CHEMICAL COMPOSITIONS OF ESSENTIAL OILS FROM *Zanthoxylum piperitum* AND *Anethum graveolens* L. SEEDS AND THEIR ANTIFUNGAL ACTIVITIES ON *Aspergillus flavus*

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MASTER OF SCIENCE
IN BIOTECHNOLOGY

GRADUATE SCHOOL
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A THESIS SUBMITTED TO CHIANG MAI UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN BIOTECHNOLOGY

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IN BIOTECHNOLOGY

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ACKNOWLEDGEMENT

I would like to express my deepest gratitude to Asst. Prof. Dr. Hataichanoke Niamsup, my supervisor, for her kind advice, supervision, encouragement, very thoughtful guidance, and great support throughout the course of this research. I also wish to express my heartfelt thanks to Asst. Prof. Dr. Nuchnipa Nuntawong, my coadvisor, for her kind recommendation, encouragement and very kind-hearted suggestion throughout the course of this research. I also wish to express my sincere appreciation to Assoc. Prof. Dr. Griangsak Chairote, the chairman and Dr. Worraanong Leewattanapasuk, the examining committee for their valuable comments and suggestion.

I am grateful to Graduate School and Bruno Welderman Foundation for partial financial supports of my research, great opportunities, and invaluable experiences. I also wish to express my grateful thank to Dr. Prapimpuk Thaunsukon, Dr. Yaowapa Suvathi and my senior at Biotechnology research group, the Government Pharmaceutical Organization (GPO), for their kind advice, preliminary technique and encouragement throughout training. I am grateful to Dr. Angkana Inta at Department of Biology, Chiang Mai University, for identified botanically of seed spice in this study and I thank Department of Biology, Chiang Mai University, for Light microscope CH 30 RF-200. Furthermore, I thank Department of Chemistry, Chiang Mai University, for knowledge and facilities.

I would like to thank my senior, junior and my friends from Biochemistry and Biochemical Technology Branch, the Department of Chemistry at Chiang Mai University, who have cheered me up, encouraged me, and gave very thoughtful comments on my study and research.

And last but most important, I am deeply indebted to my family, whose love, support and sacrifice are the source of the energy, enabling me to accomplish this goal.

Chorpaka Phuangsri

Thesis Title	Chemical Compositions of Essential Oils from *Zanthoxylum piperitum* and *Anethum graveolens* L. Seeds and Their Antifungal Activities on *Aspergillus flavus*
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ABSTRACT

The aims of present study were to determine antifungal activity of the essential oils extracted from *Anethum graveolens* L. and *Zanthoxylum piperitum* seeds and to evaluate their potential as a food preservative. The chemical compositions of the oils were determined by GC–MS. *Trans*-isodillapiole (33.1%) , (+) -carvone (26.0%) , (-) -limonene (19.2%) and dihydrocarvone (12.3%) were the main components of *A. graveolens* L. oil. Whereas, the main components of *Z. piperitum* oil were β-phelladrene (23.2%), sabinene (14.6%) and brevifolin (11.9%).

The antifungal activity of *A. graveolens* L. and *Z. piperitum* oils were tested both in solid and liquid cultures of *Aspergillus flavus*. The minimum inhibitory concentrations of *A. graveolens* L. and *Z. piperitum* oils, when determined on the 9th day were 2.0 µL/mL and 4.5 µL/mL, respectively. The possible mechanism of the oils antifungal activity against *A. flavus* is considered to be distortion of the fungal conidial heads and the reduction of hyphal diameters which could be observed by light microscopes. To confirm their target in fungal plasma membrane, their effect on ergosterol synthesis was assessed. The result indicated that the ergosterol content in the plasma membrane of *A. flavus* when treated with 0.25 µL/mL *A. graveolens* L. oil was reduced to 32%. Whereas *Z. piperitum* oil at 1.0 µL/mL concentration decreased the ergosterol content by 28% compared to the control.

The potential use of essential oils from *A. graveolens* L. and *Z. piperitum* as an antifungal coat was tested on dried bird chili, a model product. The essential oil from *A. graveolens* L. at 2.0 and 4.0 µL/mL of reduced fungal development on chili peel by 84.00% and 93.30%, respectively. While *Z. piperitum* oil reduced to 45.20% and 89.32% at 4.5 and 9.5 µL/mL, respectively, compared with the control. Thus, the essential oils of *A. graveolens* L. and *Z. piperitum* may be applied as a natural food preservative.

หัวข้อวิทยานิพนธ์	องค์ประกอบทางเคมีของน้ำมันหอมระเหยจากเมล็ดพริกหอมและผักชีลาวและฤทธิ์การต้านเชื้อราต่อ *Aspergillus flavus*	
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บทคัดย่อ

วัตถุประสงค์ในการศึกษาครั้งนี้เพื่อศึกษาฤทธิ์การต้านเชื้อราของน้ำมันหอมระเหยจากเมล็ดพริกหอมและผักชีลาวและประเมินศักยภาพการเป็นสารกันบูดในอาหาร การศึกษาองค์ประกอบทางเคมีของน้ำมันหอมระเหยโดย GC-MS พบว่า *Trans*-isodillapiole (33.1%), (+)-carvone (26.0%), (-)-limonene (19.2%) และ dihydro-carvone (12.3%) เป็นองค์ประกอบหลักของน้ำมันผักชีลาว ขณะที่องค์ประกอบหลักของน้ำมันพริกหอมคือ β-phelladrene (23.2%), sabinene (14.6%) และ brevifolin (11.9%) การศึกษาฤทธิ์การต้านเชื้อรา *Aspergillus flavus* ของน้ำมันหอมระเหยจากเมล็ดพริกหอมและผักชีลาวพบว่าค่าความเข้มข้นที่น้อยที่สุดในการยับยั้งเชื้อรา คือ 2.0 ไมโครลิตรต่อมิลลิลิตรและ 4.5 ไมโครลิตรต่อมิลลิลิตรตามลำดับ การศึกษาผลของน้ำมันที่มีต่อเชื้อรา *A. flavus* ภายใต้กล้องจุลทรรศน์พบว่าส่วนหัวของเชื้อราถูกทำลายและขนาดเส้นผ่านศูนย์กลางของเส้นใยลดลง เพื่อเป็นการยืนยันว่าเยื่อหุ้มเซลล์เป็นเป้าหมายของสารต้านเชื้อรา จึงได้ศึกษาปริมาณของเออร์โกสเทอรอล จากการทดลองพบว่าเออร์โกสเทอรอลที่อยู่ในเยื่อหุ้มเซลล์ของเชื้อรา *A. flavus* เมื่อเลี้ยงในอาหารเหลวที่มีน้ำมันผักชีลาวเข้มข้น 0.25 ไมโครลิตรต่อมิลลิลิตรมีปริมาณลดลง 32% ขณะที่น้ำมันพริกหอมเข้มข้น 1.0 ไมโครลิตรต่อมิลลิลิตรทำให้ปริมาณเออร์โกสเทอรอลลดลง 28% เมื่อเทียบกับเชื้อราควบคุมที่ไม่มีน้ำมันหอมระเหย

การใช้น้ำมันหอมระเหยจากผักชีลาวและพริกหอมในการป้องกันการเจริญเติบโตของเชื้อรา *A. flavus* โดยการเคลือบบนพริกแห้งที่ใช้เป็นตัวอย่างผลิตภัณฑ์จำลอง โดยน้ำมันผักชีลาวใช้ความเข้มข้น 2 และ 4 ไมโครลิตรต่อมิลลิลิตร สามารถลดจำนวนสปอร์เชื้อราได้ 84.00% และ 93.30% ตามลำดับ ขณะที่น้ำมันพริกหอมลดจำนวนสปอร์เชื้อราได้ 45.20% และ 89.32% ที่ความเข้มข้น 4.5

และ 9.0 ไมโครลิตรต่อมิลลิลิตรตามลำดับ ดังนั้นน้ำมันหอมระเหยจากเมล็ดผักชีลาวและพริกหอมสามารถนำไปประยุกต์เป็นสารกันบูดธรรมชาติ

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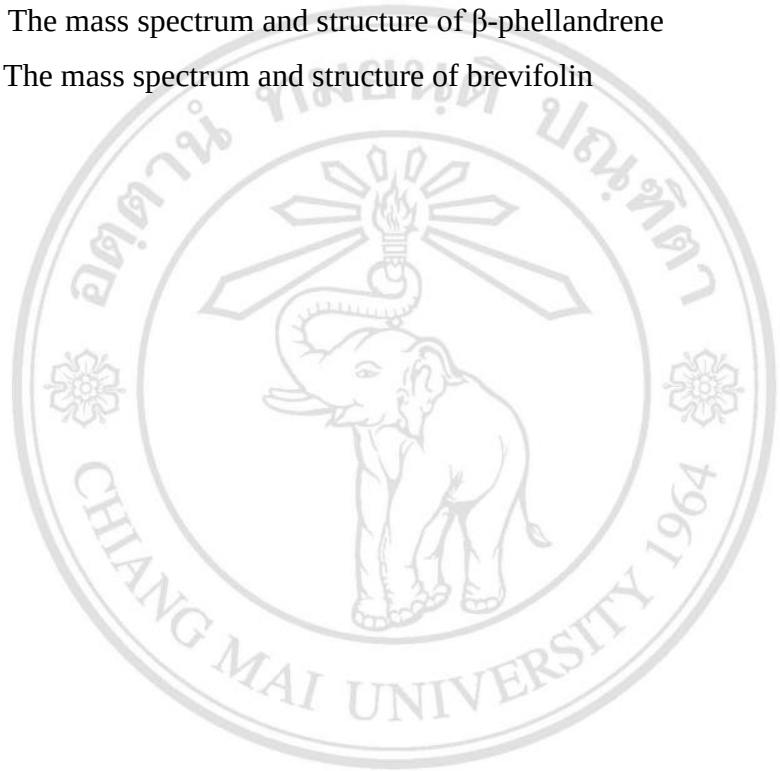


ABBREVIATIONS AND SYMBOLS

A	Absorbance
ºC	degree of Celcius
g	gram
mg	milligram
cm	centimeter
mm	millimeter
µm	micrometer
nm	nanometer
mL	milliliter
µL	microliter
kJ	kilojoule
ppm	one part per million
%	percentage
h	hour
min	minute
KOH	potassium hydroxide
rRNA	ribosomal deoxyribonucleic acid
SEM	Scanning electron microscope
TEM	Transmission electron microscopy
v/w	volume/mass
w/w	mass/mass
w/v	mass/volume
v/v	volume/volume

CHAPTER 1

INTRODUCTION

1.1 Statement background

Protection of food commodities during processing, transportation and storage from spoilage caused by microorganisms is challenging for food industries. Toxigenic fungi produce mycotoxins leading to significant qualitative losses of commodities and potentially inducing various health problems in consumers. Aflatoxins are a group of very hazardous and common toxic metabolites produced by *Aspergillus flavus* and *Aspergillus parasticus* (Chun et al., 2007). Aflatoxins are widely known for carcinogenic, teratogenic, hepatotoxic, mutagenic, and immunosuppressive properties and can inhibit several metabolic systems (Joseph et al., 2005). The production of the toxin affects agricultural commodities, e.g. dried chili powder, which is a widely used flavoring ingredient in Thai food. In addition, the high humidity of tropical and subtropical countries promotes fungal growth (Prakash et al., 2014). Aflatoxins are highly thermostable in nature and do not degrade during the cooking process (Kim, 2007). Chili is grown worldwide as a vegetable and spice. In Thailand, it is an important food crop grown for local consumption and food industry (Kraikruan et al., 2008). Bird chili (*Capsicum frutescens* Linn.) is one of the two chili types widely used in Thailand, known in Thai as "Prik Khee Nu" (Wangcharoen et al., 2009). Bird Chili was processed to chili powder with drying method in the open air followed by grinding. It is used as a flavoring ingredient in Thai food. The product becomes susceptible to fungal attack and the possible development of aflatoxin because of high humidity during prolonged storage. Thus, control of aflatoxin-producing fungi is essential. The use of synthetic chemical preservatives to control aflatoxigenic fungi in food has led to a number of environmental and health problems because of their carcinogenicity, teratogenicity, high and acute toxicity, and long degradation periods (Lingk, 1991). The concern of consumers and food processors about these risks has led to alternative

preservative to replace chemical pesticides. Natural products such as essential oils are interesting alternatives because they are safe for consumers, and have a low risk of developing resistance by pathogenic microorganisms (Cardile et al., 2009). However, there is no known report of the antifungal activity of the essential oils from *Zanthoxylum piperitum* and *Anethum graveolens* L. seeds. Thus, we have conducted research on the development of essential oils from spices as a potential source of eco-friendly antifungal agent.

In this study, we extracted the essential oils from spices and evaluated their effects on the mycelial growth and biomass of the fungus, *A. flavus*, previously isolated from dried chili powder. Furthermore, we studied morphological changes in hyphae of the fungus caused by the essential oils and their possible mechanism of action. We also assessed their potential application to control postharvest spoilage on stored chili, as an agricultural product model.

1.2 Food spoilage

The food spoilage has been a big problem for consumers and food processors. The contamination of food commodities by microorganisms especially *Aspergillus* had led to economic losses and health problems in consumers, particularly caused from secondary metabolite (mycotoxins) that they produce. The temperature and moisture are main factors for fungal growth especially in tropical and subtropical regions. Foods and agricultural products are infected by spoilage fungi such as peanuts, maize, rice, corn, cotton seed, fruits, vegetables, cereals, grains, nuts, legumes, pistachio nuts and oil seeds (Abyaneh et al., 2009; Kim, 2007; Shukla et al., 2012). In Thailand, rural Thai people are agriculturists dependent on agriculture as their main source of income. In Thailand, chili (*Capsicum frutescens* L.) is an economically important in local vegetable consumption and food industry. It is widely planted in the Northeast (warm climate area) of Thailand including Ubon Ratchathani, Sisaket and Khon Kaen provinces (Boonlue et al., 2012). They are normally used in dried powder form as a flavoring ingredient in Thai food shown in Figure 1.1. Dry chilies were ordinarily processed to chili powder with drying method under sun drying and open air. These processes are easily colonized by mycotoxigenic fungi. Thus, control of water activity (aw) to be lower than 0.75 can reduce contamination by spoilage fungi (Sardiñas et al.,

2011). Therefore, good harvesting and processing are key steps to reduce mycotoxins in food which make it safe for consumers.

