao, W., Wolfunder, J. L., Hostettmann, K., Xu, R. and Qin, G. 1998. Antifungal alkaloids and limonoid derivative from *Dictamnus dascarpus*. *Phytochem.* 47: 7-11.

Ziaei, S., Kazemnejad, A. and Zareai, M. 2007. The effect of vitamin E on hot flashes in menopausal women. *Gynecol. Obstet. Invest.* 64(4): 204-207.

APPENDICES

APPENDIX A

1. Media

1.1 Nutrient agar (NA)

Beef extract	3 g
Peptone	5 g
Agar	15 g

Dissolved in distilled water up to 1 L and autoclaved at 121^{o}C, 15 psi for 20 min.

1.2 Yeast malt extract agar (YME)

Yeast extract	10 g
Malt extract	5 g
Peptone	5 g
Glucose	10 g
Agar	15 g

Dissolved in distilled water up to 1 L and autoclaved at 121^{o}C, 15 psi for 20 min.

1.3 Potato Dextrose Agar (PDA)

Potato	200 g
Dextrose	20 g
Glucose	20 g
Agar	15 g

Dissolved in distilled water up to 1 L and autoclaved at 121^{o}C, 15 psi for 20 min.

1.4 Mueller Hinton broth (MHB)

Beef extract	20 g
Acid digest of casein	17.5 g
Soluble starch	1.5 g

Dissolved in distilled water up to 1 L and autoclaved at 121^{o}C, 15 psi for 20 min.

1.5 Sabouraud dextrose broth (SDB)

Special peptone	10 g
Dextrose	20 g

Dissolved in distilled water up to 1 L and autoclaved at 121^{o}C, 15 psi for 20 min.