Figure 1.1 Varieties of chili
(http://variety.teenee.com/foodforbrain/46783.html)
(Date: November 18, 2014)

1.3 *Aspergillus*

1.3.1 Classification and characterization of *Aspergillus* section *Flavi*

Aspergillus is a large genus which consist more than 250 anamorphic species. Special characteristic of the aspergillum is spore-bearing structure shown in Figure 1.2. Some members of the genus produce teleomorphs (sexual states) in nine genera and the genus is subdivided into 7 subgenera, each of which has been divided into sections (Klich, 2007 and Rodrigues et al., 2007). Taxonomy of *Aspergillus* genus is easily identified by conidiophore. Macromorphological characters are mainly dependent on conidial and mycelial color, colony diameter, colony reverse color, production of exudates and soluble pigments. While, micromorphological characterizations include seriation, shape and size of vesicle, conidia and stipe morphology, presence of Hülle cells, and morphology of cleistothecia and ascospores (Rodrigues et al., 2007). *Aspergillus flavus* (Circumdati subgenus) belonging to the section *Flavi* causes a serious problem for agricultural commodities. *A. flavus* and *A. parasiticus* are the major species producing aflatoxin. While *A. nomius*, *A. pseudotamarii*, *A. bombysis* and *A. parvisclerotigenus* are minor aflatoxin producers in this section. Other sections capable of producing aflatoxins include *A. ochraceoroseus*, *A. rambellii*, *Emericella venezuelensis* and *E. astellata* (Frisvad et al., 2005). While, *A. oryzae*, *A. sojae* and *A. tamari* are used in fermented foods in Asia

(Klich, 2007) . In addition, the rDNA target regions, the internal transcribed spacer regions 1 and 2 (ITS1 and ITS2) and the variable regions at the 5' end of the 28S rRNA gene (D1-D2) region are used to distinguish *Aspergillus* species. Genes that regulate aflatoxin biosynthesis include locus *aflR* from *A. flavus* and *A. parasiticus*, and several structural genes, e.g. *pks* A, *nor*-1, *ver*-1, *uvm* 8 and *omt* A shown in Figure 1.3 (Rodrigues et al., 2007).

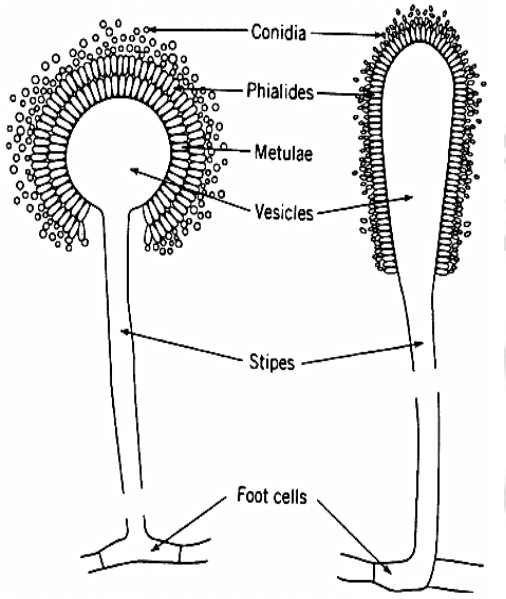


Figure 1.2 Characteristic conidiophores of *Aspergillus* (Klich, 2007)

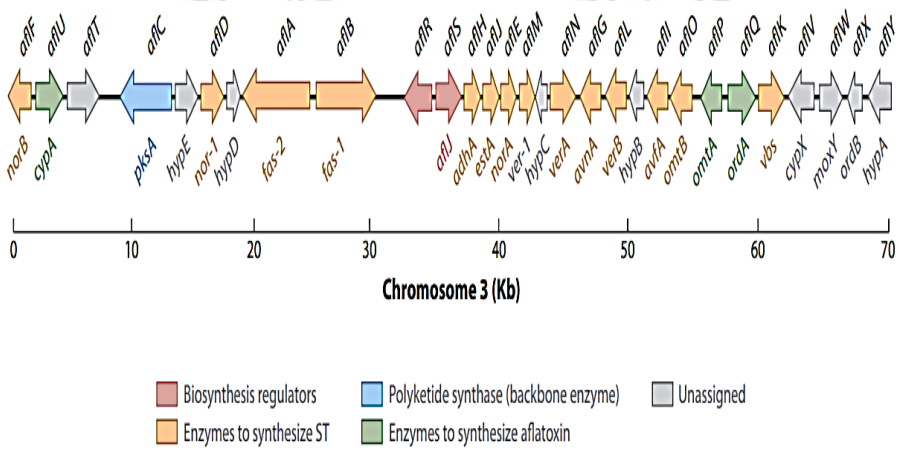
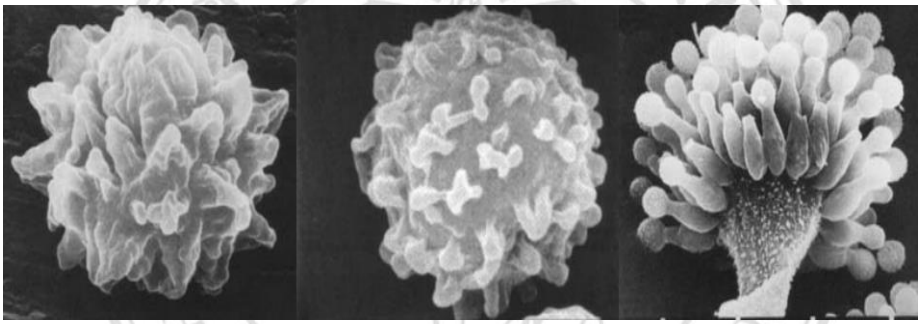


Figure 1.3 The DNA target region regulating aflatoxins production (Amaike and Keller, 2011)

1.3.2 Morphology of *Aspergillus* producing aflatoxin

The food spoilage fungi (*A. flavus*, *A. parasiticus*) are main aflatoxin producers. Scanning electron microscopy was used to study morphology of the aflatoxigenic fungi as shown in Figure 1.4. The conidial head is considered the primary character for separation of the two species. Conidia head of *A. flavus* has thin, finely roughened wall, phialides (biseriate conidial head) and spherical to elliptical shapes. While, conidia head of *A. parasiticus* are more spherical and noticeably echinulate or spinulose and phialides only (uniseriate conidial head) (Figure 1.4c). The differentiation of *Aspergillus* species are summarized in Table 1.1 (Rodrigues et al., 2007).



(A) (B) (C)

Figure 1.4 Scanning electron microscopy pictures of (A) *A. parasiticus* and (B) *A. flavus* spores, where spore ornamentation differences are clearly seen; and of (C) *A. parasiticus* conidial head (Rodrigues et al., 2007)

Table 1.1 Morphological differentiation of *A. flavus* and *A. parasiticus* (Rodrigues et al., 2007)

Fungi	Colony colour	Seriation	Conidia	
			shape	texture
A. flavus	Yellow/ green	biseriate/uniseriate	globose/elliptical	smooth/ finely
A. parasiticus	Ivy green	uniseriate/biseriate	globose	rough

1.4 *Aspergillus flavus*

Aspergillus flavus grows rapidly and the diameter will reach 65–70 mm in 7 days. The colony is yellow–green as shown in Figure 1.5 (A-B). More details can be observed by light and scanning electron micoroscopy shown in Figure 1.5 (C-E): conidiophores are usually long (400–800 µm), rough, pitted and spiny. The shape of conidia head has globose to subglobose (3.5 to 4.5 mm in diameter) and phialides (biseriate conidial head).The vesicles are globose to elongate usually 20–45 µm in diameter (Klich, 2007).

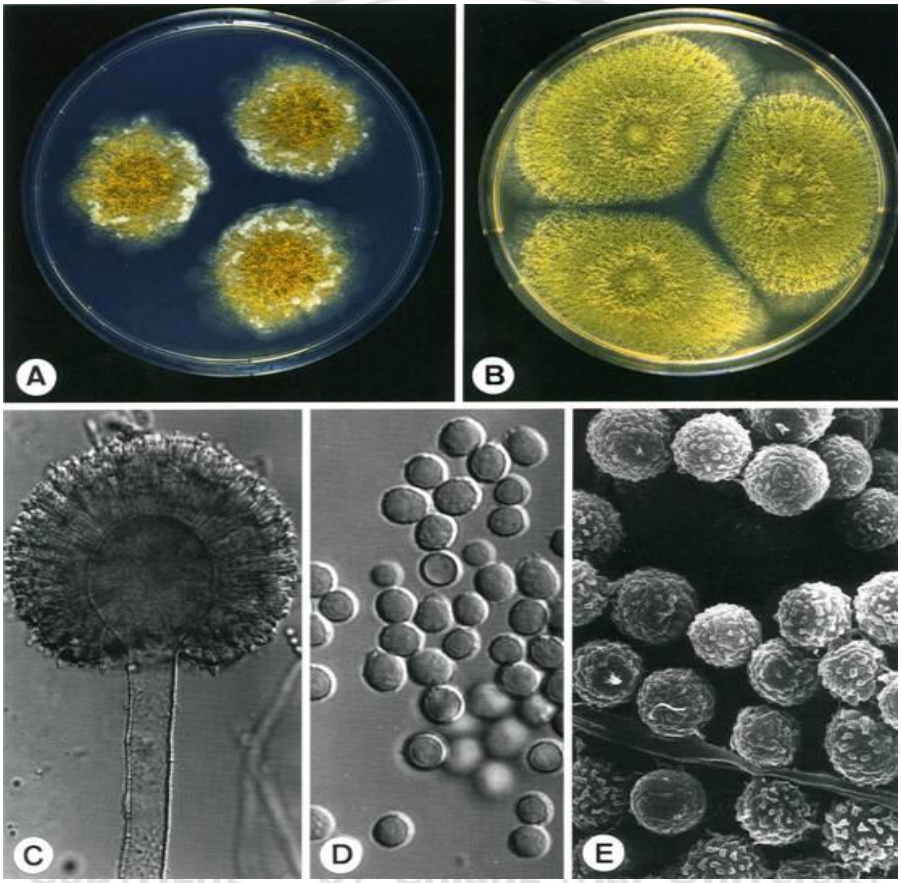


Figure 1.5 The colony diameters on Czapek's Agar (A)**,** on Malt Extract Agar (B) in 14 days 25°C, aspergillum X530 (C) and conidia X1820 (D) under light microscy, conidia (SEM) X8655
(http://www.bcrc.firdi.org.tw/fungi/showImage.jsp?id=IM200802210058)
(Date: November 18, 2014)

1.5 Mycotoxin

Mycotoxins are low molecular weight metabolites produced from the secondary metabolism of some fungi, or molds during processing and storage of harvested agricultural products. It has led to significant economic losses of commodities and potentially induced serious risks for human and animal health. Mycotoxins are frequently found in temperature and humid climate area suitable for the growth of fungi or molds. Toxigenic fungi or molds can produce one or more one of secondary metabolites and not all fungi or molds are toxigenic (Zain, 2011). Specific environment is important for growth and secondary metabolism of fungi and molds. For example, some fungi can grow in warm and humid area including *A. flavus*, *A. parasiticus* and *A. ochraceus*. While *Penicillium expansum* and *P. verrucosum* are essentially temperate fungi. Mycotoxins group are produced by *Aspergillus* stains in crop plant products, while the cereal grains are infected by *Penicillium* mycotoxins and *Fusarium* fungi contaminate cereals in warm countries (Mello, 2000) . Table 1.2 shows that the mycotoxins were found in different global locations (Iheshiulor et al., 2011) and the principal mycotoxins occurring in foods and feeds are shown in Table 1.3. (Mello, 2000).

Table 1.2 Geographic occurrences of mycotoxins (Iheshiulor et al., 2011)

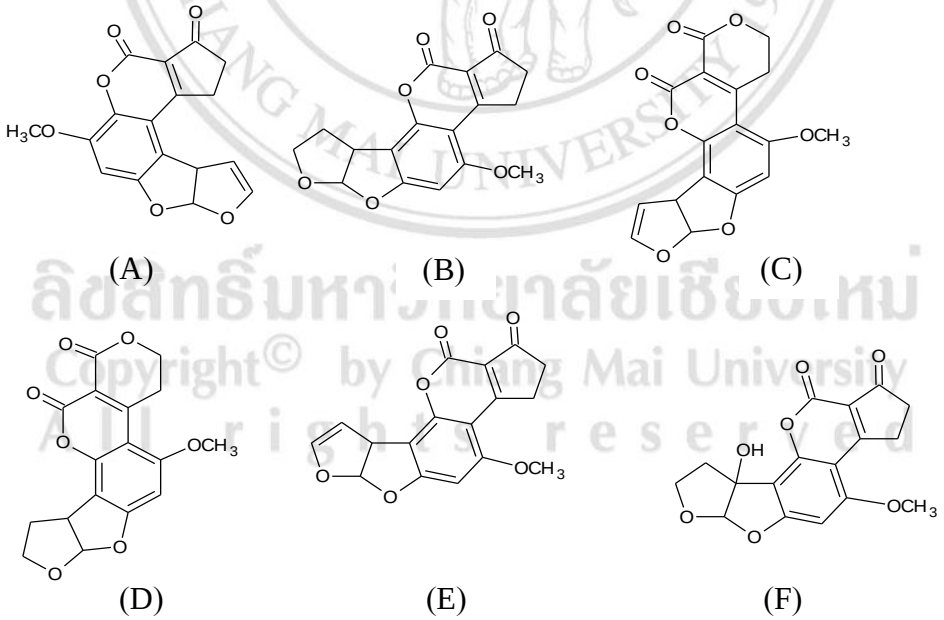
Location	Mycotoxins
Western Europe	Ochratoxin, Vomitoxin, Zearalenone
Eastern Europe	Vomitoxin, Zearalenone
North America	Ochratoxin, Vomitoxin, Zearalenone, Aflatoxins
South America	Aflatoxins, Fumonisins, Ochratoxin, Vomitoxin, T-2 toxin
Africa	Aflatoxins, Fumonisins, Zearalenone
Asia	Aflatoxins
Australia	Aflatoxins, Fumonisins

Table 1.3 Origin of principal mycotoxins occurring in common foods and feeds (Mello, 2000)

Mycotoxins	Fungal species
Aflatoxins	*Aspergillus flavus; A. parasiticus*
Cyclopiazonic acid	*A. flavus*
Ochratoxin A	*A. ochraceus; Penicillium viridicatum; P. cyclopium*
Citrinin	*P. citrinum*; *P. expansum*
Patulin	*P. expansum*
Citreoviridin	*P. citreo-viride*
Deoxynivalenol	*Fusarium culmorum*; *F. graminearum*
T-2 toxin	*F. sporotrichioides*; *F. poae*
Diacetoxyscirpenol	*F. sporotrichioides; F. graminearum; F. poae*
Zearalenone	*F. culmorum*; *F. graminearum; F. sporotrichioides*
Fumonisins; moniliformin; fusaric acid	*F. moniliforme*
Ergopeptine alkaloids	*Neotyphodium coenophialum*
Tenuazonic acid; alternariol; alternariol methyl ether; altenuene	*Alternaria alternata*
Lolitrem alkaloids	*N. lolii*
Ergot alkaloids	*Claviceps purpurea*
Phomopsins	*Phomopsis leptostromiformis*
Sporidesmin A	*Pithomyces chartarum*

1.5.1 Aflatoxin

Aflatoxins produced by *Aspergillus* species especially *A. flavus* and *A. parasiticus* are the most toxic, mutagenic, teratogenic, and carcinogenic. "Aflatoxin" word comes from the combination of "a" for the *Aspergillus* genus and "fla" for the species *flavus*, and toxin meaning poison. The aflatoxins were found in 1960s after the death of more than 100000 turkey poults (in the U.K.). Aflatoxins have six groups in nature (B1, B2, G1, G2, M_1 and M_2) showed in Figure 1.6. UV light and thin-layer chromatography methods are used to differentiate aflatoxins groups. Groups B refer to fluorescence under UV light blue color and groups G refer to green color. Aflatoxins M1 and M2 type produced in milk and dairy products. Aflatoxin B1 is the most toxic and carcinogen produced by toxigenic strains especially *A. flavus* and *A. parasiticus* (Zain, 2011). The suitable temperature is the main factor for growth of fungi and aflatoxin production. For example, the low temperature range of *A. parasiticusis* is 6 to 8 ^{o}C and high is 44 to 66 ^{o}C, optimum being 25 to 35 ^{o}C. While, *A. flavus* could produce toxin between 12 and 42 ^{o}C and its optimum is 28 to 30 ^{o}C (Bhat et al., 2010). Thus, tropical and subtropical regions have conditions favoring the fungal growth.



(A) (B) (C)

(D) (E) (F)

Figure 1.6 Structures of aflatoxins A) AFB1, B) AFB_2, C) AFG_1, D) G2, E) AFM_1 and F) AFM_2

1.5.2 Aflatoxins and their consequences to health

Aflatoxins are produced by *A. flavus* and *A. parasiticus* in food commodities including maize, oilseeds, spices, groundnuts, tree nuts, milk, and dried fruit during processing, transportation and storage of harvested products. Aflatoxin B1 (AFB1) is one of the most hazardous mycotoxins for human health as shown in Figure 1.7. Aflatoxins mechanism is passing to digestion system in the body. The liver is where transformation of aflatoxins B1 by the cytochrome P 450 enzyme system (CYP1A2, 3A4, 3A5, 3A7) to aflatoxin-8, 9-epoxide occurs. The product can bind to liver proteins resulting in acute aflatoxicosis or to DNA and induce hepatocellular carcinoma (HCC). HCC as a result of chronic aflatoxin exposure has been well presenting most often in persons with chronic hepatitis B virus (HBV) infection. When hepatitis B virus (HBV) infects liver cell, it causes higher liver cancer risk. In addition, aflatoxins have an effect to immune suppression (diminished weight and height) and stunted growth in children (Wu et al., 2011).

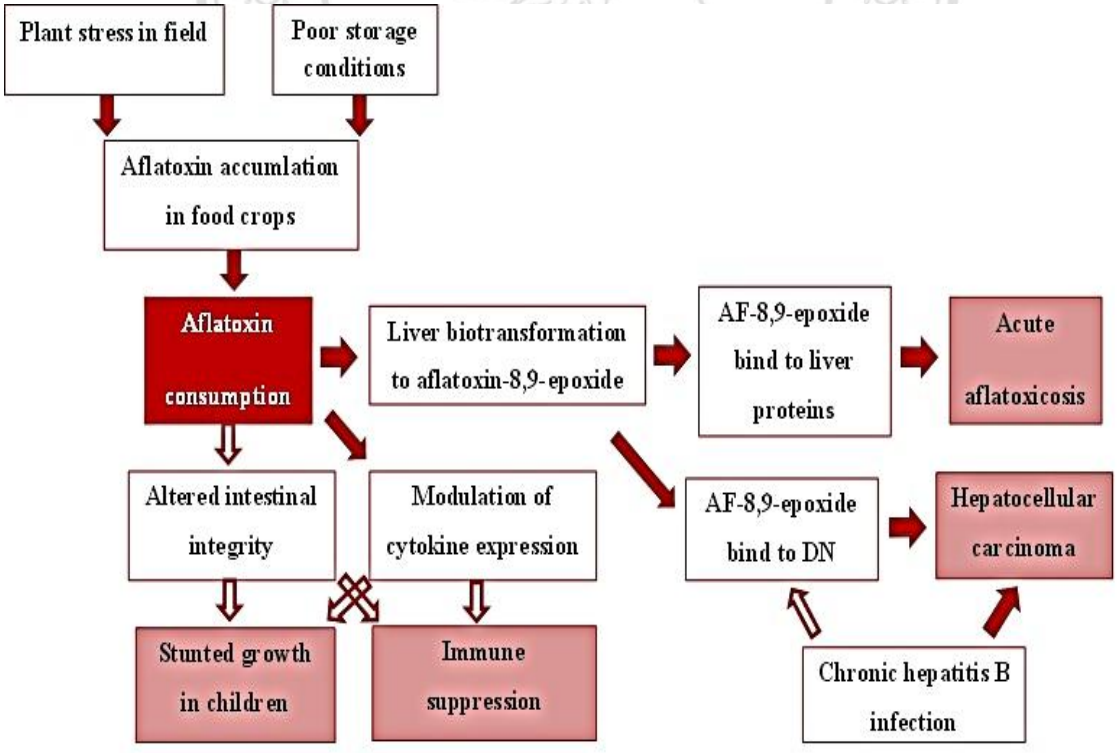


Figure 1.7 Aflatoxin and disease pathways in humans

1.6 Fungal cell

1.6.1 Structure of fungal cell

The fungal cell structurally consists of nucleus, ribosome, lysosome, golgi bodies, endoplasmic reticulum, mitochondria, septum, cell membrane and cell wall. The cell wall is an important organelle for the growth, survival, host–pathogen interactions and structural barrier of fungi (Latge´ and Beauvais, 2014; Schachtschabel et al., 2012). It consists of polysaccharides, mainly polymers of glucose (β-1, 3 and β-1, 6 -glucans), N-acetyl-glucosamine (chitin), galactofuranose (galactomannan), galacto-aminogalactan and cell wall glycoproteins (galactomanno-proteins) (Schachtschabel et al., 2012). The chitin, a polymer of β-1, 4-linked N-acetylglucosamine (GlcNAc), is necessary for structural integrity, viability and virulence (Rogg et al., 2011). The plasma membrane of fungi contains sterols that are impotent for the organization and functions of this structure. The structure of cell membrane and cell wall is shown in Figure 1.8.

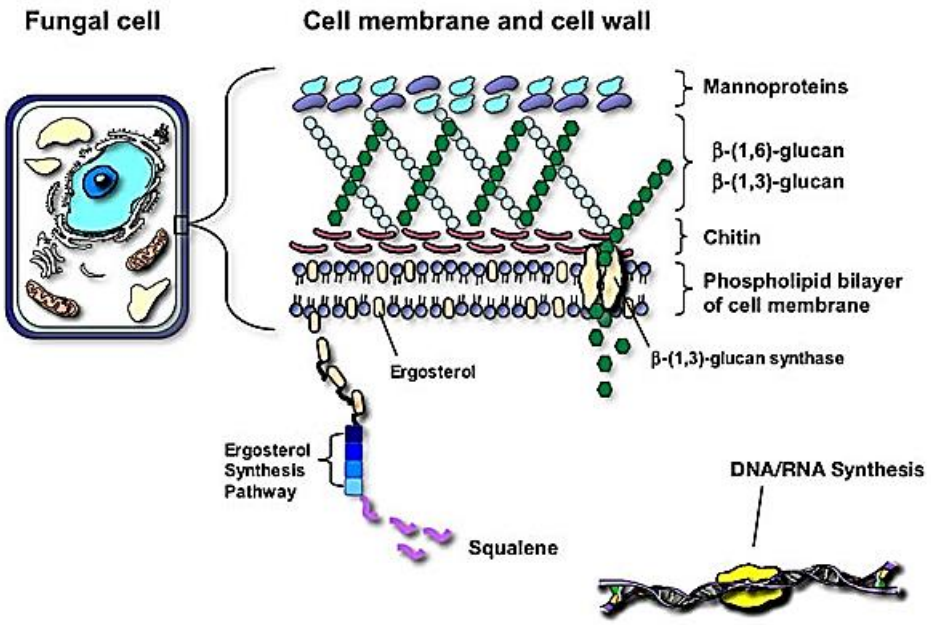


Figure 1.8 The structure of fungal cell
(http://www.doctorfungus.org/thedrugs/images/drug-targets.jpg)
(Date: November 18, 2014)

1.6.2 Ergosterol

The major sterols are found in three predominant forms: cholesterol in animal, phytosterols (sitosterol, stigmasterol, campesterol) in plants, and ergosterol in fungi as summarized in Figure 1.9 (Dupont et al., 2011) . Ergosterol is an important constituent of fungal membrane lipid. It is most abundant in the fungal plasma membrane and the main sterol in eukaryotic cells, which is responsible for structural membrane character, such as fluidity, permeability and pheromone signaling same as cholesterol pathway of mammalian cells (Veen and Lang 2005; Yun et al., 2014) . Each of these sterols is the end-product of a long multistep biosynthetic pathway that derives from a common initial pathway (acetyl CoA to squalene epoxide) (Figure 1.9). The detailed ergosterol biosynthetic pathway is shown in Figure 1.10 and genes and corresponding enzymes of ergosterol biosynthesis are listed in Table 1.4. Several enzymes pathway, including Erg6, Erg2, Erg3 and Erg4, are unnecessary, while others (e.g. Erg9, Erg1, Erg7, Erg11 and Erg24) are important for fungal viability (Yun et al., 2014) . Ergosterol biosynthesis is the most important biochemical difference between opportunistic fungi and their human host. Thus, it is investigated as a target of antifungal drugs (Nes et al., 2009). Several researchers previously reported the blockage of key enzyme ergosterol synthesis (Kagan et al., 2005; Nes et al., 2009; Yun et al., 2014).

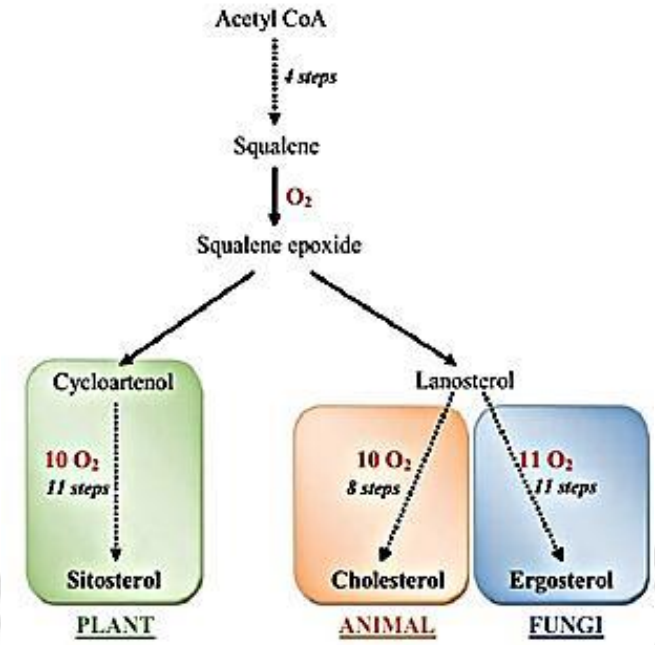


Figure 1.9 Biosynthesis pathways of sterols in plants, animals, and fungi (Dupont et al., 2011)

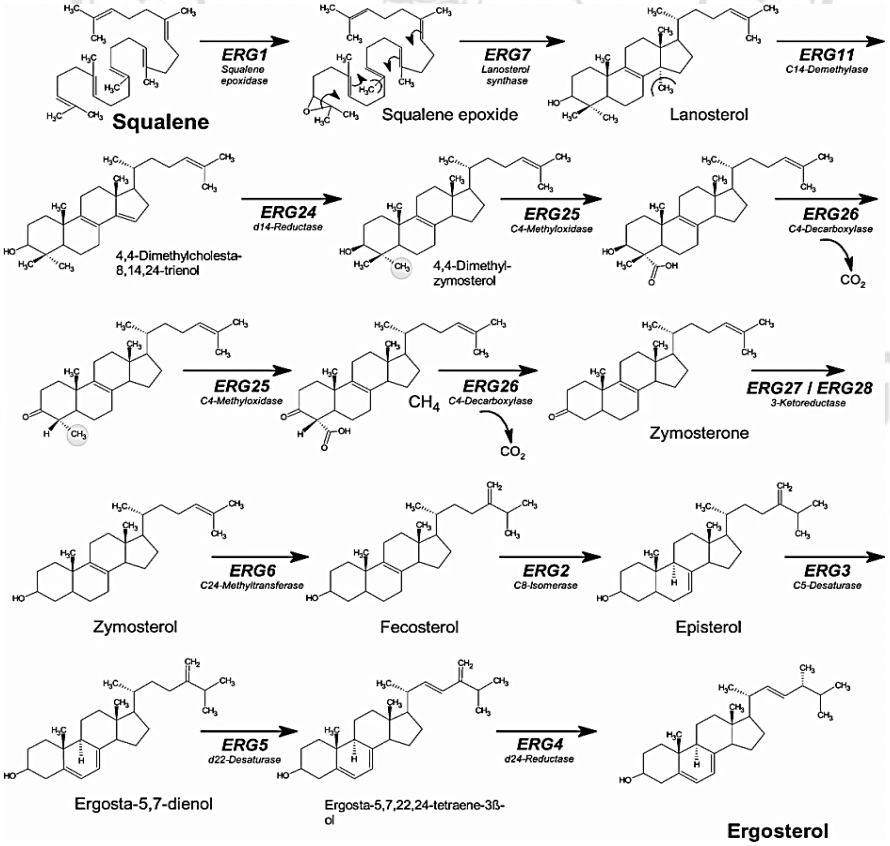


Figure 1.10 Ergosterol biosynthetic pathways (Veen and Lang, 2005)

Table 1.4 Genes and corresponding enzymes of ergosterol biosynthesis, in order of occurrence in the biosynthetic pathway (Kagan et al., 2005)

Gene	Enzyme encoded	Targeting SBI**
ERG10	Acetoacetyl-CoA thiolase	
ERG13	HMG-CoA synthase	
HMG1, HMG2	HMG-CoA reductase	
ERG12	Mevalonate kinase	
ERG8	Phosphomevalonate kinase	
ERG19	Mevalonate pyrophosphate decarboxylase	
IDI1	Isopentenyl diphosphate isomerase	
ERG20	Geranyl pyrophosphate synthase	
ERG9	Squalene synthase	
ERG1	Squalene epoxidase	Class IV SBI (medical fungicides)
ERG7	Lanosterol synthase	
ERG11	Lanosterol C-14 demethylase	Class I SBI
ERG24	Sterol C-14 reductase	Class II SBI
ERG25	Sterol C-4 methyloxidase	
ERG26	Sterol C-3 dehydrogenase	
ERG27	Sterol C-3 keto reductase	Class III SBI
ERG28	Unknown function	
ERG6	Sterol C-24 methyltransferase	
ERG2	Sterol C-8 isomerase	Class II SBI
ERG3	Sterol C-5 desaturase	
ERG5	Sterol C-22 desaturase	
ERG4	Sterol C-24 reductase	

**SBI: Sterol biosynthesis inhibitor

1.7 Spice

Etymologies of spices were the Latin language, meaning “specific kind”. It is called in each countries by other names such as beharat (Arabic), besamim (Hebrew), epices (French), especerias (Spanish), kimem (Ethiopian), Kruen tet (Thai), Krooder (Norwegian), masala (Hindi), rempah (Malaysian and Indonesian), specie (Italian), sheng liu (Mandarin), specerjien (Dutch) (Uhl, 2000).