Appendix B

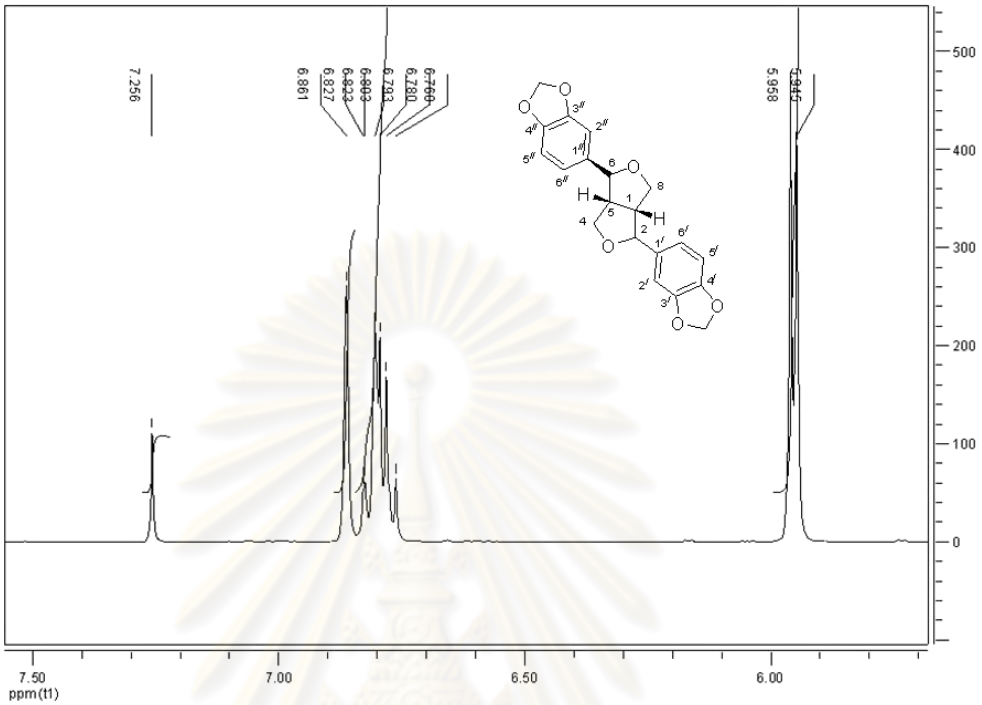


Figure 1 ^{1}H NMR spectrum ($CDCl_3$) of Compound **2**

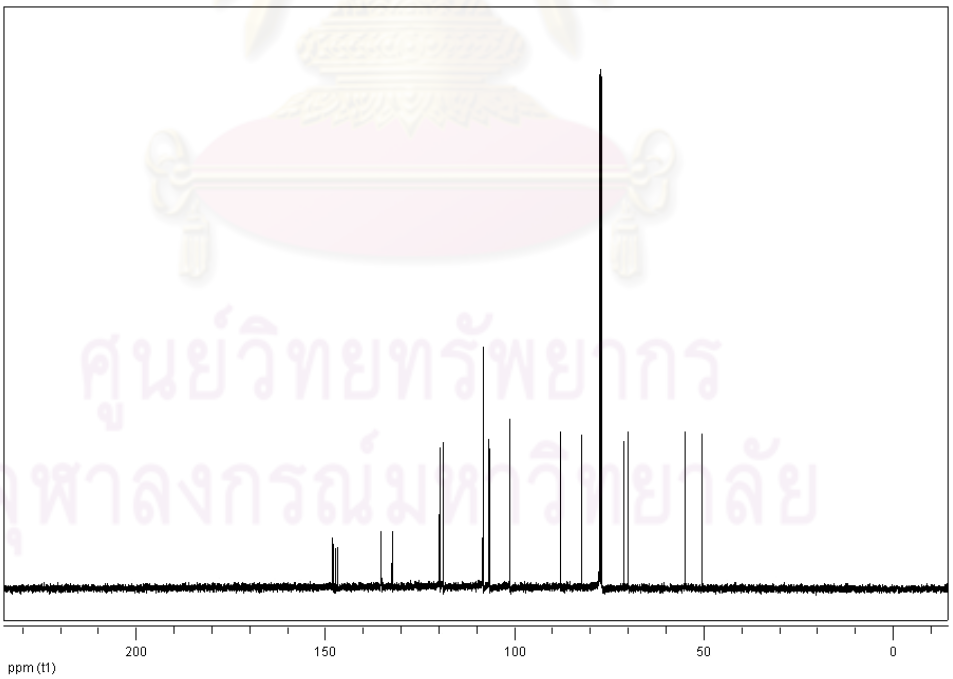


Figure 2 The ^{13}C NMR spectrum ($CDCl_3$) of Compound **2**

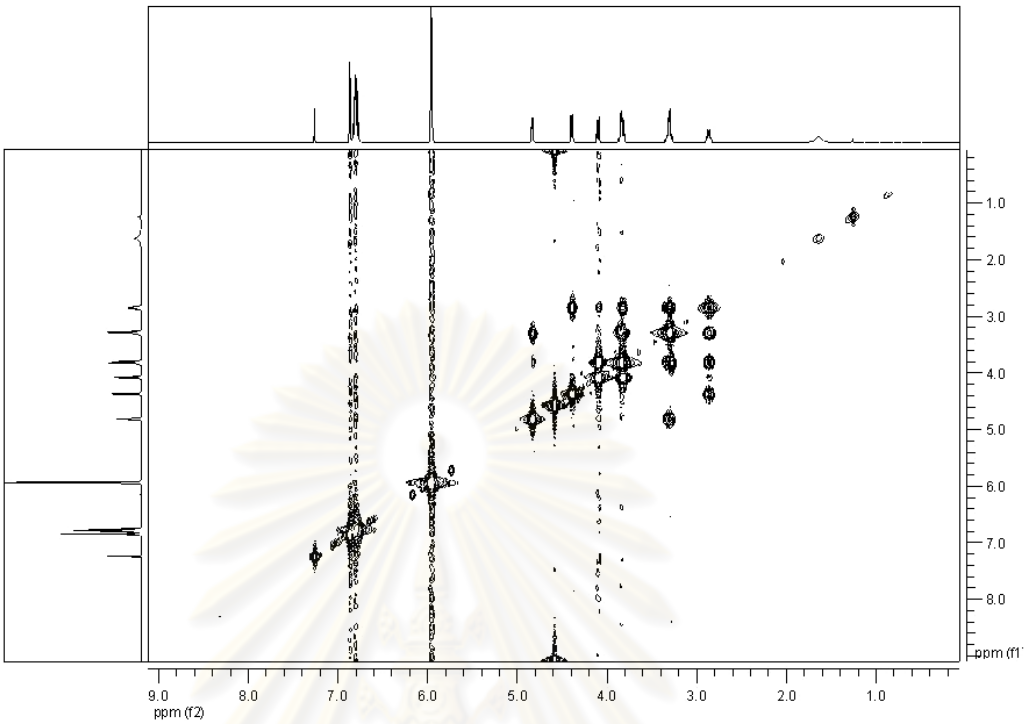


Figure 3 The COSY spectrum ($CDCl_3$) of Compound **2**

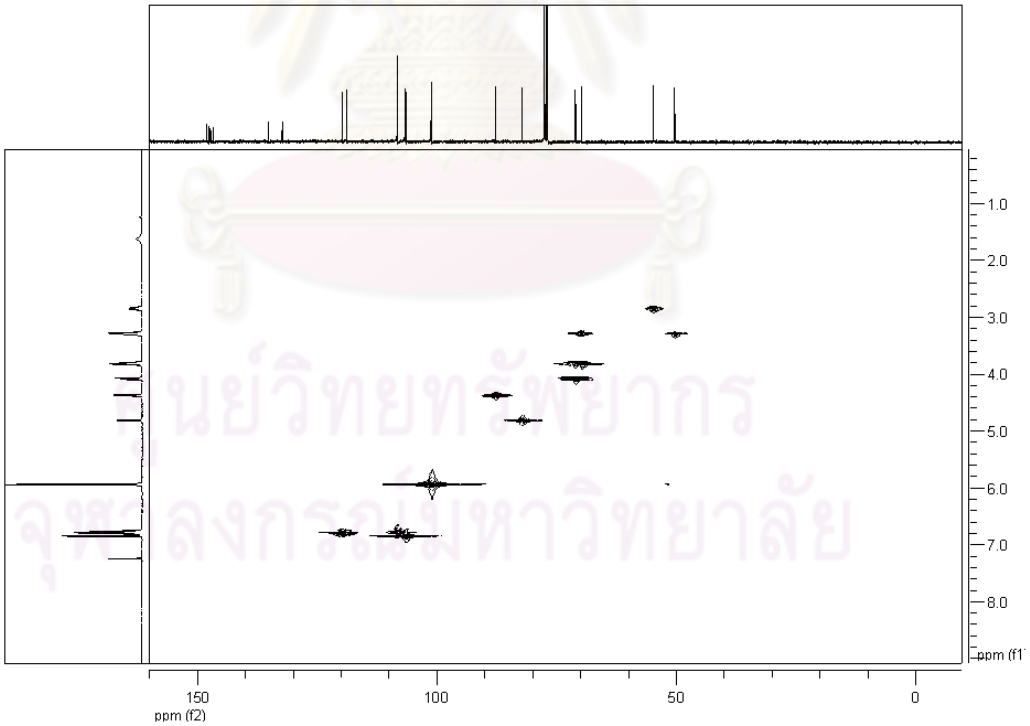


Figure 4 The HSQC spectrum ($CDCl_3$) of Compound **2**

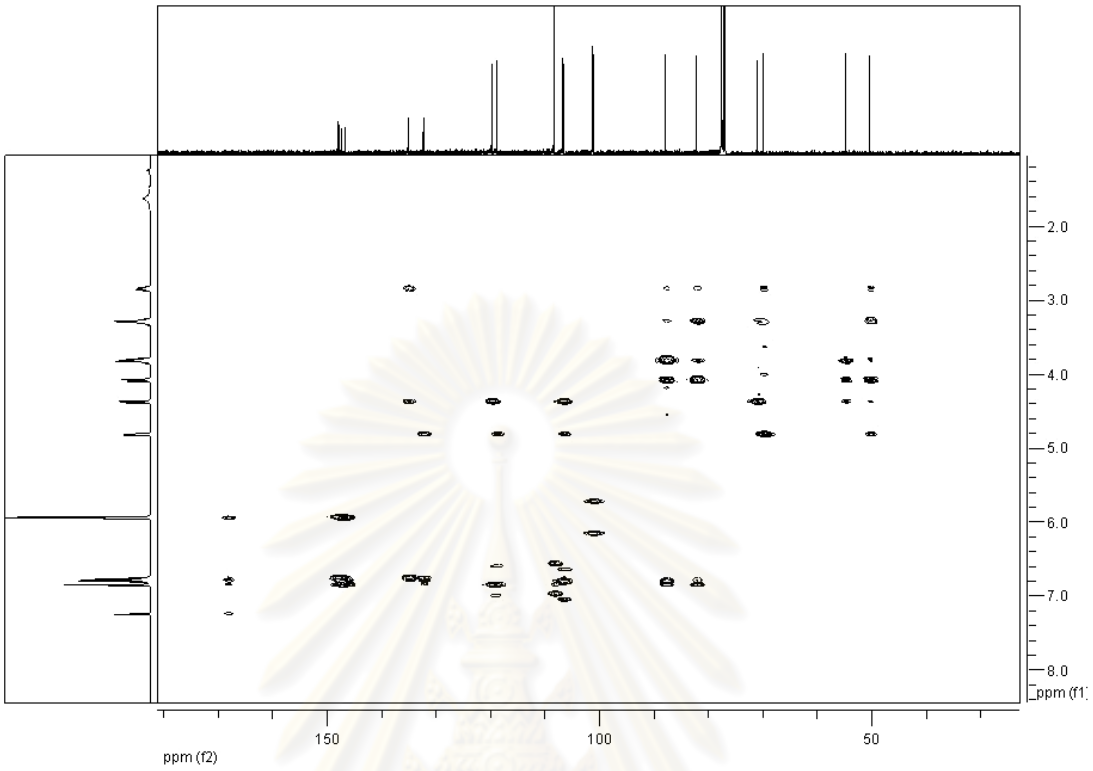


Figure 5 The HMBC spectrum ($CDCl_3$) of Compound **2**

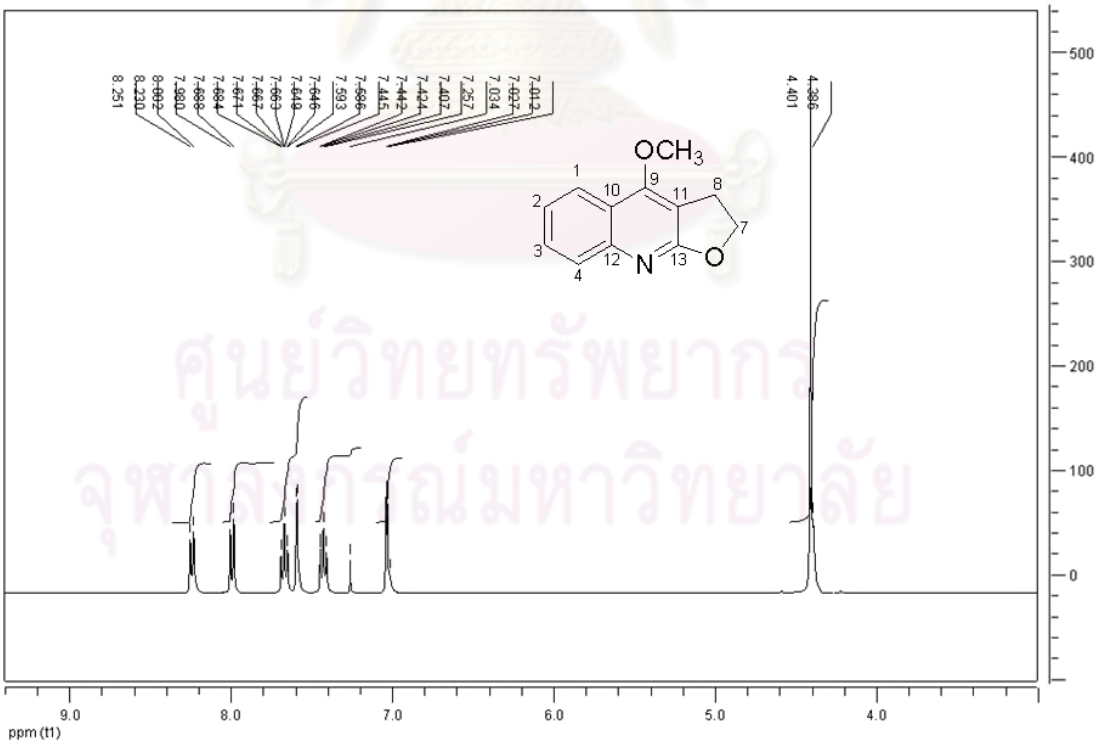


Figure 6 The ^1H NMR spectrum ($CDCl_3$) of Compound **3**

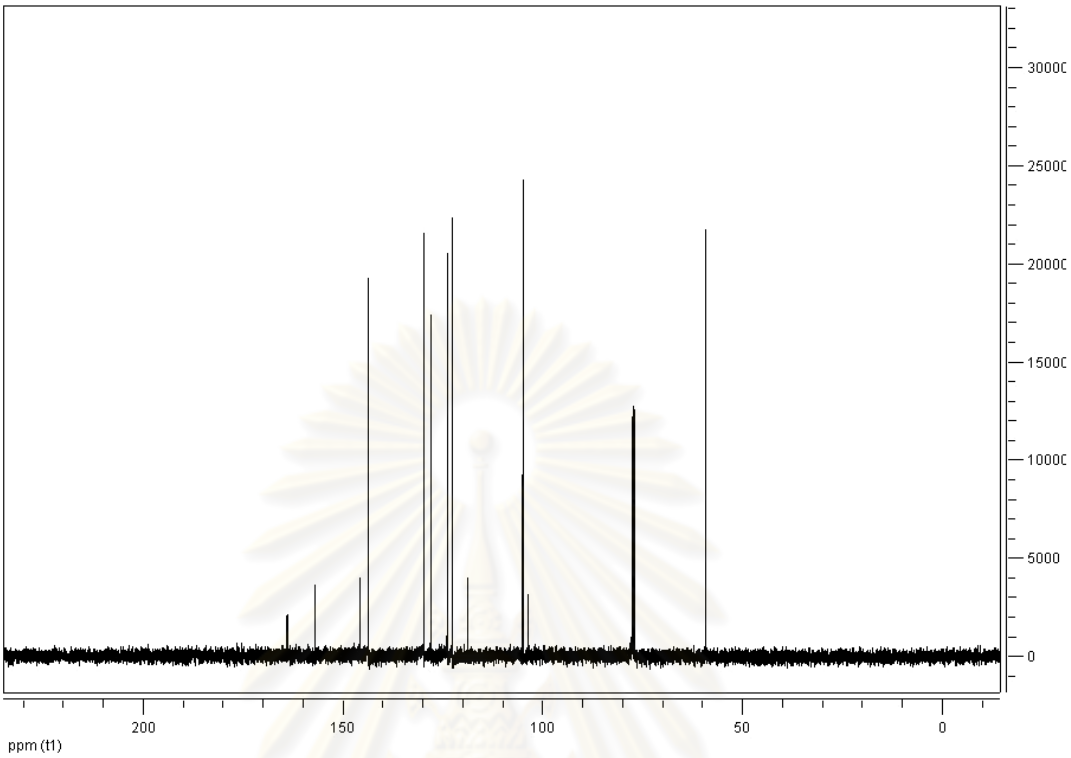


Figure 7 The ^{13}C NMR spectrum ($CDCl_3$) of Compound **3**

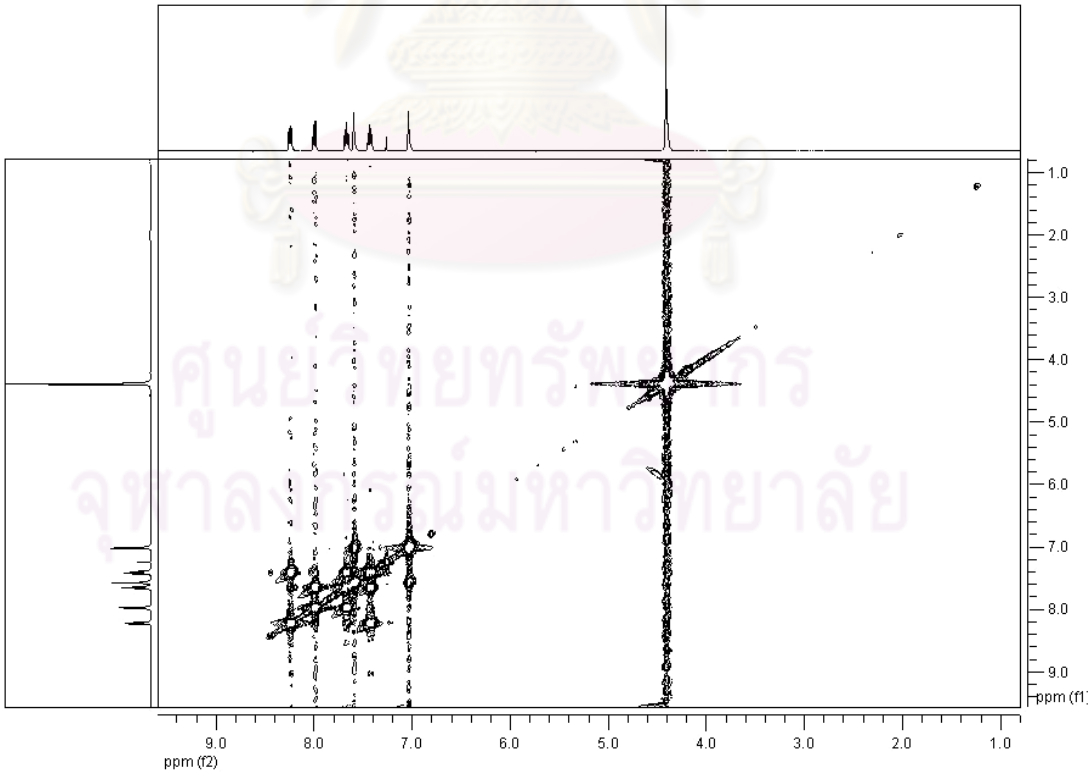


Figure 8 The COSY spectrum ($CDCl_3$) of Compound **3**

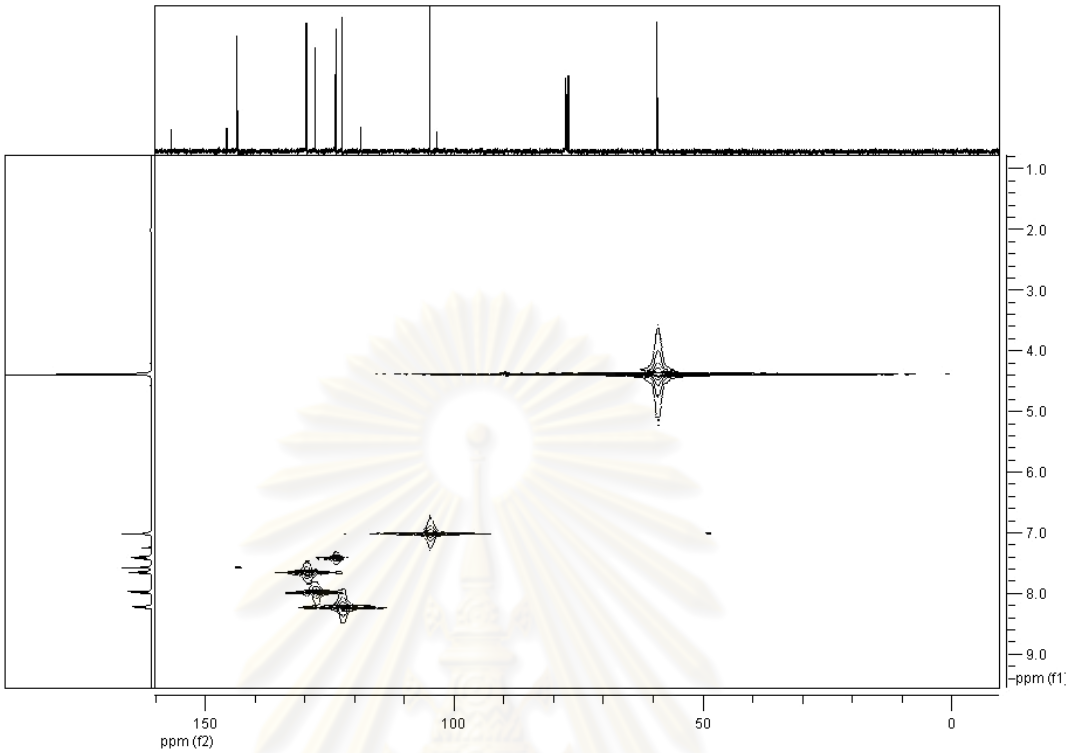


Figure 9 The HSQC spectrum ($CDCl_3$) of Compound **3**

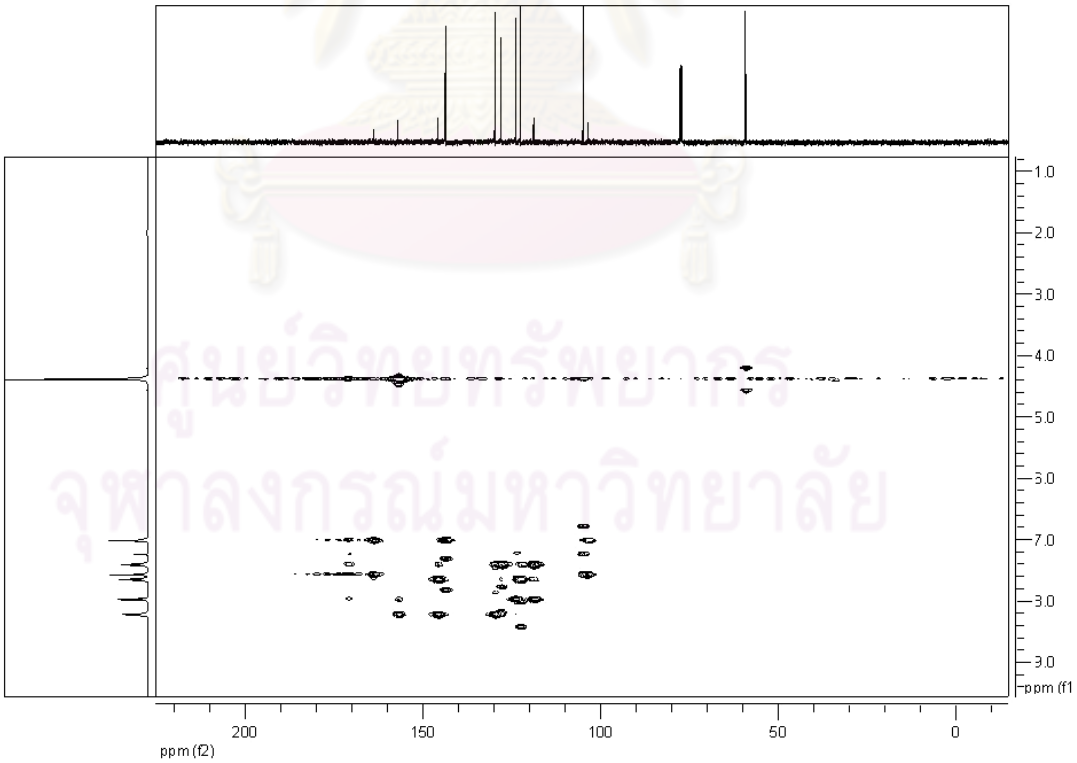


Figure 10 The HMBC spectrum ($CDCl_3$) of Compound **3**

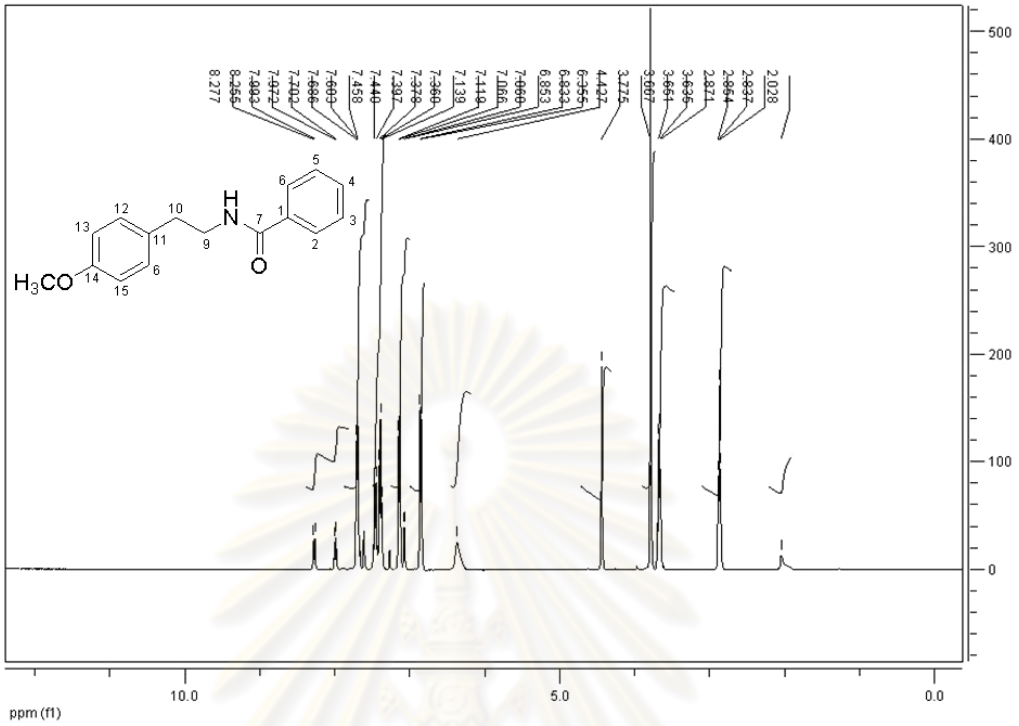


Figure 11 The ^{1}H NMR spectrum ($CDCl_3$) of Compound **4**