1.7.1 Spices in history

Spices consisted survey, adventure, religious missions, commerce, and victory in history. In ancient and medieval times, spices had prodigious commercial value. Spices and flavorings are most found in the tropics or subtropics. The origin of most popular spices and flavorings is east. Spices (anise, basil, cardamom, cinnamon, clove, garlic, ginger, mace, mustard, nutmeg, onion, peppers, star anise, tamarind, and turmeric) are found in India, Southeast Asia and China. The Middle East, North Africa, and other parts of the Mediterranean have bay leaf, coriander, cumin, dill, fennel, fenugreek, rosemary, sage, sesame, and thyme. The winter zones of Europe are origin of spices such as juniper and horseradish, while annatto, chile peppers, chocolate, epazote and sassafras come from the Americas. Indians, Middle Easterners, Chinese, Aztecs, and Incas have used spices since ancient time. Spices were used to enhance lusciousness and flavor for food in modern age. In addition, spices have been applied for food preservation such as meats or fish, protection for off-flavor, scent and cosmetics. Furthermore, spices have pharmaceutical value and are used for treatment and to prevent diseases, antidotes for potion. In early civilizations, cinnamon, garlic, and oregano were used to control distribution of the plague. Many cultures used spices for religious, ceremony and belief because of their amazing properties (Raghavan, 2007).

1.7.2 Origin and major areas of cultivation of herbal spices

The herbal spices were found in Mediterranean countries and used since ancient Egyptian and Roman times. The Mediterranean zone is the major source of herbal spices. While, cultivation of herbs were vastly found in Germany, France and the USA. The countries of origin of herbal spices and major areas of cultivation are given in Table 1.5.

Table 1.5 Origin and major areas of cultivation of herbal spices (Peter, 2004)

Spice	Origin	Major areas
All spice	Central America, Mexico	Jamaica, Honduras, Guatemala, and West Indies Leeward Islands
Basil, sweet	India, Iran, Africa	Belgium, France, Bulgaria, Hungary, India, Italy, Poland, Spain and USA
Bay leaves (laurel)	Countries bordering the Cyprus, France, Greece, Italy, Mediterranean	Israel, Morocco, Portugal, Spain, Turkey and Yugoslavia
Caraway	Europe	Netherlands, Bulgaria, Canada, Germany, India, Morocco, Poland, Romania, Russia, Syria, UK and USA
Celery	Europe, Africa	France, Hungary, India, Japan, Netherlands, UK and USA
Chervil	Russia and Western Asia	France, Italy, Russia, Spain, UK and USA
Chive	Northern Europe	Austria, Canada, France, Germany, Italy, Netherlands, Switzerland, UK and USA
Coriander	Africa, Europe	Argentina, Bulgaria, China, France, India, Italy, Morocco, Mexico, Netherlands, Romania, Russia, Spain, Turkey, UK, USA and Yugoslavia
Dill	France, Spain and Russia	Canada, Denmark, Egypt, Germany, Hungary, UK India, Netherlands, Mexico, Pakistan, Romania, and USA
Fenugreek	Europe and West Asia	Algeria, Argentina, Cyprus, Egypt, France, Germany, Greece, India, Italy, Lebanon, Morocco, Portugal, Spain, USA and Yugoslavia
Oregano	Greece, Italy and Spain	Albania, France, Greece, Italy, Spain Mexico, Turkey and Yugoslavia
Leek	Mediterranean region	Europe, Africa, Near East and USA
Spearmint	England and UK	Germany, Japan, Netherlands, Russia

Fennel	Europe and Asia Minor	Bulgaria, China, Denmark, Egypt, France, Germany, India, Italy, Japan, Morocco, Netherlands, Romania, Russia, Syria, UK and USA
Marjoram	Saudi Arabia and Western Asia	Italy, Morocco, Portugal, Spain, France, Germany, Grenada, Hungary, South America, Tunisia, UK and USA
Parsley	Sardinia	Algeria, California, Louisiana, Italy Belgium, Canada, France, Germany, Greece, Japan, Lebanon, Netherlands, Portugal, Spain, Turkey and UK
Rosemary	Europe	Algeria, France, Germany, Italy, USA Morocco, Portugal, Romania, Russia, Spain, Tunisia, Turkey and Yugoslavia
Sage	Albania and Greece	Albania, Cyprus, Dalmatian Islands, UK Canada, Southern France, Italy, Spain Portugal, Turkey, Yugoslavia and USA
Thyme	China and East Indies	Bulgaria, Canada, France, Germany, Greece, Italy, Morocco, Portugal, USA Russia, Spain, Tunisia, Turkey and UK

1.7.3 Emerging global spices and flavoring

Spices were used to enhance flavor, color, or texture in foods and beverages. Some of the emerging global spices and flavoring are shown in Table 1.6.

Table 1.6 Emerging global spices and flavoring (Raghavan, 2007)

Seeds/fruits/bark	Ajowan, black cumin, canela, green cardamom, asafoetida, fennel seed, fenugreek, star anise, sumac, annatto, mustard seed
Leaves/stalks/stems	Thai basil, cilantro, kari leaf, kaffir lime leaf, epazote, shiso, lemongrass, recao leaf
Chile peppers	Chipotle, aji, ancho, bird pepper, cayenne, guajillo, habanero, poblano, rocoto, Cachucha
Fruits	Citrus, cranberry, guava, mango, persimmon, green papaya, kalamansi, pomegranate, olive, kokum
Vegetables	Mushrooms, tomatoes, squash, taro, yams
Nuts	Almond, candlenut, cashew, peanut, pine nut, pistachio, gingko biloba
Seeds	Pumpkin, sesame, sunflower, lotus
Food wrappers	Corn husk, lotus leaf, pandan leaf, banana leaf, nori, hoba leaf, hoja santa
Flowers	Jasmine, orange blossom, rose, squash blossom, violet, roselle, lavender, ginger flower
Roots	Coriander, ginseng, lotus, turmeric, galangal, wasabi, licorice, daikon
Fish and shrimp	Blacan, nam pla, nuoc mam, trasi, guedge, dashi (dried, fermented, and smoked)
Legumes	Miso, oyster sauce, soybean pastes (fermented, pickled, and salted), dals, beans
Other	Balsamic vinegar, coconut milk, ghee, dende oil, palm sugar, chocolate, vanilla, coffee, tea

1.7.4 Form, functions and applications of spices

Spices are available in many forms based on the specific application, processing parameters, and shelf life such as the fresh, dried, or frozen, ground, crushed, pureed, as pastes, extracts, or infusions. The summary of the advantages and disadvantages of various spice forms is shown in Table 1.7.

1.7.4.1 Fresh spices

Fresh tastes of spices in foods are frequently demanded by consumers and chefs. It is the overall flavor, aroma, and texture of some spices such as ginger, cilantro, sweet basil, or chili peppers. Consumers seek fresh taste of spices which cause their aroma. Aroma could be lost during harvesting, storing, processing, or handling. For example, fresh ginger has more pungency than dried ginger because pungent-producing gingerol was higher than in dried ginger. Fresh spices provide crispy textures and colorful appeal. Fresh spices become aromatic when roasted or fried in oil. However, consistency of fresh spices is more difficult to control because their origin, age, and storage conditions cause flavor variations. Therefore, dry spices and spice extractives are the forms most often used in foods or beverages processes. Fresh spices are not often used in foods industries, but are usually found in restaurants and household.

1.7.4.2 Dried Spices

Spices are frequently used in dried form because they are easier to process, have longer shelf life and lower cost. Dried spices (finely or coarsely ground and cracked) are ground by milling to various-sized particulates. Pounding is manufacture rapid air movement and heat that dissipates some of the volatile oils and even change some natural flavor notes through oxidation. Distribution of ground spices is more than fresh whole spices in the market. Some volatile oils are released through grinding. While, some spices flavor is scented through drying because of disposal of humidity. Dried spices can better withstand higher temperatures and processing conditions than fresh spices. Sensory characteristics of spices were different depending on planting location and harvesting, storing, and process. For example, dried ginger of India has a slim lemon-like flavor. While, that of southern China comes with slightly bitter notes.

1.7.4.3 Spice extractives

Spice extractives are highly concentrated forms and combination between taste (non-volatile compounds) and aroma (volatile compounds) based on type of spices and their characteristic flavor. The sensations can be sweet, piney, sour, bitter, spicy, sulfury, earthy and pungent derived from all parts of the plant such as buds, flowers, leaves, stems, fruits, wood and bark. The ratio of volatile to non-volatile portions varies among spices causing similar or different flavors within a genus or even within varieties. Volatile oils or essential oils characterized by a strong aroma are formed by aromatic plants as secondary metabolites. The major compounds of essential oil from spice belong to terpene group including monoterpenes, diterpenes, triterpenes and sesquiterpenes. The main volatile compounds of spices are monoterpenes, which give strong aroma when heating, crunching, or cutting of spice cells. Spice extractives (essential oils, oleoresins and dry encapsulated oil) are more concentrated and stable than fresh or dried spices. Thus, it is used at little level and favorable for food industries.

Table 1.7 A summary of advantages and disadvantages of different spice forms (Raghavan, 2007)

Spice Form	Advantages	Disadvantages
Fresh whole	Fresh flavor Release of flavor slowly at high temperatures Label friendly	Variability in flavor and color Short shelf life High microbes Unstable to heat Seasonal availability
Dried ground	Process friendly Longer shelf life Easy handling and weighing Stronger taste intensity than fresh	Less aroma Hot spots—flavor and color Takes more storage space Variability in flavor and color Undesirable specks Seasonal availability Spice dust contamination during production
Extractives	Standardized flavor and color Uniform appearance, color and flavor Low usage Low microbes Less storage space Available throughout the year	Difficult to handle and weigh Aroma and taste usually not typical of natural spice Loss of volatiles at high temperatures

1.8 Essential oils from some spices

Essential oils are produced by grinding, crushing, distillation (using water, steam or steam and water, solvent) from the overall plant (leaf, seed, stem, root, flower and bark). Composition of essential oils depends on extraction method and type of spices. Essential oils are very complex natural mixtures which may contain about 20-60 components. Some popular spices examble of essential oils are shown in Table 1.8. The major chemical components are terpenes (monoterpenes, and sesquiterpenes), and other aromatic and aliphatic constituents. The essential oils have biological activity, hence being used for new applications in human health and agriculture alternatives or complements to synthetic compounds of the chemical industry (Bakkali et al., 2008).

Table 1.8 Example of characterized essential oil components in some popular spices (Przygodzka et al., 2014)

Spices	Components in essential oils
Nutmeg	Sabinene, α-pinene, β-pinene, terpinen-4-ol, myristicin, campfene, myrcene, α -hellandrene, α -terpinene, limonene, safrole, copaene, *p*-cymene, 1,8-cineole, terpinolene, transsabinene hydrate, linalool, *cis*-sabinene hydrate, *cis*-*p*-menth-2-en-ol, *cis*-piperitol, methyl eugenol, eugenol, elemicin
Fennel	*trans*-Anethole, fenchone, estragole, limonene, camphene, dillapiole, α-pinene, fenchyl alcohol, anisaldehyde, myristicin
Anise	Chlorogenic acid isomers, *trans*-anethole, estragole, anise ketone, β-caryophyllene, linalool, anisaldehyde, anisic acid, limonene, creosol, α-pineneacetaldehyde, β-farnasene hydroquinine, *p*-cresol, γ-himachalene *ar*-curcumene
Clove	Eugenol, eugenol acetate, β-caryophyllene, α-cububene, acopaene, isoeugenol, nerolidol, farnesol
Star anise	Kaempferol, quercetin and their glycosides, *trans*-anethole, α-pinene, camphene, β-pinene, linalool, safrole, anisaldehyde, acetoanisole, estragole, limonene, *p*-allylanisole, anisyl methyl ketone, 1,8-cineole, *p*-cymene, terpinen-4-ol, α-terpineol,d-3-carene,aphellandrene, β-phellandrene

Cardamom	1,8-Cineole, α-terpinyl acetate, limonene, borneol, α-pinene, methyl eugenol, β-pinene, sabinene, myrcene, α-phellandrene, γ-terpinene, *p*-cymene, terpinolene, linalool, linalyl acetate, α-terpineol, α-terpinyl acetate, citronellol, nerd, geraniol, *trans*-nerolidol
White pepper	α-Pinene, linalool, β-damascenone, eugenol, skatole, mcresol, guaiacol, piperonal
Ginger	α-Zingiberene, geranial, geraniol, β-bisabolene, nerol, borneol, 1,8-cineol, α-terpineol, β-phellandrene, linalool, methyl nonyl ketone, camphene
Cinnamon	Cinnamaldehyde, eugenol, eugenol acetate, cinnamyl acetate, cinnamyl alcohol, linalool, methyl eugenol, cymene, cineol, safrole, pinene, benzaldehyde, cinnamaldehyde, benzyl benzoate, monoterpene, hydrocarbon, caryophyllene, phyllandrene
Coriander	Linalool, α-pinene, γ-terpinene, geranyl acetate, camphor, myrcene, geraniol, β-pinene, camphene, limonene, *p*-cymol, borneol, dipentene, α-terpinene, *n*-decylaldehyde, acetic acid esters
Vanilla	Vanillin, vanillic acid, *p*-hydroxybenzaldehyde, linoleic acid, phydroxybenzoic acid, acetic acid, hexadecanoic acid, anisic acid, acetovanillone, *p*-hydroxybenzyl alcohol, vanillyl alcohol,

1.9 Functions of spices

Spices have many functions in food products (primary functions: flavor, aroma, texture, color and secondary effects: preservative, nutritional, health functions)

1.9.1 Primary function of spices

1.9.1.1 Coloring

Consumers demand natural color from spice in foods and beverages such as saffron, paprika, turmeric, parsley, and annatto. The colorings are extracted from essential oils using organic solvents. For example, extracted natural colors (bright yellow, red, and orange) from paprika, saffron, red pepper, parsley, sweet pepper ginger, mustard and turmeric are used in foods and beverages. Coloring components, type of color of spices are shown in Table 1.9.