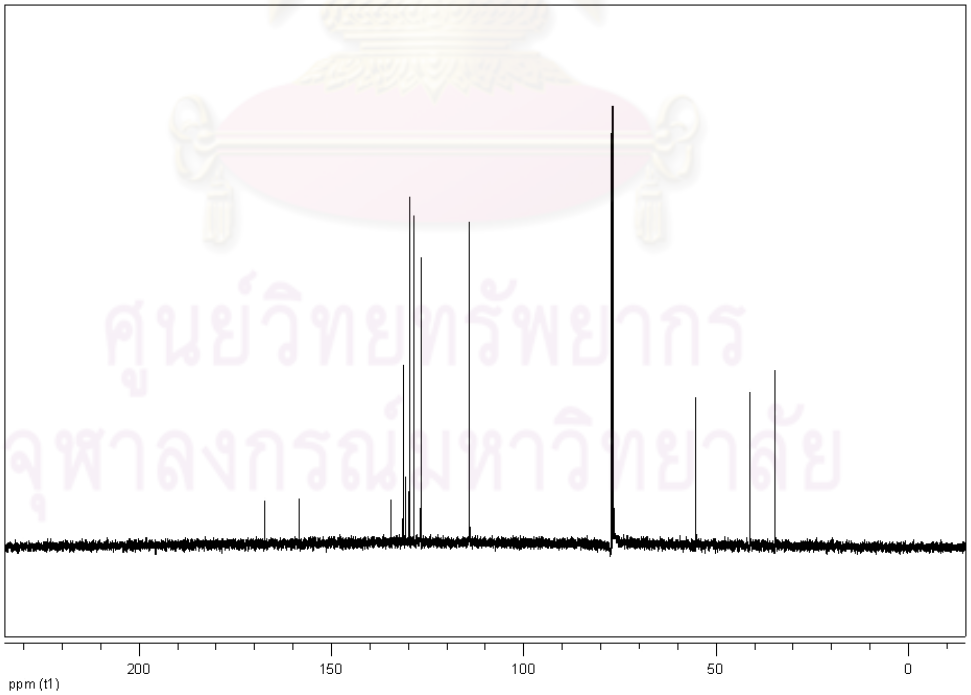


Figure 12 The ^{13}C NMR spectrum ($CDCl_3$) of Compound **4**

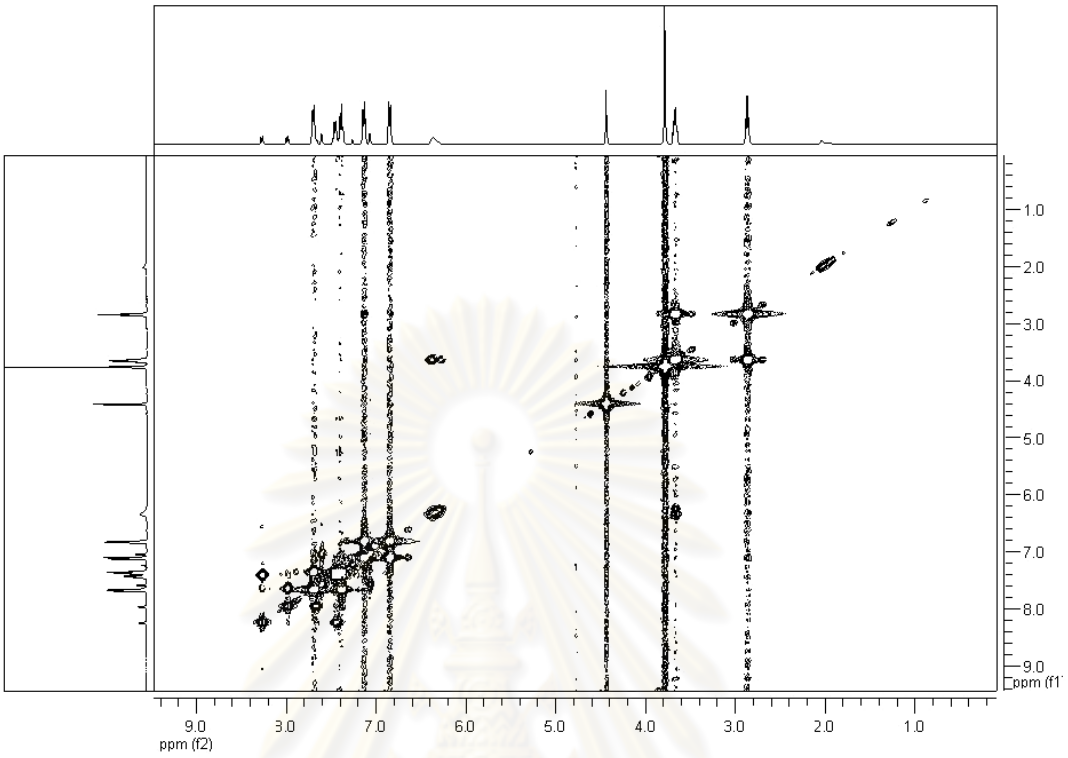


Figure 13 The COSY spectrum ($CDCl_3$) of Compound **4**

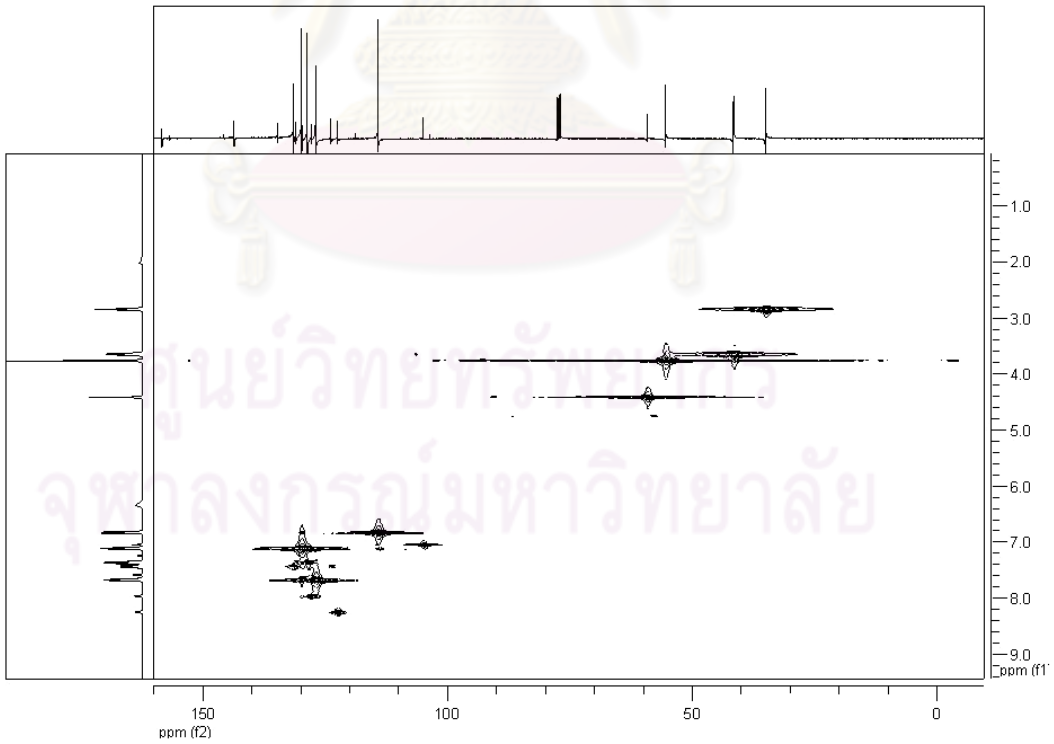


Figure 14 The HSQC spectrum ($CDCl_3$) of Compound **4**

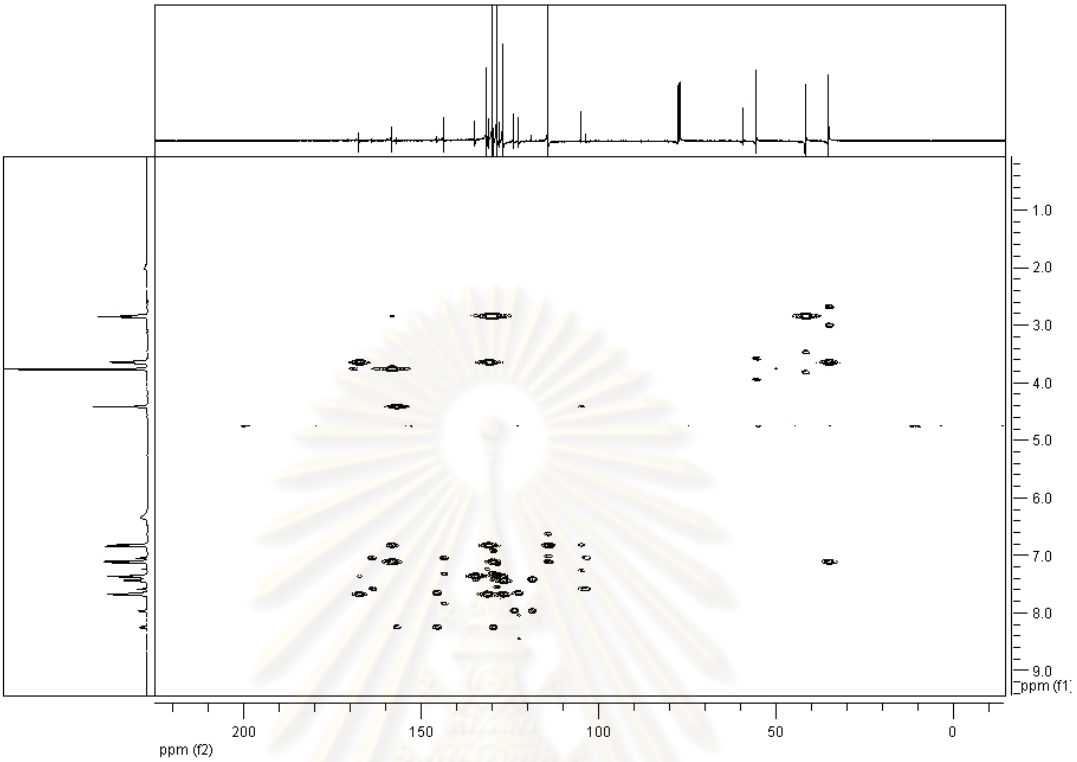


Figure 15 The HMBC spectrum ($CDCl_3$) of Compound **4**

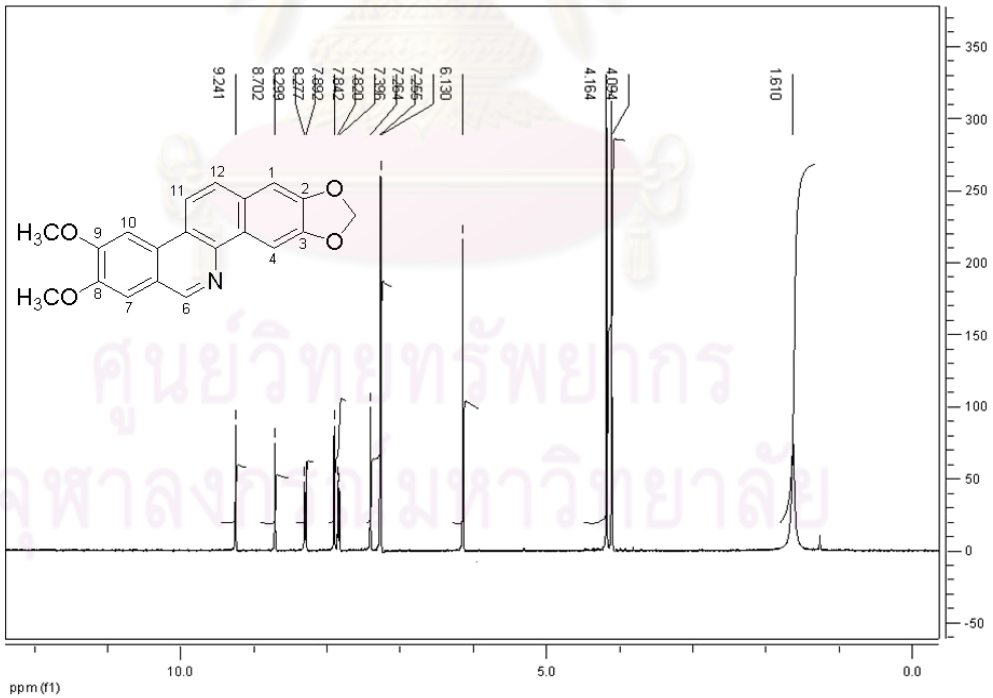


Figure 16 The ^1H NMR spectrum ($CDCl_3$) of Compound **5**

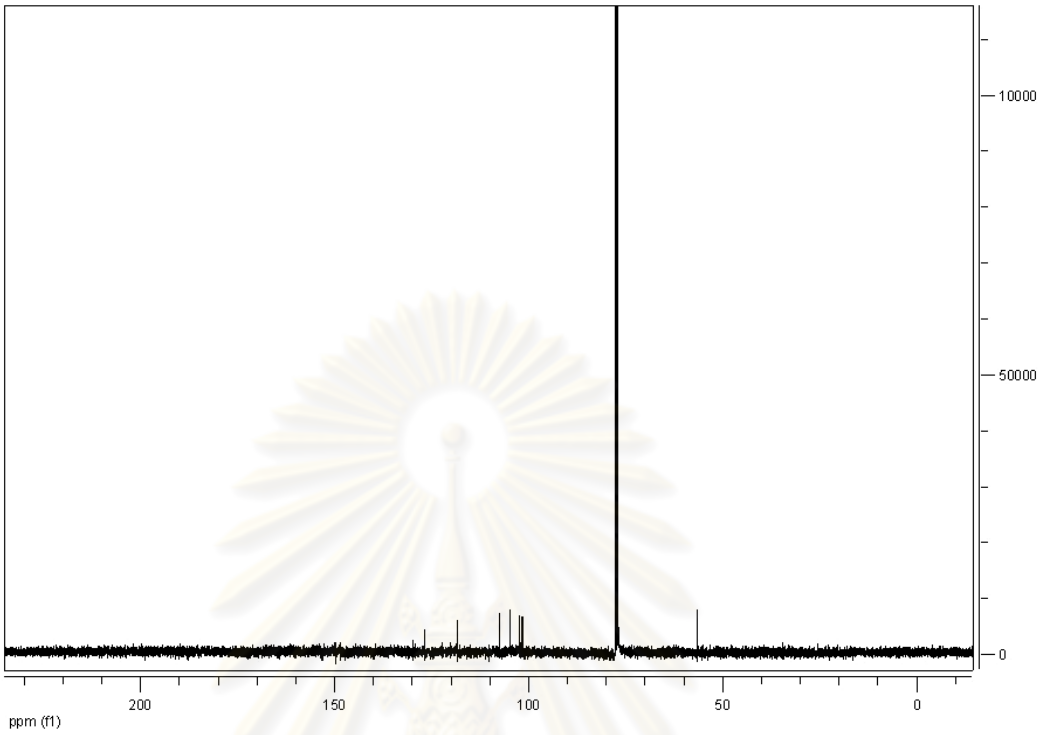


Figure 17 The ^{13}C NMR spectrum ($CDCl_3$) of Compound **5**

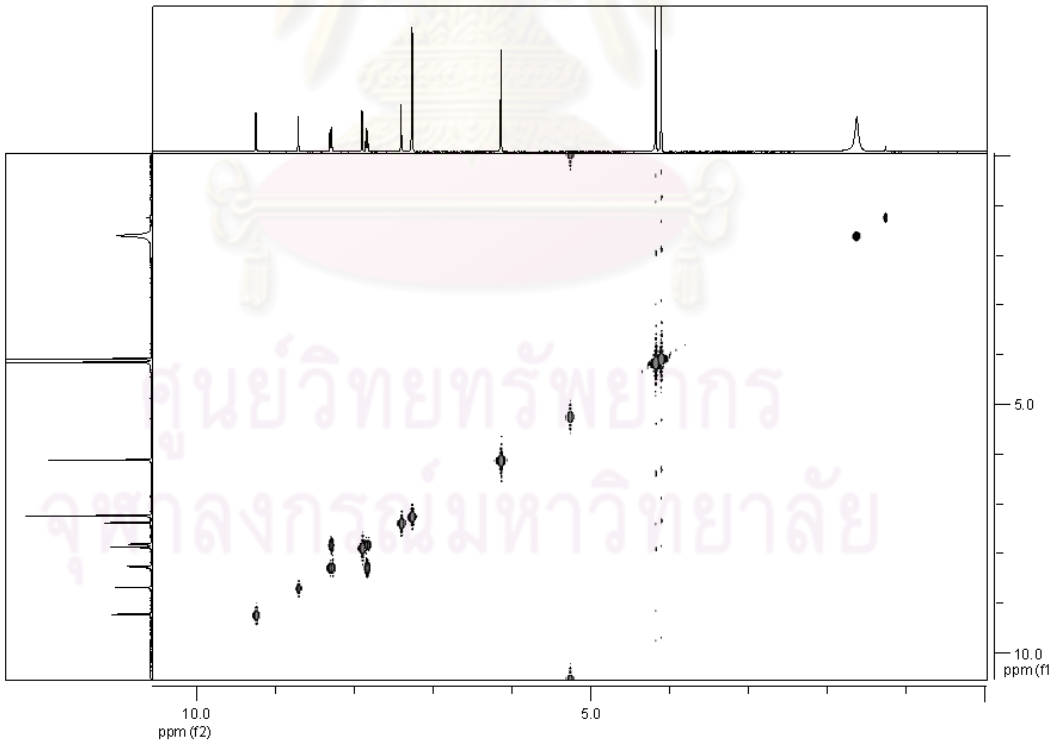


Figure 18 The COSY spectrum ($CDCl_3$) of Compound **5**

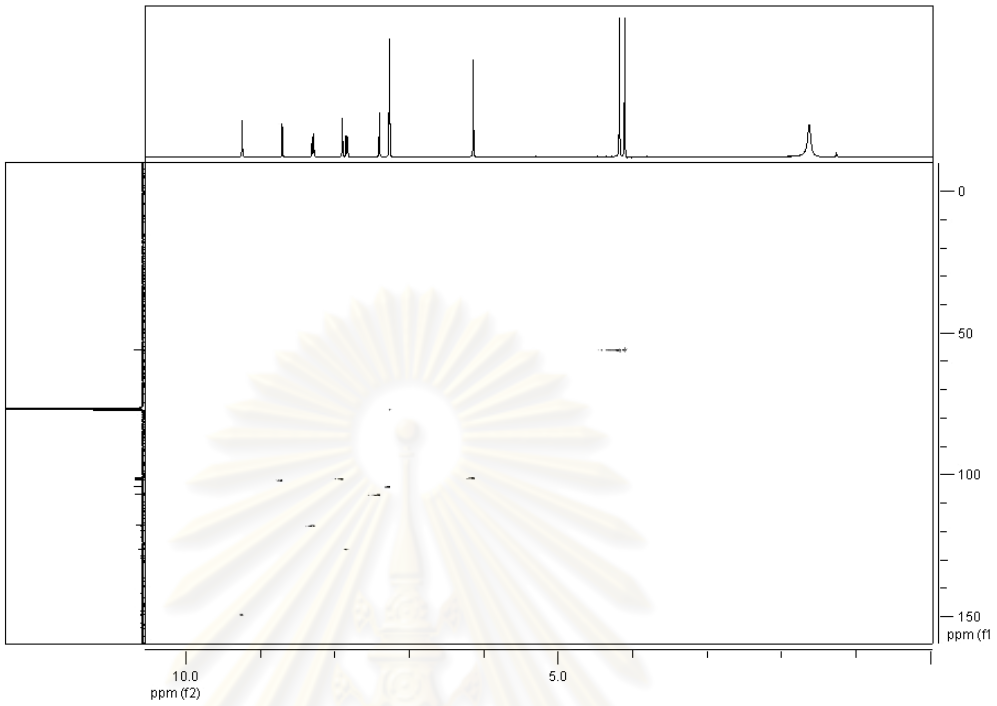


Figure 19 The HSQC spectrum ($CDCl_3$) of Compound **5**

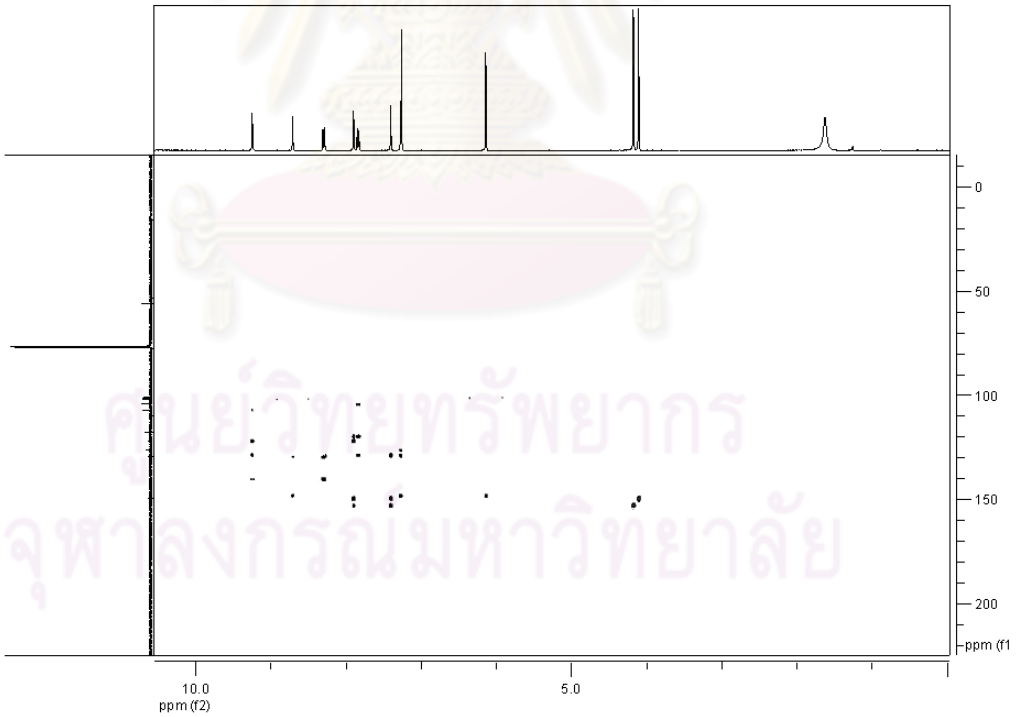


Figure 20 The HMBC spectrum ($CDCl_3$) of Compound **5**

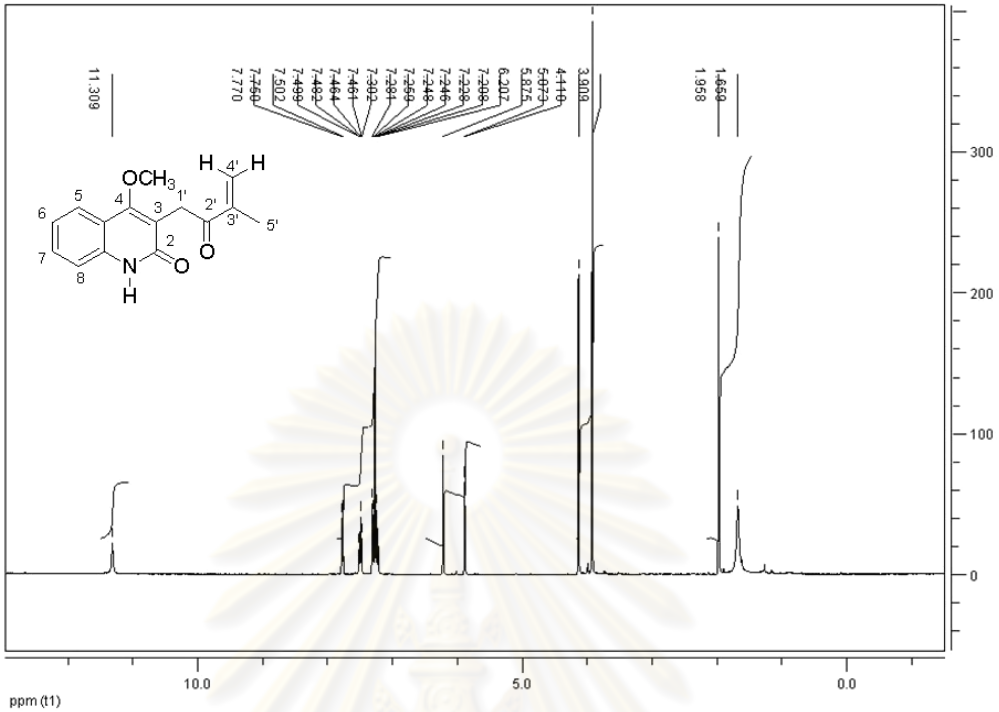


Figure 21 The ^{1}H NMR spectrum ($CDCl_3$) of Compound **6**

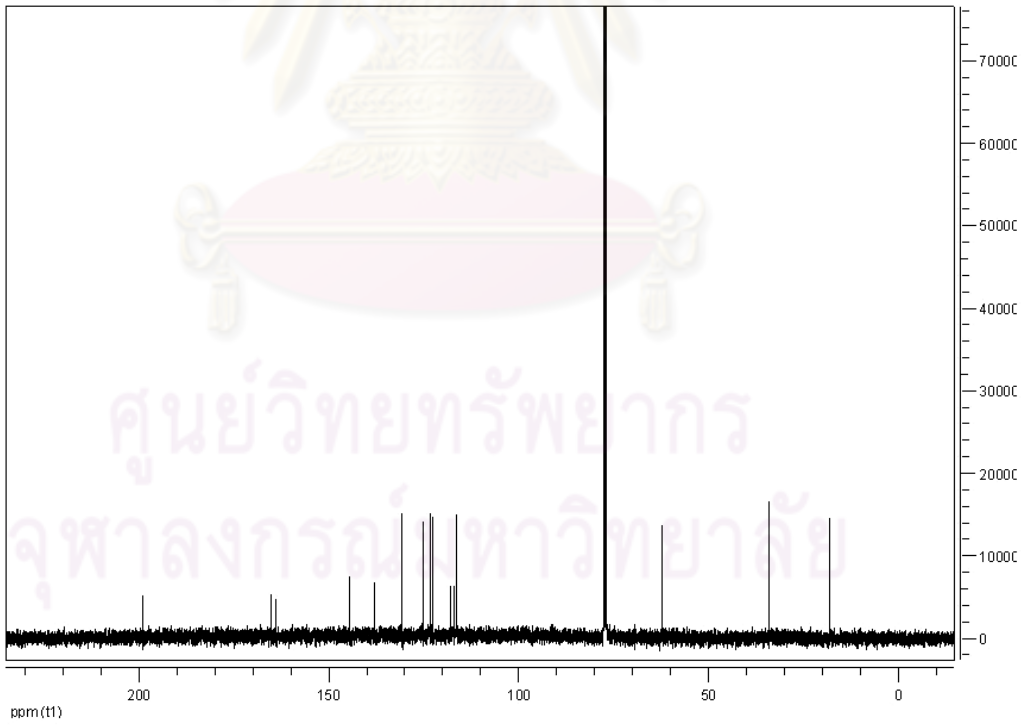


Figure 22 The ^{13}C NMR spectrum ($CDCl_3$) of Compound **6**