Table 1.9 Coloring components of selected spices (Peter, 2004)

Coloring Component	Type of Color	Spices
Carotenoids;		
Beta-Carotene	Reddish orange	Red pepper, mustard, paprika, saffron
Cryptoxanthin	Red	Paprika, red pepper
Lutein	Dark red	Paprika, parsley
Zeaxanthin	Yellow	Paprika
Capsanthin	Dark red	Paprika, red pepper
Capsorubin	Purple red	Paprika, red pepper
Crocetin	Dark red	Saffron
Neoxanthin	Orange yellow	Parsley
Violaxanthin	Orange	Parsley, sweet pepper
Crocin	Yellowish orange	Saffron
Flavonoids	Yellow	Ginger
Curcumin	Orange yellow	Turmeric
Chlorophylls	Green	Herbs

1.9.1.2 Flavor, taste, aroma, and texture

The flavor, taste, aroma and texture of spices determine efficacy of food and beverage recipe. Spices can be grouped into six tastes with sweet, salty, spicy, bitter, sour, and hot shown in Table 1.10. Some spices have more than a single flavor profile. For example, fennel does not have only sweet notes but also bitter and fruity notes. Quality of texture is depending on specific physical characteristics, (e.g., whole or ground), and cooking techniques of spices.

Table 1.10 Typical sensory characteristics of spices (Raghavan, 2007)

Sensory characteristic	Spices and other flavorings
Sweet	Green cardamom, anise, star anise, fennel, allspice, cinnamon
Sour	Sumac, caper, tamarind, sorrel, kokum, pomegranate
Bitter	Fenugreek, mace, clove, thyme, bay leaf, oregano, celery, epazote, ajowan
Spicy	Clove, cumin, coriander, canela, ginger, bay leaf
Hot	Chile peppers, mustard, fagara, black pepper, white pepper, wasabi
Pungent	Mustard, horseradish, wasabi, ginger, epazote, garlic, onion, galangal
Fruity	Fennel, coriander root, savory, tamarind, star anise
Floral	Lemongrass, sweet basil, pandan leaf, ginger flower
Woody	Cassia, cardamon, juniper, clove, rosemary
Piney	Kari leaf, rosemary, thyme, bay leaf
Cooling	Peppermint, basil, anise, fennel
Earthy	Saffron, turmeric, black cumin, annatto
Herbaceous	Parsley, rosemary, tarragon, sage, oregano, dill weed
Sulfury	Onion, garlic, chives, asafetida
Nutty	Sesame seed, poppy seed, mustard seed, whole seeds (ajowan, cumin)

1.9.2 Secondary functions of spices

The essential oils of spices have biological activity, hence being used for new applications in human health, nutrition and agriculture alternatives or complements to synthetic compounds of the chemical industry. Thus, spices may be used as antimicrobial agents for food preservation, which are considered natural and eco-friendly. The summary of the medicinal properties of herbal spice is listed in Table 1.11.

Table 1.11 Medicinal properties of herbal spice (Peter, 2006)

Spice	Medicinal properties
Black pepper	Carminative, antipyretic, diuretic, anthelminthic, anti-flammatory and antiepileptic
Cardamom	Anti-depressive, carminative, appetizer, diuretic
Ginger	Carminative, anti-nauseant, diuretic, anti-flatulence, antihistaminic, aphrodisiac and cholesterol lowering
Turmeric	Carminative, antibiotic, anti-flatulence, antiseptic and anti-inflammatory
Garlic	Antimicrobial, diuretic, diaphoretic, anti-flatulence, cholesterol lowering and anti-inflammatory
Clove	anti-flatulence, analgesic, stimulant, carminative and anti-nauseant
Nutmeg	Stimulant, carminative, astringent, aphrodisiac, anti-inflammatory
Cinnamon	Stimulant, carminative, astringent, aphrodisiac, anti-inflammatory
Chilli	Carminative and anti-rheumatic
Allspice	Stimulant, digestive and carminative
Basil, sweet	Stomachic, anthelmintic, diaphoretic, expectorant, antipyretic carminative, stimulant, diuretic, demulcent
Bay leaves	Stimulant, narcotic
Caraway	Stomachic, carminative, anthelmintic, lactagogue
Celery	Stimulant, tonic, diuretic, carminative, emmenagogue, anti-inflammatory
Chive	Stimulant, diuretic, expectorant, aphrodisiac, emmenegogue, antiinflammatory

Coriander	Carminative, diuretic, tonic, stimulant, stomachic, refrigerent, aphrodisiac, analgesic, anti-inflammatory
Cumin	Stimulant, carminative, stomachic, astringent and antiseptic
Dill	Carminative, stomachic, antipyretic,stomachic, emmenagogue carminative, fennel stimulant
Fenugreek	Carminative, tonic, aphrodisiac
Leek	Stimulant, expectorant
Marjoram	Carminative, expectorant, tonic, astringent
peppermint	Stimulant, stomachic, carminative, antiseptic
spearmint	Stimulant, carminative and antispasmodic
Oregano	Stimulant, carminative, stomachic, diuretic, diaphoretic and emmenagogue
Parsley	Stimulant, diuretic, carminative, emmenagogue, antipyretic, antiinflammatory
Rosemary	Mild irritant, carminative, stimulant, diaphoretic
Sage	Mild tonic, astringent, carminative
Tarrgon	Aperient, stomachic, stimulant, febrifuge
Thyme	Antispasmodic, carminative, emmenagogue, anthelmintic, spasmodic, laxative, stomachic, tonic, vermifuge

1.10 Spices as antimicrobials

The investigation of new spices as antimicrobial agents for preservative has increased awareness of food products and demand of new self-preservatives that are biodegradable and eco-friendly. The inhibitory effects against specific microorganisms are shown in Table 1.12.

Table 1.12 Plant source and major components of some essential oils and tested antioxidant and antimicrobial properties (Gonza´lez et al., 2011)

Common name of essential oils	Major components	Antioxidant properties	Antifungal properties	Antibacterial properties
Bergamot	Limonene Linalool	ABTS[a]	-	Pathogenic bacteria
Cinnamon	*trans*-Cinnamadehyde	ABTS[a]	*Aspergillus* *Fusarium* *Penicillium*	Pathogenic bacteria
Coriander	Linalool	DPPH[b]	*Saccharomyces*	Pathogenic bacteria
Clove	Eugenol Eugenyl acetate	DPPH[b]	*Aspergillus*	Pathogenic bacteria
Eucalyptus	Eucalyptol	Thiobarbiuric acid DPPH[b]	*Candida* *Saccharomyces* *Rhodotorula*	Pathogenic bacteria
Thyme	Borneol Viridiflorol Thymol Carvacrol Terpinene Cymene	Aldehyde-Carboxylic acid ABTS[a] DPPH[b]	*Aspergillus* *Candida* *Saccharomyces* *Rhodotorula*	Pathogenic bacteria

Oregano	Carvacrol Thymol Terpinene Cymene	Thiobarbituric acid DPPH[b]	*Botrytis* *Fusarium* *Clavibacter* *Candida* *Saccharomyces* *Rhodotorula*	Pathogenic bacteria
Rosemary	Pinene Bomyl acetate Cymene	ABTS[a] DPPH[b]	*Candida* *Saccharomyces* *Rhodotorula*	Pathogenic bacteria
Sage	Camphor Pinene Pinene 1,8-Cineole Thujone	ABTS[a]	*Candida* *Aspergillus* *Penicillium* *Fusarium*	Pathogenic bacteria
Tea Tree	Terpinen-4-ol Terpinene Terpinene 1,8-Cineole	DPPH[b]	*Fusarium* *Pyrenophora* *Candida*	Pathogenic bacteria
Lemon	Limonene Valencene Ocimene	ABTS[a]	*Aspergillus* *Penicillium*	Pathogenic bacteria

a : 2,2'-Azino-*bis*(3-ethylbenzthiazoline-6-sulphonic acid)

b : 2,2-Diphenyl-1-picrylhydrazyl

Many spices possess antimicrobial activity due to their essential oil fractions. As in the previous study, the essential oil from *Coriandrum sativum* seed exhibited antimicrobial against *Candida spp.* (Silva et al., 2011) and the essentail oil from seeds of fennel (*Foeniculum vulgare* Mill) exhibited antimicrobial activity against *Staphylococcus albus, Bacillus subtilis, Salmonella typhimurium, Shigella dysenteriae* and *Escherichia coli* (Diao et al., 2014). The essential oils from caraway seed, garlic and origanum were tested for their antifungal activity against *Eurotium herbariorum, E. amstelodami, A. flavus and A. sydowii* (Dimic et al., 2007). Moreover, the essential oils of thyme and cinnamon (≤500 ppm), marigold (≤2000 ppm), spearmint, basil,

quyssum (3000 ppm) completely inhibited *A. flavus*, *A. parasiticus*, *A. ochraceus* and *Fusarium moniliforme*. Caraway showed inhibitory effect at 2000 ppm against *A. flavus*, *A. parasiticus* and 3000 ppm against *A. ochraceaus* and *F. moniliforme*. All the test fungi were completely inhibited by anise at ≤500 ppm (Soliman et al., 2002). Moreover, essential oil extracted from the bark of *Cinnamomum jensenianum* Hand-Mass exhibited antifungal activity against *A. flavus*, explained based on the alteration of fungal plasma membrane, mycelial growth, spore germination and aflatoxin B1 production (Tian et al., 2012). Some phenolic compounds isolated from plant essential oils such as eugenol from cloves and thymol from thyme caused complete growth inhibition of both *A. flavus* and *A. versicolor* at 0.4 mg/mL or less. Anethol extracted from star anise seeds inhibited the growth of all test fungi strains at a concentration of 2 mg/mL (Hitokoto, 2014).

1.11 Spice labeling, standards, regulations and quality specification

Consumers require spices of the highest quality. Safety of spices is the major consideration spices of buyers and users such as sanitation, pesticides, labeling and sterilization method used. The United States and the European Union (EU) have acquired the international standards for quality specifications. Importation of spice has survey quality standards by American Spice Trade Association (ASTA). In Europe, the minimum specification qualities for spice are set by European Spice Association (ESA). In addition, many origin countries of spices have quality specifications, including the Indian Spice Board (ISB) and International Pepper Community (IPC). ASTA sets general quality tests including cleanliness, ash level, volatile oil, moisture content, water activity, pesticide levels, mycotoxin/aflatoxin levels, and particle size. Further, ASTA tests color values, piperine levels (peppers), capsaicin level (chile peppers), pungency and other sensory values. Quality standards are set again by FDA before distribution. Several methods test for microbial contamination and insect growth during storage used fumigation (methyl bromide), sterilizing (ethylene oxide, irradiation, and heat treatment). Ethylene oxide (EtO) is toxic to human health and affects the sensory profiles of spices. FDA allowed irradiation for spices in 1983 because it destroyed microbial more efficiently than EtO. Consumer concern on irradiation and chemical treatments has led to steam heat using. High-pressure steam controlled low temperature and moisture content for storage to prevent mold growth and aflatoxin that they

produced. So, nitrogen or carbon dioxide substitute oxygen method used for maintaining. Spices must be clean from pathogen microorganism and micro toxin that their produced including *E. coli*, *Salmonella* and *Aspergillus*. Thus, good preservation and monitoring are important quality specification of spices (Raghavan, 2007).

1.12 *Anethum graveolens* L.

1.12.1 Scientific classification

Kingdom: Plantae

Order: Apiales

Family: Apiaceae or Umbelliferae

Genus: *Anethum*

Species: *A. graveolens*

Binomial name: *Anethum graveolens* L.

Common name: insilal (Amharic), shabath (Arabic), samit (Armenian), samin (Burmese), see loh (Cantonese), dill (Danish), dille (Dutch), shivit and sheveed (Farsi), aneth odorant (French), dill (German), anitho (Greek), shamir (Hebrew), suwa/suwa patta (Hindi), adas manis/adas cina (Indonesian/Malaysian), aneto (Italian), diru (Japanese), phak si (Laotian), shih-lo (Mandarin), dill (Norwegian, Swedish), endro (Portuguese), ukrop (Russian), enduru (Sinhalese), eneldo (Spanish), satakuppi sompa (Tamil), pak chee lao (Thai), dereotu (Turkish), and tieu hoi hurong (Vietnamese) (Uhl, 2000).

Local name: ผักชีลาว (Pak chee lao), เทียนตาตั๊กแตน (Tian ta tukatan)

1.12.2 History and origin

Mediterranean region, as early as 3000 BC, was believed to be the origin of *Anethum graveolens*. Culinary and medicinal herb has been used, since ancient time, in many countries including Assyrians and later Egyptians, Romans, Greeks, and northern Europeans. Dill is referred to as a “soothing medicine” because parents use it to reduce stomach pains of babies when crying and to lull them to sleep (Raghavan, 2007).

1.12.3 Botanical characteristic of plant

Dill (*Anethum graveolens*) is an annual or biennial herb of the parsley family (Apiaceae or Umbelliferae). A stem is slender branched, hollow and grows up to a height of 90–120 cm. Leaves are softly delicate and finely divided 10–20 cm long. The flowers are yellow, in small umbels (2–9 cm diameter) and long spindle-shaped roots. The seeds are 4–5 mm long and straight to slightly curve with a longitudinally ridged surface (Shyu et al., 2009). Characteristics of *A. graveolens* L. plant is shown in Figure 1.11. The somatic number of *A. graveolens* is diploid 2n = 22 (Weiss, 2002). It is native in Southwest Asia but is cultivated in Europe, India and the United States (Doymaz et al., 2006). Dill seeds contain calcium, magnesium, phosphorus, potassium, and vitamin A. Dill leaves contain calcium, iron, potassium, sodium, and folic acid (Raghavan, 2007). The composition of dry dill herb is water 7 g, protein 20 g, fat 4 g, carbohydrayes 44 g, fibre 12 g, ash 12 g, ascorbic acid 60 mg; energy value about 1060 KJ per 100 g. While, dry dill seeds have water 7-9 %, protein 16-18 %, fat 15-20 %, carbohydrayes 25-35 %, pectin 5-7 %, fibre 20-30 %, energy value about 1275 KJ per100 g (Weiss, 2002).

Figure 1.11 Characteristics of *A. graveolens* L. plant (http://upload.wikimedia.org/wikipedia/commons/1/10/Illustration_Anethum_graveolens0.jpg)
(Date: November 13, 2014)

1.12.4 Composition of essential oil

The essential oil of pale yellow color was extracted from European dill seeds with yield of 2.5% to 4% (w/v). The main compositions were carvone (30% to 60%), limonene (33%), and α-phellandrene (20.61%). Indian dill seeds oil yield was 1% to 4% (w/v), which is light brown in color. The essential oil of Indian dill seeds has high dillapiole (52%) but has less carvone (21%) than the European dill seeds (Raghavan, 2007). The main constituent of dill herb oil is α-phellandrene. While, carvone and limonene predominate the main component in seeds oil (Chemat and Esveld, 2013). However, the composition and yields of essential oils depending on harvesting season and different extraction methods. The odor of phellandrene was dill-like, fragrant, and fresh. While carvone odor was caraway-like, cooling and the odor of dill ether were dill-like, floral, and fragrant. Citrus-like, fresh and pine-like were odors of limonene and α-pinene compounds, respectively (Callan et al., 2007).

1.12.5 Benefits

The dill seeds were widely used in cakes and pastries, soups, salads, meats, potatoes, flavouring pickles, bread, and soups. While, dill leaves were used in eggs, meats, salads, seafood and soups. Dill oil was used in chewing gums, candies and pickles (Orhan et al., 2013; Shyu et al., 2009). It has been used traditionally for gastrointestinal ailments such as flatulence, indigestion, stomachache colic and to tract intestinal gas (Yazdanparast et al., 2008). Its pharmacological properties, such as its antimicrobial (Delaquis et al., 2002; Tanruean et al., 2014), antihypercholesteromic (Yazdanparast et al., 2008), cancer chemo-preventive effects (Zheng et al., 1992), antioxidant (Shyu et al., 2009; Tanruean et al., 2014) and toxicological efficacy against the flesh fly (Khalaf, 2004) have been reported. As a folk remedy, dill is considered for some gastrointestinal ailments such as mucosal protective and antisecretory effects of the gastric mucosa in mice (Hosseinzadeh et al., 2002). In females, as traditional medicine, it increases mother's milk production and promotes menstruation. While high doses of dill have been mentioned to weaken sexual and decreases spermatogenesis in males (Monsefi et al., 2006). Moreover, Some studies have shown that dill weed oil suppressed some fungi such as *A. niger*, *Trichoderma viride*, and *Penicillium chrysogenum* (Yang and Clausen, 2007).

1.13 *Zanthoxylum piperitum*

1.13.1 Scientific classification

Kingdom: Plantae

Order: Sapindales

Family: Rutaceae

Genus: *Zanthoxylum*

Species: *Z. piperitum*

Binomial name: *Zanthoxylum piperitum* (L.) DC

Common name: fagara, Chinese pepper, Japanese pepper, anise pepper, Nepalese pepper, Indonesian lemon pepper, Japanese prickly ash and flower pepper. Yan chiao, chi fa chiao (Cantonese) , sechan peber (Danish) , sechuan peper (Dutch), poivre de setchuan (French), ksantosilum (Hebrew), anizs bors (Hungarian) , Szechuan-pfeffer (German) , tilfda (Hindi) , andalimon (Idonesian), sansho (Japanese), tippal (Konkani), chopi (Korean), kok mak met (Laotian), katmurikku (Malayalam), tirphal (Marathi), timur (Napali), hua chiao, gan jiao (Mandarin), piment sechuan (Portuguese), sychuan skij perates (Russian), Szechuan pepper (Swedish), chiit (Tagalog), ma lakh, mak kakh (Thai), yermah (Tibet), and dan cay (Vietnamese)

Origin: South China, Vietnam, Japan, and North and Western India (Raghavan, 2007)

Local name: พริกหอม (Prik hom)

1.13.2 Botanical characteristics of plant

Zanthoxylum piperitum species is a medicinal plant belonging to the Retaceae family and distributes mainly in tropical and subtropical regions including Korea, China, Japan and Taiwan (Hwang and Kim, 2012). *Z. piperitum* is the ornament of the prickly ash group. It is a dense shrub or small tree 8-15 feet high, spiny trunks, glossy leaves and dark green. The flowers are greenish yellow, in small corymbs (1-2 inches) and fruits are reddish with shiny black seeds. Characteristics of *Z. piperitum* plant is shown in Figure 1.12.

Figure 1.12 Characteristics of *Z. piperitum*: Stem (A), Flowers (B), Fruits (C), Dry fruits (D)
(http://sureaux.blogspirit.com/media/02/02/176683153.jpg) (A)
(http://hosho.ees.hokudai.ac.jp/~tsuyu/top/plt/citrus/zanthoxylum/pip_04.jpg) (B)
(http://www.deeproot.co.uk/photo/images360/z/Zanthoxylum%20piperitum.jpg) (C)
(http://sybaritica.files.wordpress.com/2012/02/sichuan-peppercorns-2.jpg) (D)
(Date: November 13, 2014)

1.13.3 Composition of essential oil

Previous chemical study of the genus *Zanthoxylum* (bark, stems, wood and leaves) has been shown to produce a diverse range of secondary metabolites including alkaloids, aliphatics and aromatic amides, lignans, coumarins, sterols and carbohydrate residues (Sreelekha et al., 2014). *Z. piperitum* dried fruit has been shown to produce a diverse range of aromatic agents including β-myrcene, limonene, citronellal, cryptone, phellandral and geranyl acetate (Chung, 2005). Jiang and Kubota (2004) reported that *d*-limonene, β-phellandrene, myrcene, citronellal, geraniol, and geranyl acetate were major components of *Z. piperitum* dried fruit.

1.13.4 Benefits

Zanthoxylum piperitum plants have been used for the treatment of stomach ache, vomiting, diarrhoea, abdominal pain and moist dermal ulcer (Jeong et al., 2011). Traditionally, the bark has been used in treatment for rheumatism, arthralgia, stasis, contusions, snakebite, and common cold in China. In Taiwan, the leaves are used for healing common cold (Cheng et al., 2005). It is used as a food additive to mask fishy flavor in Korean food (Chung, 2005) . Its pharmacological properties have been associated with antibacterial, antilipid peroxidative, antiviral activities, antioxidant and as a cholesterol acyltransferase inhibitor (Chio et al., 2008; Hur et al., 2003; Jeong et al., 2011; Kusuda et al., 2006; Park et al., 2007; Yamazaki et al., 2007) . In addition, *Z. piperitum* derived essential oil has been shown to repel mosquitoes (Kamsuk et al., 2007; Yang et al., 2004) and inhibit growth of pathogens such as *Pityrosporum ovale* and *Candida albicans* (Jang et al., 2003).

1.14 Research objectives

1.14.1 To extract and chemically characterize essential oils from seeds of *Zanthoxylum piperitum* and *Anethum graveolens* L.

1.14.2 To investigate antifungal activity of the essential oils against *Aspergillus flavus*

1.14.3 To preliminarily study the antifungal mechanism in the plasma membrane of treated *A. flavus*

1.14.4 To apply the essential oil to control postharvest spoilage on stored chili.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals

Chemicals	Company
Absolute Ethanol	QRec, New Zealand
Acetone	QRec, New Zealand
Anhydrous sodium sulfate	Carlo erba, France
n-heptane	RCI Lab scan, Thailand
Potassium hydroxide	QRec, New Zealand
Potato dextrose agar	HIMEDIA, India
Potato dextrose broth	RCI Lab scan, Thailand
Tween-20	BDH, India

2.2 Instrument

Name	Model	Company
Analytical balance	PB 1502-S	Mettler Toledo, Thailand
Autoclave oven	M LS-3780	Sanyo, Japan
Blender	-	National, Japan
Counting chamber	-	Hausser Scientific, USA
Heating mantle	-	Electrothermal, UK
Hot plate and magnetic stirrer	Clifton Cerastir	Torrey Pines, California Scientific, USA
HP gas chromatograph 5890 series II	-	Hewlett-Packard, California
Incubator	WB M15	Falc, Italy

Name	Model	Company
Laminar flow	Telstar Bio-II-A	Terrassa, Spain
Light microscope	CH 30 RF-200	Olympus, Japan
Micropipette	-	Gilson, France
Oven 400	-	Germany
Pipette tip	-	Axygen, USA
Shaker	-	Gallenkamp, USA
Spectrophotometer	Thermo-spectronic	Thermo Fisher, USA
Vortex mixer	Genie-2-G-560E	Bohemia, USA
Water bath	Eco Temp Tw 20	Julobo, USA

2.3 Plant materials and microorganism

2.3.1 Seed spice

The seeds of *Anethum graveolens* L. and *Zanthoxylum piperitum* were purchased from a local market in Chiang Mai, Thailand. They were identified botanically at Department of Biology and kept at Biochemistry and Biochemical Technology Laboratory, Department of Chemistry, Chiang Mai University at room temperature (25-30 ^{o}C) (Figure 2.1).

Figure 2.1 The seeds used in this study

A) *Anethum graveolens* L. and B) *Zanthoxylum piperitum*

2.3.2 *Aspergillus flavus*

An aflatoxigenic fungi, *Aspergillus flavus*, was previously isolated from dried chili powder (Thakaew and Niamsup, 2013). The fungus was stored on potato dextrose agar (PDA). The stock culture was grown at 30 ºC and maintained at 4 ºC for further test.

Figure 2.2 An aflatoxigenic fungi, *Aspergillus flavus*

2.4 Extraction of essential oils

All seeds were ground into powder using blender. The essential oils were extracted from 100 g of each spice powder by hydrodistillation for approximately 4 h using a Clevenger-type apparatus. The collected oils were dried over anhydrous sodium sulfate. It was stored in amber glass bottle covered with aluminum foil at approximately 4 ºC for further analysis and antifungal testing.

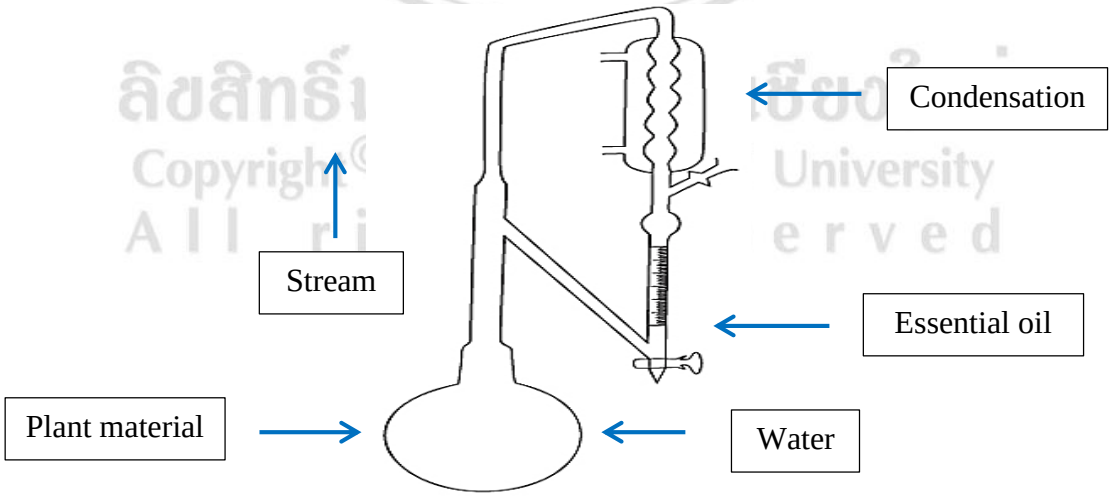


Figure 2.3 Clevenger-type apparatus

(http://popups.ulg.ac.be/1780-4507/docannexe/image/830/img-1.jpg)

(Date: November 25, 2014)

2.5 Essential oil analysis

The analysis of the oil was performed using a Gas Chromatography (GC) 6890 Agilent Technology/MSD 5973 Hewlett Packard, equipped with a FID detector and HP-5MS capillary column (Bond and cross-link 5%phenyl-methylpolysiloxane 30 m × 0.25 mm, film thickness 0.25 μm). The injector and detector temperatures were set at 220 and 280 ºC, respectively. The oven temperature was held at 50ºC for 3 min, then programmed to 240 ºC at a rate of 3 ºC/min. Helium was the carrier gas, at a flow rate of 1 mL/min. One microlitre of diluted oil (50/100 in acetone, v/v) was injected manually and in the split- less mode. For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used. The components were identified based on the comparison of their relative retention time and mass spectra with those of standards, W8N05ST (Wiley ver. 8.0) library data of the GC-MS system and literature data (Adams, 2001).

Figure 2.4 Gas Chromatography (GC) 6890 Agilent Technology
(http://www.st2-service.com/images/catalog/5973inert.jpg)
(Date: November 13, 2014)

2.6 Antifungal activity of essential oils against *A. flavus*

The essential oils of *A. graveolens* L. and *Z. piperitum* seeds were tested for the antifungal activity against an aflatoxigenic fungus, *A. flavus*, by poisoned food technique on agar plate and mycelial mass in liquid medium.

2.6.1 Antifungal activity of essential oils on agar plate

Antifungal activity of the essential oils was evaluated against the *A. flavus* using the poisoned food technique (Tian et al., 2011). The essential oils were dissolved in 0.5 mL of 5% (v/v) Tween-20 and 9.5 mL melted PDA then mixed by vortex to obtain the final concentrations of 0.25, 0.5, 1.0, 1.5, 2.0 for *A. graveolens L.* oils and 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 4.5 µL/mL for *Z. piperitum* oils and pour into autoclaved petri dish. The plate without essential oil was used as a control. After solidifying, a 4 mm disc from a three-day-old fungus using a cork borer was placed in the middle of the plate. The plates were sealed with polyethylene film and incubated at a temperature of 28±2 ºC. The efficacy of treatment was evaluated every day for 9 days as described and the mycelial inhibition percentage was calculated by the formula below (Albuquerque et al., 2006) . All tests were performed in triplicates. The entire experiment was repeated twice.

$$\text{Mycelial inhibition percentage} = [(dc-dt)/dc] \times 100$$

Where dc is the mean diameter of fungal colony in the control sets and dt is the mean diameter of fungal colony in the treatment sets. The lowest concentration which completely inhibited fungal growth was considered as the minimum inhibitory concentration (MIC).