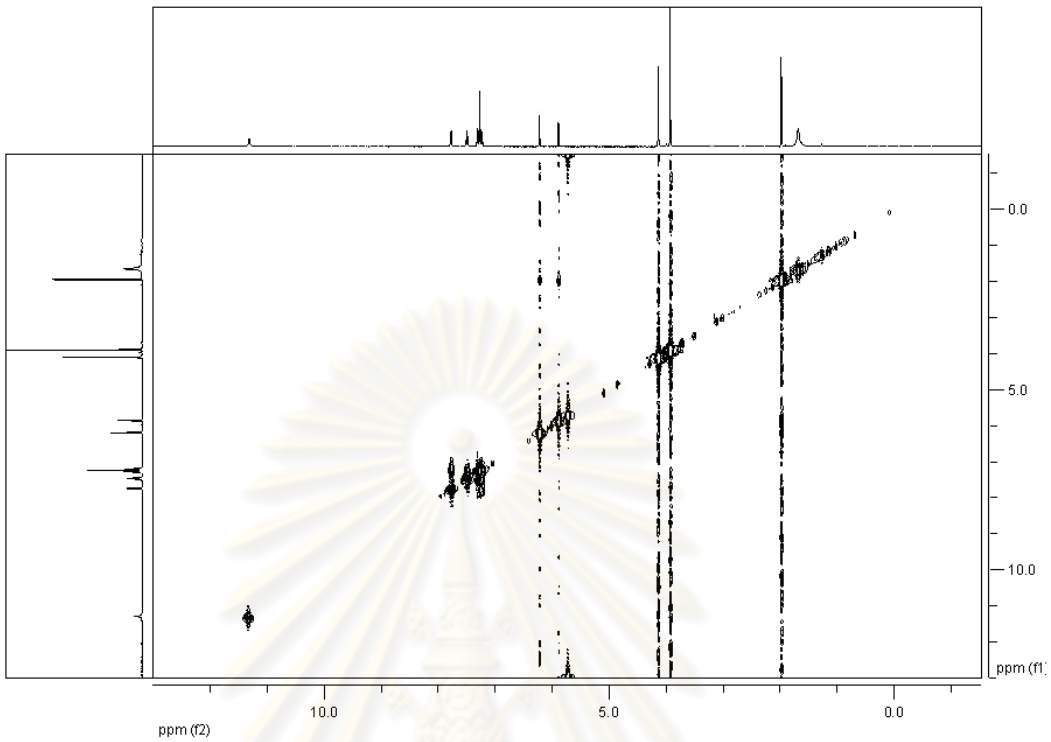


Figure 23 The COSY spectrum ($CDCl_3$) of Compound **6**

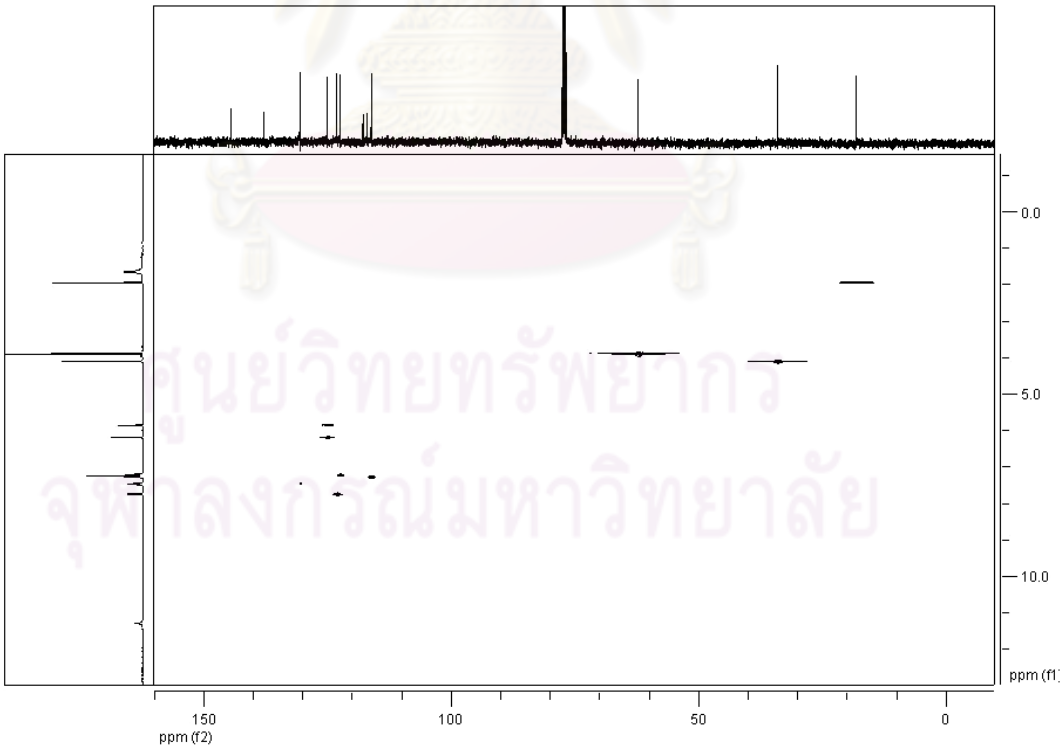


Figure 24 The HSQC spectrum ($CDCl_3$) of Compound **6**

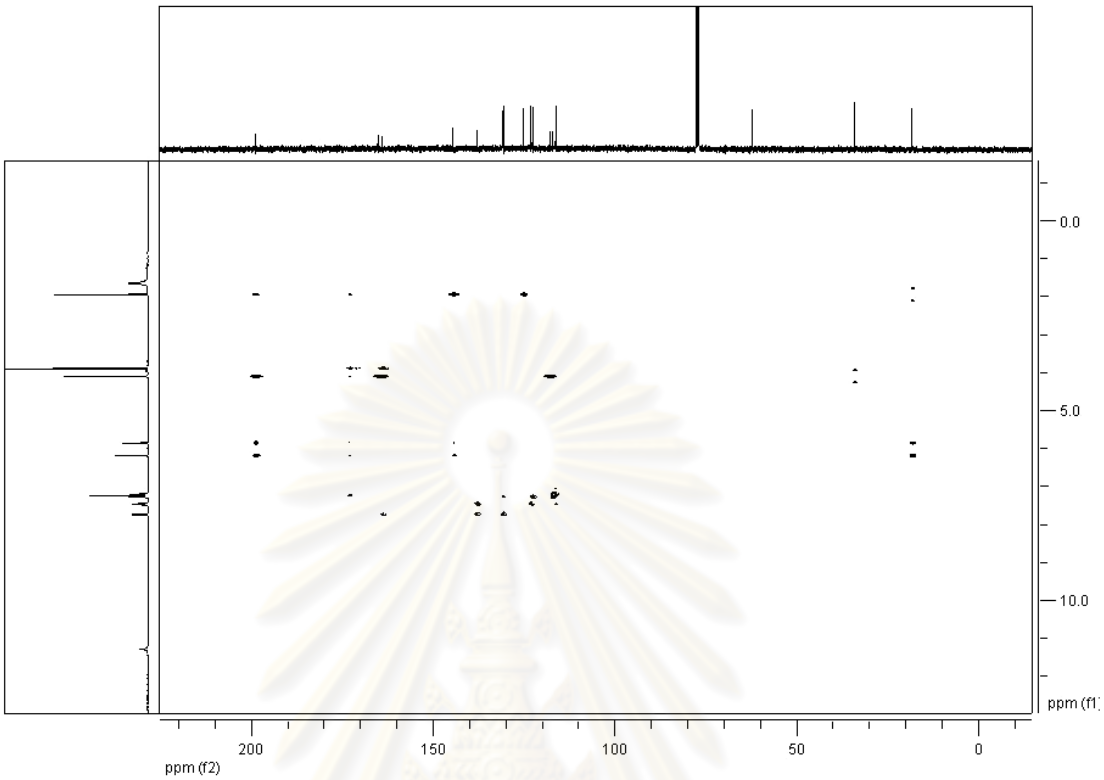


Figure 25 The HMBC spectrum ($CDCl_3$) of Compound **6**

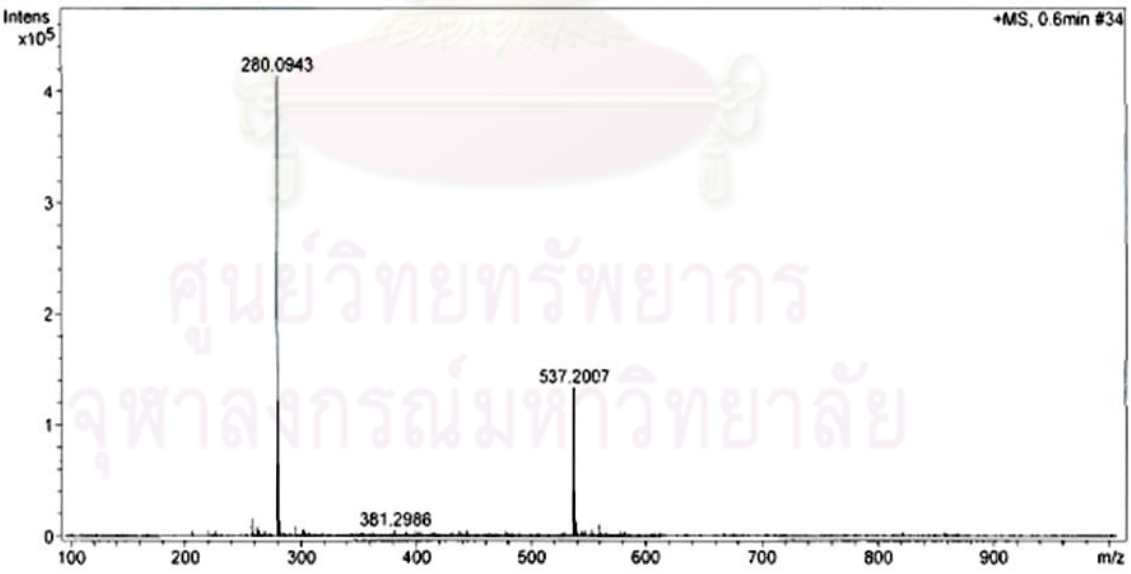


Figure 26 The High resolution mass spectrum of Compound **6**

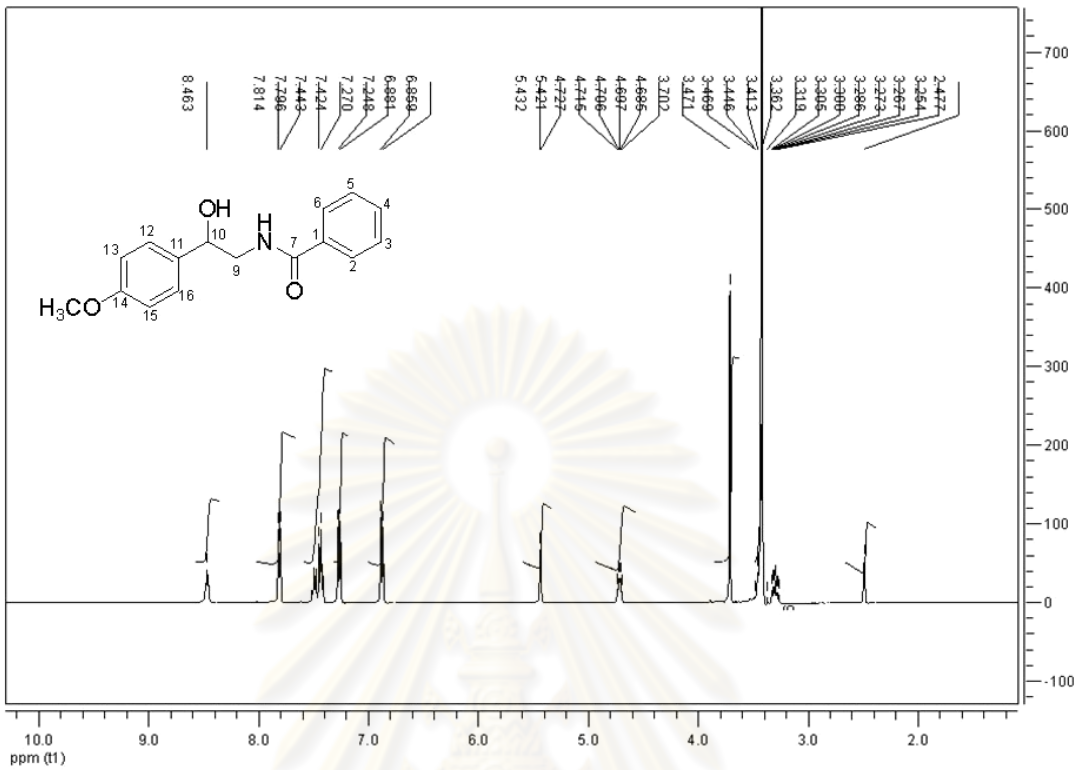


Figure 27 The ^{1}H NMR spectrum (methanol-d_6) of Compound **7**

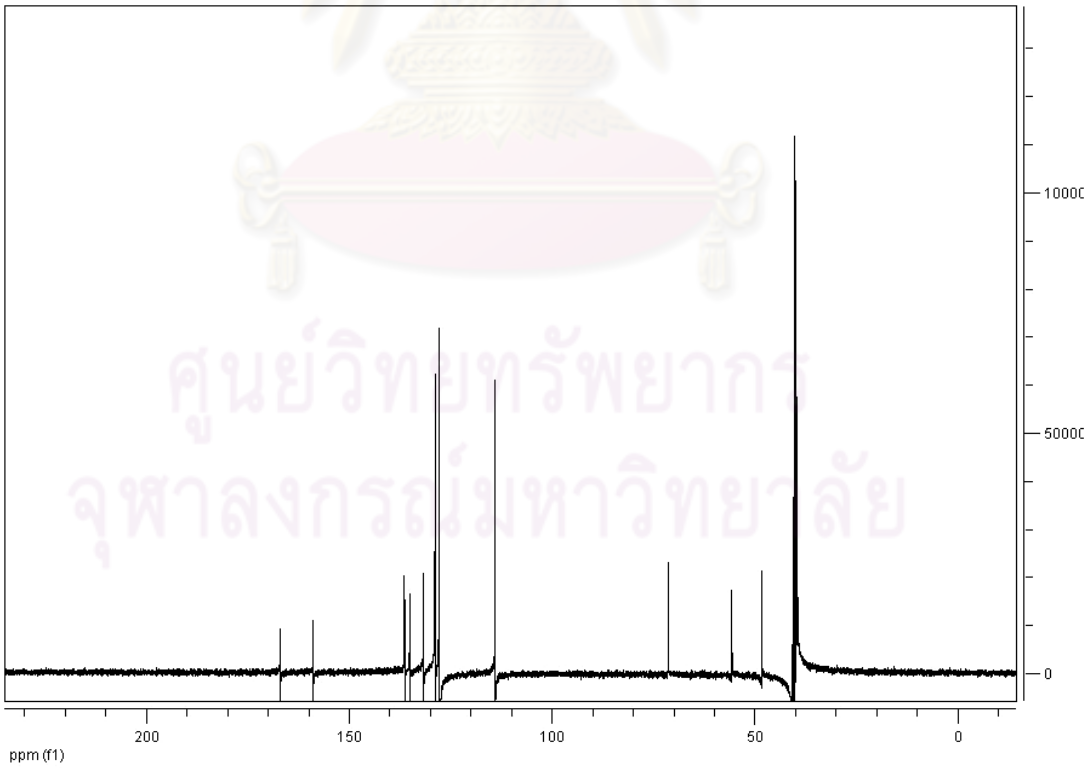