2.6.2 Antifungal activity of essential oils in liquid culture

The antifungal activity of the essentials oils on the wet and dry mycelial weight of *A. flavus* was determined following the method of Dikbas et al., (2008). Here, 20 mL of Potato Dextrose Broth (PDB) medium was transferred to each Erlenmeyer flask, to which a requisite amount of the essential oil was added to obtain 0.25, 0.5, 1.0, 1.5, 2.0 for *A. graveolens L.* oil and 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 4.5 µL/mL for *Z. piperitum* oil. Next, 100 µL of the fungal inoculum containing 10^7 spores/mL was inoculated into each flask at 28±2 ºC in an incubator shaker. Spore number was counted

under a hemocytometer. Sample without essential oil was used as a control. The flasks containing mycelia mass after 9 days were harvested and washed twice with sterile distilled water. The mycelia were allowed to dry at 70 ^{o}C before determining the dry weight. Percentage inhibition of the mycelial growth was calculated by the formula below. All tests were performed in triplicates. The entire experiment was repeated twice.

Inhibition percentage = [(dwc-dwt)/dwc]×100

Where dwc and dwt are the mean dry mycelial weights of control and treatment sets, respectively.

2.6.3 Light microscope examination of essential oil-treated *A. flavus*

Fungal hypha morphology was observed by slide culture. The PDA plates with and without 0.25 and 1.0 µL/mL for *A. graveolens L.* and *Z. piperitum* essential oil, respectively were prepared as stated before (2.6.1). Agar from treatment and control plate was cut into 1×1 cm size by sterile blade. The square agar was transferred to sterile glass slide and inoculated with mycelial fungi by a loop on 4 sides of square agar of both treatment and control. The plates were sealed with polyethylene film and incubated at a temperature of 28±2 ^{o}C for 3 days. Morphology of test and control was observed under microscope with the magnification at 40x.

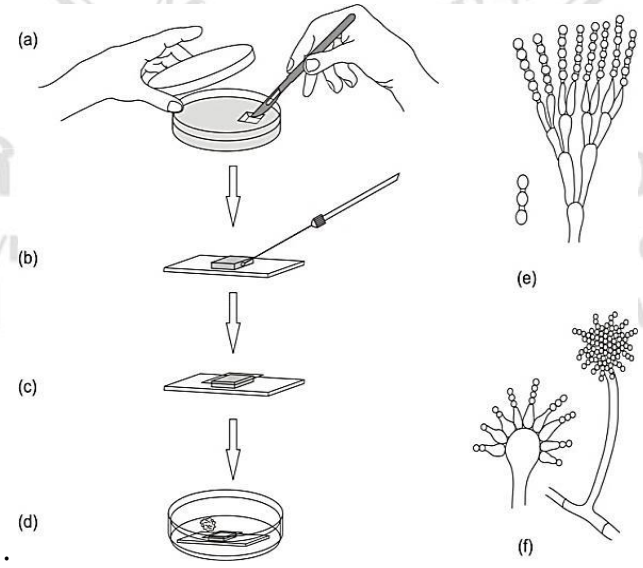


Figure 2.5 Slide Culture Method

(http://elte.prompt.hu/sites/default/files/tananyagok/microbiology/images/522987c2.jpg)

(Date: November 13, 2014)

2.6.4 Effect of essential oils on ergosterol content in plasma membrane of *A.flavus*

The ergosterol content in *A. flavus* plasma membrane was detected following Tian et al., (2012). A 100 µL spore suspension of spoilage fungi (10^7 spores/mL) was inoculated into PDB medium containing 0.25 and 1.0 µL/mL for *A. graveolens L.* and *Z. piperitum* essential oils, respectively. After 4 days of incubation at 28±2°C, mycelia were harvested and washed twice with sterile distilled water. The wet weight of mycelia was determined. Five milliliters of 25% alcoholic potassium hydroxide solution was added to cell pellet and vortex mixed for 2 min; this was followed by incubation in 85 °C water bath for 4 h. Sterols were then extracted from sample by adding a mixture of 2 mL sterile distilled water and 5 mL *n*-heptane followed by sufficient vortex-mixing for 2 min. After the layers were allowed to separate for 1 h at room temperature, the *n*-heptane layer was separated and analyzed by spectrophotometry between 230 and 282 nm. The calculated formula of the ergosterol amount is as follows:

% ergosterol + % 24(28) dehydroergosterol = (A_{282}/290)/pellet weight

% 24(28) dehydroergosterol = (A_{230}/518)/pellet weight;

where 290 and 518 are the E values (in percentages per centimeter) determined for crystalline ergosterol and 24(28) dehydroergosterol, respectively, and pellet weight is the net wet weight (in grams).

2.6.5 Efficacy of essential oils as an antifungal coat in dried chili

Dried chili model 4 pieces/plate (approximately 2.2 - 2.3 g each) were sterile by autoclaving at 121 °C for 15 min. The cooked dried chilies were allowed to dried at 70 °C for 12 h before test. The essential oil dissolved in 2.0 mL of 5% Tween-20 to procure the final concentration at 2.0 and 4.0 µL/mL for *A. graveolens* L. and 4.5 and 9.0 µL/mL for *Z. piperitum*. The essential oils were coated on dried chili at room temperature and then transferred to sterile Petri dishes. Next, 10 µL of the fungal inoculum containing 10^7 spores/mL was inoculated into each piece. Sample without essential oils was used as a control. After 9-day incubation at 28±2 °C, the essential oil-coated and control dried chilies were counted for fungal colonies by pour plate method.

Figure 2.6 Dried chili model used in this study

2.7 Statistical analyses

All the measurements were replicated thrice for each treatment, and the data reported as mean ± standard deviations. Significant differences between the mean values were determined by Duncan's Multiple Range test ($p<0.05$), following one-way ANOVA. The statistical analyses were performed using SPSS, 17.0 (Chicago, USA).

CHAPTER 3

RESULTS

3.1 Yield of the essential oils

All spice seeds were ground into powder. 100 g of each spice powder was extracted for essential oils by hydrodistillation for approximately 4 h using a Clevenger-type apparatus. Essential oil of pale yellow color was extracted from *A. graveolens* L. with yield of 0.87 to 1.13 %w/w (1.00-1.30 %v/w). Whereas *Z. piperitum* provided light yellow essential oil of 0.35 - 0.44 %w/w (0.40-0.50 %v/w) yield. (Figure 3.1).

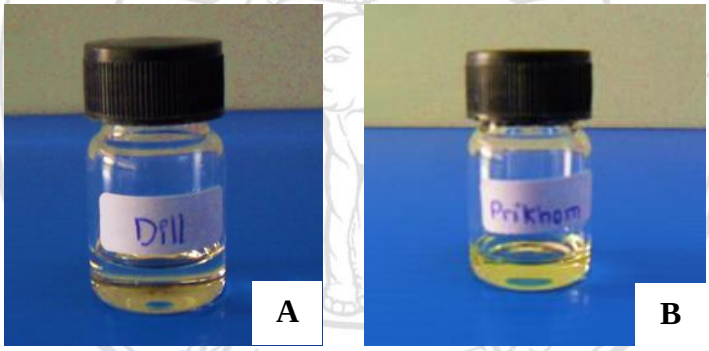


Figure 3.1 The essential oils from A) *A. graveolens* L. and B) *Z. piperitum*

3.2 Chemical composition of the essential oils

The chemical compositions of *A. graveolens* L. and *Z. piperitum* essential oils were analyzed by Gas Chromatography (GC) 6890 Agilent Technologies/ MSD 5973 5973 Hewlett Packard series GC-MS. The compositions were identified based on the comparison of their relative retention time and mass spectra with those of standards, W8N05ST (Wiley ver. 8.0) library data of the GC-MS system. *A. graveolens* L. and *Z. piperitum* oil mass spectral is shown in Figures 3.2 and 3.3, respectively. The amounts of components are also listed in Table 3.1 and Table 3.2

3.2.1 Chemical composition of *Anethum graveolens* L. essential oil

GC-MS analysis resulted in the identification of twenty-four components, accounting for 96.2% of the total oil shown in Table 3.1. Of these, the constituents could be grouped into three sub-classes with monoterpene hydrocarbon 21.8%, oxygenated monoterpene 40.5% and phenylpropene 33.9%. The major peaks of the essential oil constituents exhibited at 12.607, 20.892, 22.954 and 38.718 min (Figure 3.2), each with peak height of 19.2%, 12.3%, 26.0%, 33.1%, of which m/z values consistent with the mass spectral patterns of (-)-limonene (98% similarity), dihydrocarvone (98% similarity), (+)-carvone (96% similarity), *trans*-isodillapiole (90% similarity), respectively. The minor components (≥ 0.5%) were shown at 12.860 and 20.498 min (Figure 3.2) with 2.0% and 1.4% abundance, and their m/z value were consistent with the mass spectral patterns of β-ocimene (96% similarity) and (+)-isodihydrocarvone (99% similarity), respectively.

Table 3.1 Chemical composition of *A. graveolens* L. essential oil identified by GC-MS analysis

Peak no.	Compound	Retention time (min.)	Match Quality	Molecule Weight	%
1	α-(+)-Pinene	8.277	97	136	Tr
2	(+)-Sabinene	9.899	97	136	Tr
3	β-Pinene	9.999	94	136	Tr
4	β-Myrcene	10.715	91	133	0.1
5	1,5,8-*P*-Menthatriene	11.262	94	136	0.3
6	(-)-Limonene	12.607	98	136	19.2
7	β-Ocimene	12.860	96	136	2.0
8	γ -Terpinene	13.724	96	136	Tr
9	(-)-α-Thujone	15.034	95	152	Tr
10	α -4-Dimethylstyrene	15.157	96	132	0.1
11	*cis*-Limonene Oxide	17.167	97	152	Tr
12	*trans*-Limonene Oxide	17.390	95	152	0.1
13	Dill ether	19.599	95	152	0.1
14	(+)-Isodihydrocarvone	20.498	99	152	1.4
15	Dihydrocarvone	20.892	98	152	12.3
16	Dihydrocarveol	21.550	97	154	0.3
17	*cis*-Carveol	21.861	98	152	0.2
18	(+)-Carvone	22.954	96	150	26.0
19	*cis*-Dioxide carvone	23.976	90	166	Tr
20	(*E*)-Anethol	24.311	97	148	0.2
21	Thymol	24.940	93	150	Tr
22	Myristicin	34.182	98	192	0.4
23	Elemicin	35.586	98	208	0.2
24	*trans*-Isodillapiole	38.718	90	222	33.1

Tr.: trace amount <0.05%

Total 96.2%

3.2.2 Chemical composition of *Zanthoxylum piperitum* essential oil

Sixty three compounds have been identified in essential oil from *Z. piperitum*, accounting for 92.7% of the total oil composition as shown in Table 3.2. Of these, the components could be grouped into six sub-classes with monoterpene hydrocarbon 68.6%, oxygenated monoterpene 19.5%, sesquiterpene hydrocarbon 3.2%, oxygenated sesquiterpene 1.0%, phenylpropene 0.2%, and miscellaneous compounds 0.2%. The major compounds of the essential oil were exhibited at 10.097, 12.612 and 40.215 min (Figure 3.3) with 14.6%, 23.2%, and 11.9% abundance, of which their identity was consistent to the mass spectral pattern of sabinene (96% similarity) , β-phelladrene (94% similarity) and brevifolin (94% similarity). The minor compounds (≥ 0.5%) of the essential oil were exhibited at 8.053, 8.329, 10.144, 10.832, 11.267, 11.819, 12.929, 13.352, 13.799, 14.234, 15.115, 15.673, 15.926, 16.760, 17.600, 19.727, 20.062, 20.785, 22.994, 27.054, 28.523, 29.868, 32.424 and 34.146 min with 0.8%, 3.1%, 0.8%, 4.6%, 0.9%, 1.4%, 2.2%, 1.1%, 2.2%, 0.9%, 1.5%, 0.9%, 3.4%, 1.0%, 0.7%, 0.5%, 1.3%, 0.5%, 6.4%, 2.3%, 0.6%, 0.5%, 0.6% and 0.5% (Figure 3.3), of which their m/z rations were consistent to the mass spectral pattern *α*-thujene (94% similarity) , α-(+)-pinene (96% similarity) , β-*L*-pinene (94% similarity) , β-myrcene (95% similarity) , α-phellandrene (98% similarity) , α-terpinene (97% similarity) , *cis*-ocimene (98% similarity), β-ocimene (96% similarity), γ-terpinene (96% similarity), 4-thujanol (98% similarity) , α-terpinolen (97% similarity) , *cis*-β-terpineol (91% similarity) , linalool (97% similarity) , (*E*)-*p*-menth-2-en-1-ol (91% similarity) , 4-terpinenol (96% similarity), *L*-cryptone (95% similarity), β-fenchol (91% similarity), *cis*-piperitol (90% similarity) , (+)-piperitone (96% similarity) , terpinyl acetate (91% similarity) , geraniol acetate (91% similarity) , caryophyllene (99% similarity) , germacrene D (98% similarity), δ-cadinene (99% similarity).