Figure 28 The ^{13}C NMR spectrum (methanol-d_6) of Compound **7**

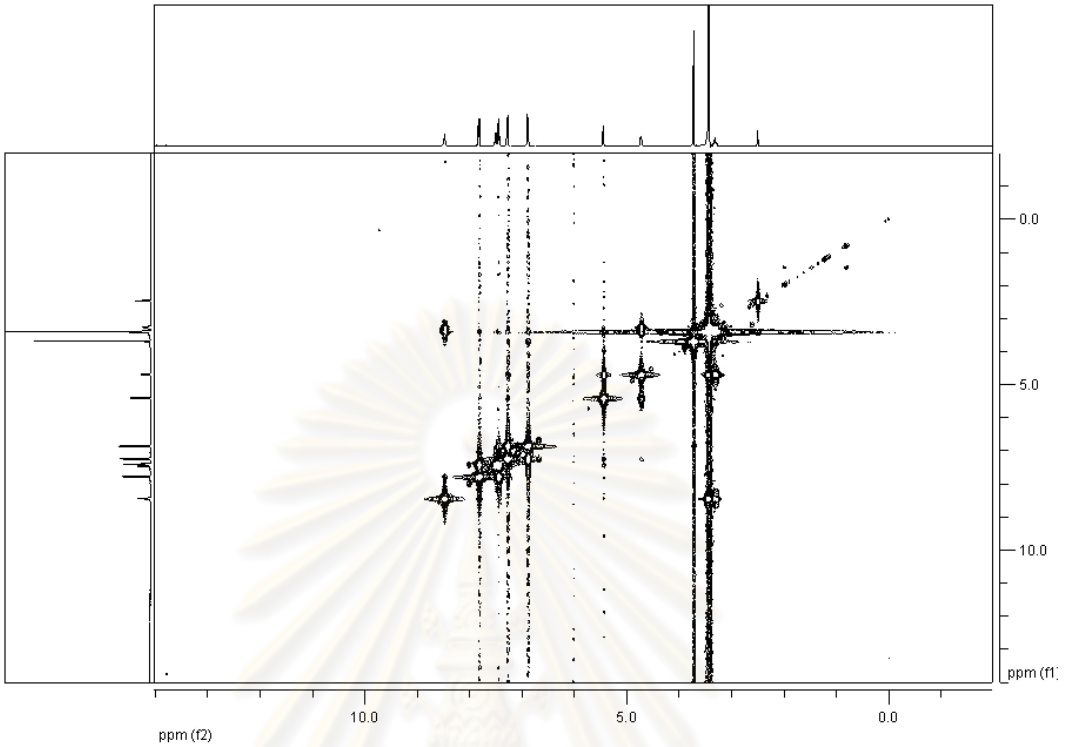


Figure 29 The COSY spectrum (methanol-d_6) of Compound **7**

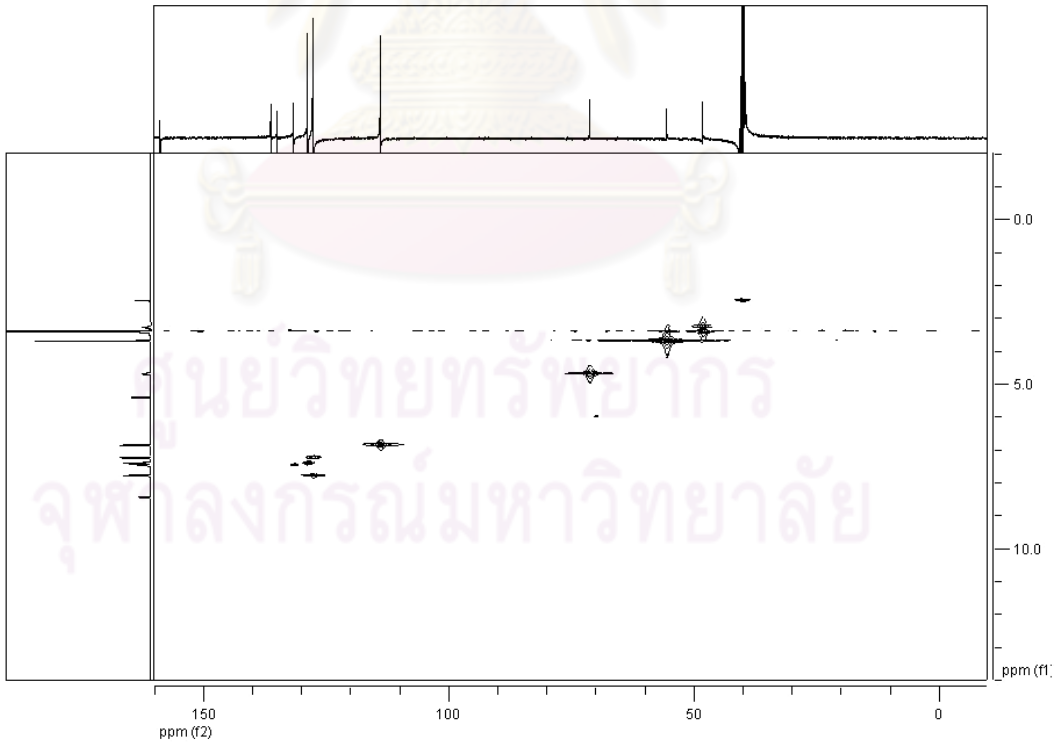


Figure 30 The HSQC spectrum (methanol-d_6) of Compound **7**

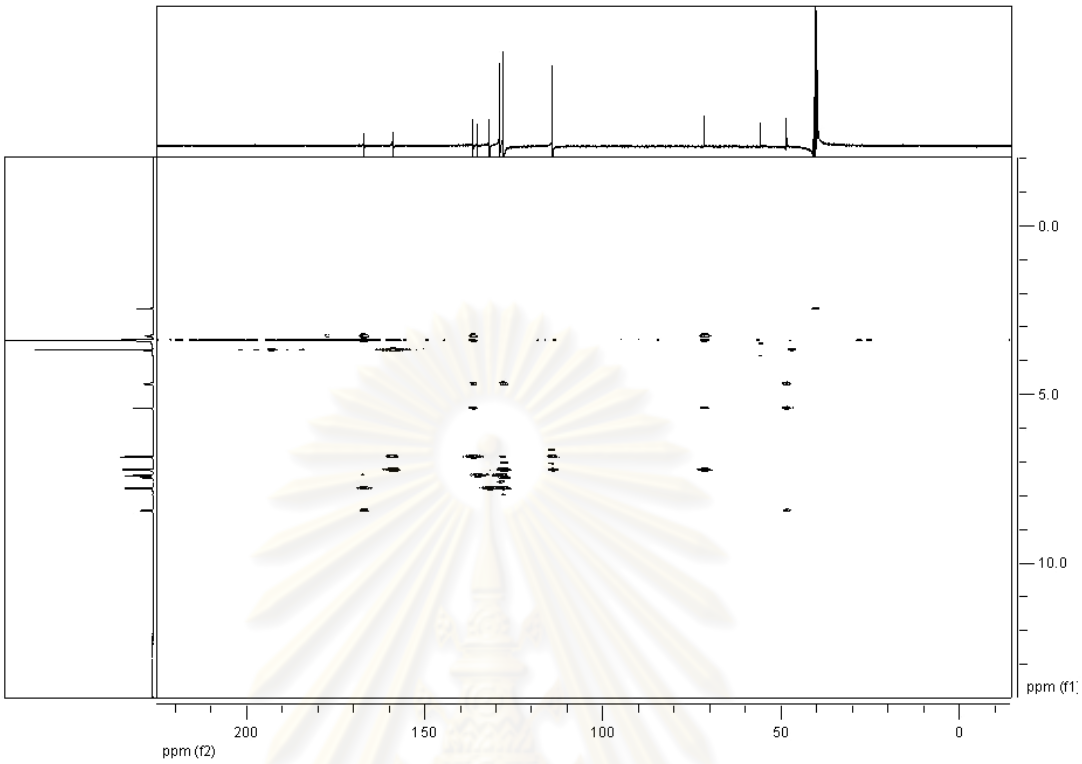


Figure 31 The HMBC spectrum (methanol-d_6) of Compound **7**

VITA

Ms. Janpen Tangjitjaroenkun was born on February 10, 1977 in Bangkok, Thailand. She graduated with Bachelor Degree of Science in Microbiolgy from Srinakarinwirot University in 1998 and Master Degree of Science in Microbiology from Kasetsart University in 2002. She worked as lecturer at Kasetsart University, Sriracha Campus for three years before pesuing her further study in 2005. Since then, she started her study on Ph.D. Degree of Program in Biotechnology, Faculty of Science at Chulalongkorn University. During her was study she received a grant fund under the program Strategic Scholarships for Frontier Research Network for the Ph.D. Program Thai Doctoral degree from Commission on Higher Education, Ministry of Education.