Table 3.2 Chemical composition of *Z. piperitum* essential oil identified by GC-MS analysis

Peak no.	Compound	Retention time (min.)	Match Quality	Molecule Weight	%
1	Hexanal	4.187	95	100	0.1
2	α-Thujene	8.053	94	136	0.8
3	α-(+)-Pinene	8.329	96	136	3.1
4	Camphene	8.852	96	136	0.1
5	(+)-Sabinene	10.097	96	136	14.6
6	β-Pinene	10.144	94	136	0.8
7	β-Myrcene	10.832	95	136	4.6
8	α-Phellandrene	11.267	94	136	0.9
9	3-Carene	11.484	97	136	Tr
10	α-Terpinene	11.819	98	136	1.3
11	β-Phelladrene	12.612	96	136	23.2
12	*cis*-Ocimene	12.929	97	136	2.2
13	β-Ocimene	13.352	98	136	1.1
14	γ -Terpinene	13.799	96	136	2.2
15	4-Thujanol	14.234	96	154	0.9
16	α-Terpinolene	15.115	98	136	1.5
17	*p*-Cymenene	15.175	97	132	0.1
18	*cis*-β-Terpineol	15.673	91	154	0.9
19	Linalool	15.926	97	154	3.4
20	(*E*)-*p*-Menth-2-en-1-ol	16.760	91	154	1.0
21	α-Campholenal	16.878	94	152	Tr
22	Neo-Allo-Ocimene	17.047	97	136	Tr
23	4-terpinenol	17.600	96	154	0.7
24	4-Isopropyl cyclohexone	18.352	96	140	0.1
25	1,4-Dimethoxybenzene	18.687	96	138	Tr
26	(+)-*P*-Menth-1-en-4-ol	19.445	97	154	0.2
27	*L*-Cryptone	19.727	95	138	0.5

28	β-Fenchol	20.062	91	154	1.3
29	*cis*-Piperitol	20.785	90	154	0.5
30	(*E*)-Carveol	21.396	98	152	Tr
31	(*Z*)-Geraniol	21.807	97	154	0.1
32	2-Methyl-3-phenyl-propanal	22.142	98	148	0.2
33	(+)-Carvone	22.336	94	150	0.1
34	(+)-Piperitone	22.994	96	152	6.4
35	Phellandral	23.699	95	152	0.2
36	Bornyl acetic ether	24.169	99	196	0.3
37	(*E*)-Anethol	24.628	98	148	0.1
38	α-(+)-Pinene	24.827	93	136	Tr
39	Carvacrol	25.344	97	150	0.1
40	Terpinyl acetate	27.054	91	196	2.2
41	Chavibetol	27.430	98	164	Tr
42	Nerol acetate	27.683	91	196	0.2
43	Copaene	28.035	99	204	Tr
44	Geraniol acetate	28.523	91	196	0.6
45	β-Elemene	28.770	99	204	0.2
46	Methyl eugenol	29.445	98	178	0.1
47	Caryophyllene	29.868	99	204	0.5
48	α-Caryophyllene	31.267	98	204	0.1
49	Naphthalene	32.254	99	204	0.1
50	Germacrene D	32.424	98	204	0.6
51	Germacrene B	33.029	95	204	0.2
52	α-Amorphene	33.217	98	204	0.1
53	β-Elemene	33.376	94	204	0.1
54	α-Farnesene	33.582	98	204	0.1
55	δ-cadinene	34.146	99	204	0.5
56	*trans*-Nerolidol	35.797	91	222	0.1
57	(+)-Spathulenol	36.296	99	220	0.3

58	β-(-)-Caryophyllene epoxide	36.431	99	220	0.4
59	Cedren-8-ol	37.166	99	222	0.4
60	Isospathulenol	38.588	99	220	0.1
61	Brevifolin	40.215	94	196	11.9
62	Farnesol	41.690	97	222	0.1
63	Farnesol acetate	45.744	97	264	0.2

Tr.: trace amount <0.05%

Total 92.7 %

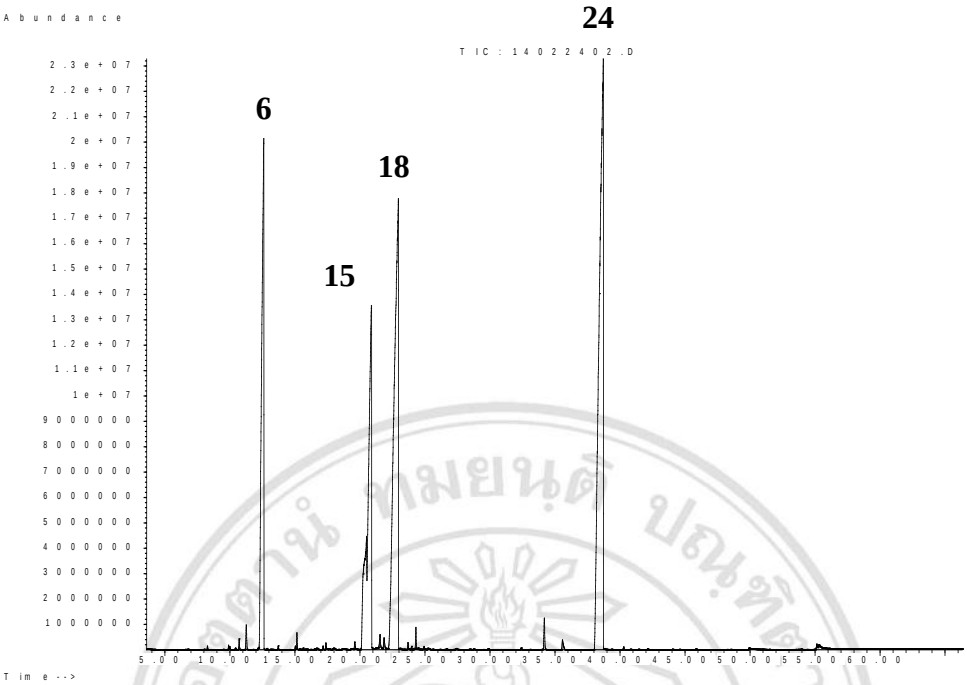


Figure 3.2 The total ion chromatogram of *A. graveolens* L. oil

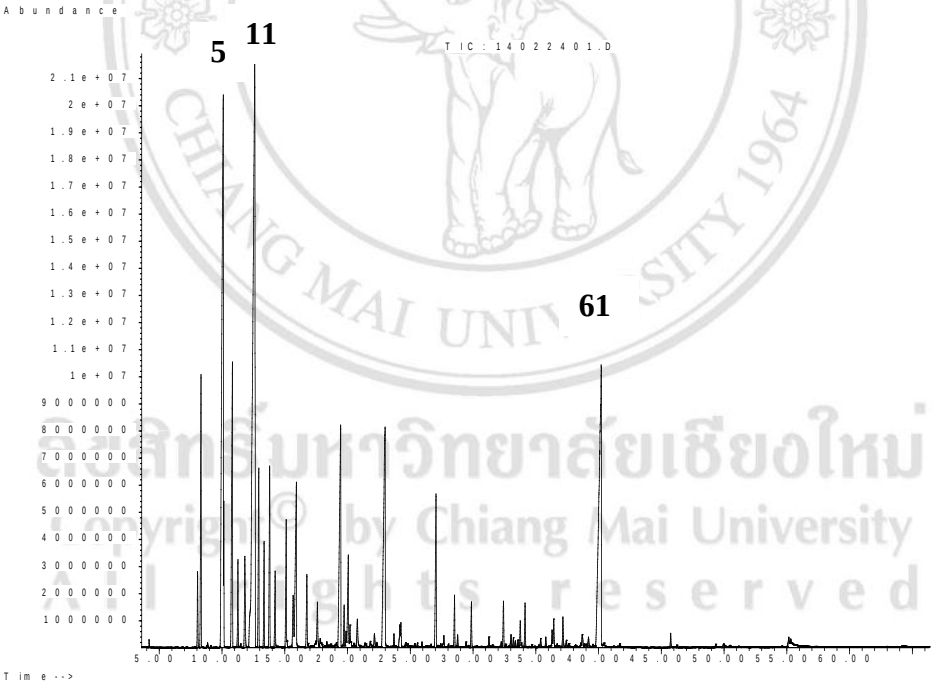


Figure 3.3 The total ion chromatogram of *Z. piperitum* oil

3.3 Antifungal activity of the essential oils on mycelial growth

The antifungal activity of essential oils from *A. graveolens* L. and *Z. piperitum* were determined by the poisoned food technique in potato dextrose agar plate against aflatoxigenic fungi (*A. flavus*) (2.6.1). The fungal growth at 28±2 ^{o}C was monitored by measuring colony diameters every day for 9 days. The results of *A. graveolens* L. oil are shown in Figures 3.4 and 3.5 and the results of *Z. piperitum* oil are shown in Figures 3.6 and 3.7.

3.3.1 Antifungal activity of *A. graveolens* L. oil on mycelial growth

Five concentrations (0.25, 0.5, 1.0, 1.5, 2.0 µL/mL) of *A. graveolens* L. essential oil were tested for their antifungal activities. The mycelial growth was retarded by 3 days for *A. flavus* at 1.0 µL/mL and 6 days at 1.5 µL/mL *A. graveolens* L. oil. The complete growth inhibition of *A. flavus* was observed at the MIC value of 2.0 µL/mL oil shown in Figure 3.4 and the fungal colony at 9th day of inoculation shown in Figure 3.5, the fungal growth colony diameter was shown in Table 3.3. The results indicated that fungal colony diameter increased with incubation time but gradually decreased when increasing concentration of *A. graveolens* L. oil. The oil significantly reduced fungal growth with inhibition percentage of 71.10%, 75.35%, 86.91% and 92.10% at 0.25, 0.5, 1.0, and 1.5 µL/mL concentrations, respectively, on the 9th day of incubation.

Table 3.3 Mean diameter of *A. flavus* colony grown in agar medium supplemented with five different concentrations of the essential oil from *A. graveolens* L.

Concentration of *A. graveolens* oil (µL/mL)	Diameter of fungal colony* (cm)
0	8.48±0.03[a]
0.25	2.45±0.08[b]
0.5	2.09±0.51[b]
1.0	1.11±0.09[c]
1.5	0.68±0.04[d]
2.0	0.40±0.00[d]

* Values are mean (n = 3) ± standard deviations. Values followed by the same letter in each column are not significantly different in ANOVA and Duncan Multiple Range Test ($p < 0.05$).

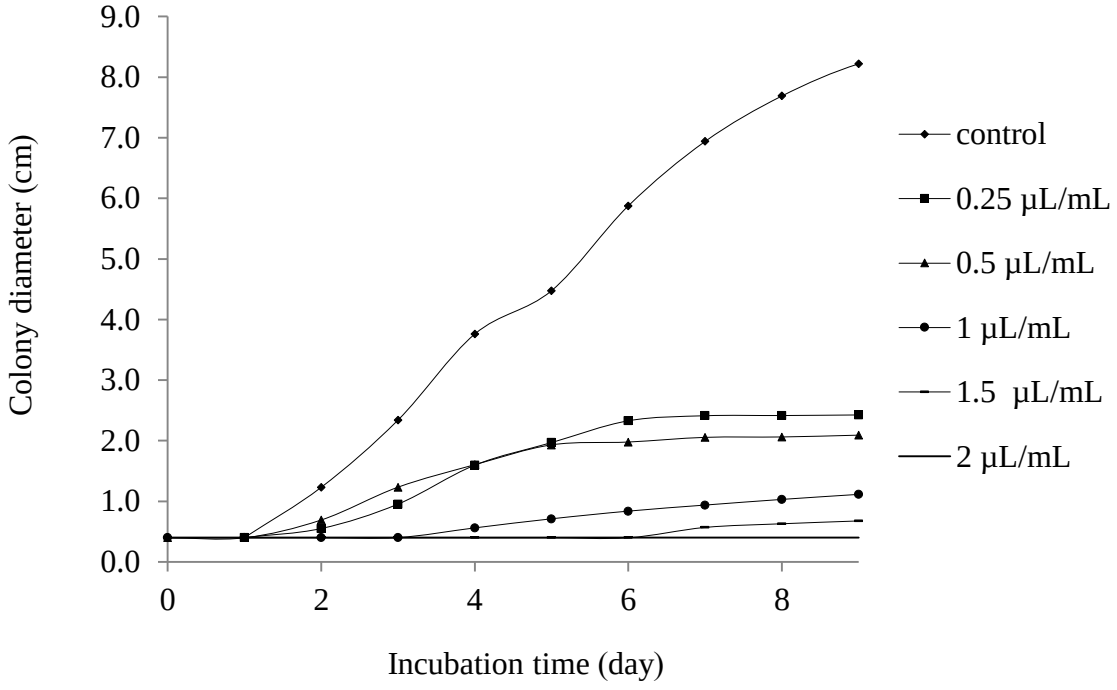


Figure 3.4 Effects of the different concentrations of *A. graveolens* oil on the colony growth of *A. flavus* in PDA medium

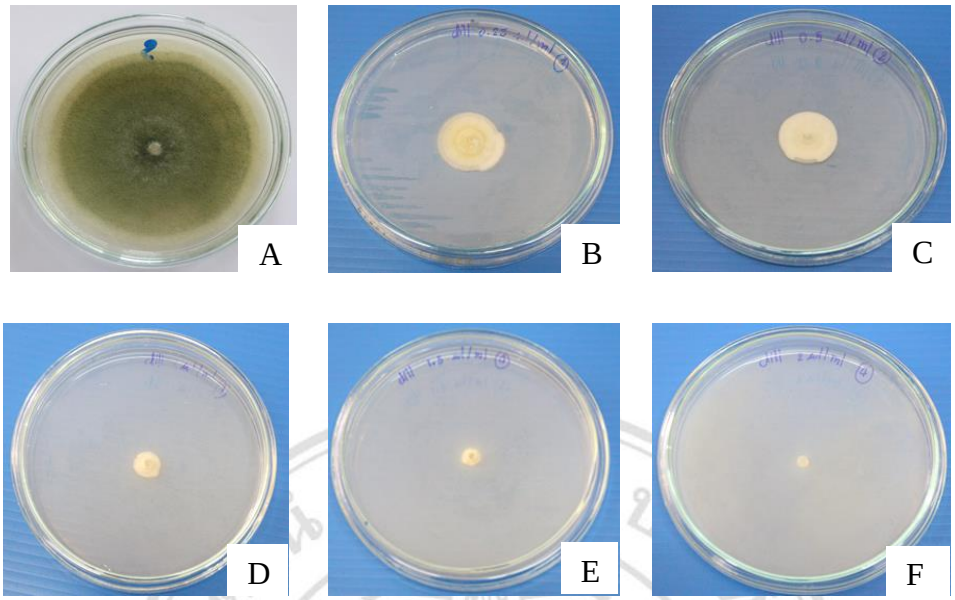


Figure 3.5 *A. flavus* colony grown on PDA control medium (A) and PDA with 0.25 µL/mL (B), 0.5 µL/mL (C), 1.0 µL/mL (D), 1.5 µL/mL (E) and 2.0 µL/mL (F) of *A. graveolens* oil after incubation at 28±2 ºC for 9 days

3.3.2 Antifungal activity of *Z. piperitum* oil on mycelial growth

Essential oil from *Z. piperitum* was tested for its antifungal activity at 8 different concentrations (1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 µL/mL) . The mycelial growth was retarded by 3 days for *A. flavus* at 2.5 µL/mL and 5 days at 3.0 µL/mL of oil. A complete growth inhibition of *A. flavus* by *Z. piperitum* oil was observed at the MIC value of 4.5 µL/mL shown in Figure 3.6 and the 9th days old fungal colonies shown in Figure 3.7, and their diameters were shown in Table 3.4. The results indicated that fungal colony diameter increased with increasing incubation time but gradually decreased when increasing concentration of *Z. piperitum* oil. The oil significantly reduced fungal growth with inhibition percentage of 46.34, 62.50, 77.83, 82.78, 86.43, 89.03 and 91.04 at 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 µL/mL concentrations, respectively.

Table 3.4 Mean diameter of *A. flavus* colony grown in agar medium supplemented with the essential oil from *Z. piperitum* at eight different concentrations

Concentration of *Z. piperitum* oil (µL/mL)	Diameter of fungal colony* (cm)
0	8.48±0.03[a]
1.0	4.55±0.01[b]
1.5	3.18±0.26[c]
2.0	1.88±0.14[d]
2.5	1.46±0.12[e]
3.0	1.15±0.06[f]
3.5	0.93±0.18[f, g]
4.0	0.76±0.07[g]
4.5	0.40±0.00[h]

* Values are mean (n = 3) ± standard deviations. Values followed by the same letter in each column are not significantly different in ANOVA and Duncan Multiple Range Test ($p < 0.05$).

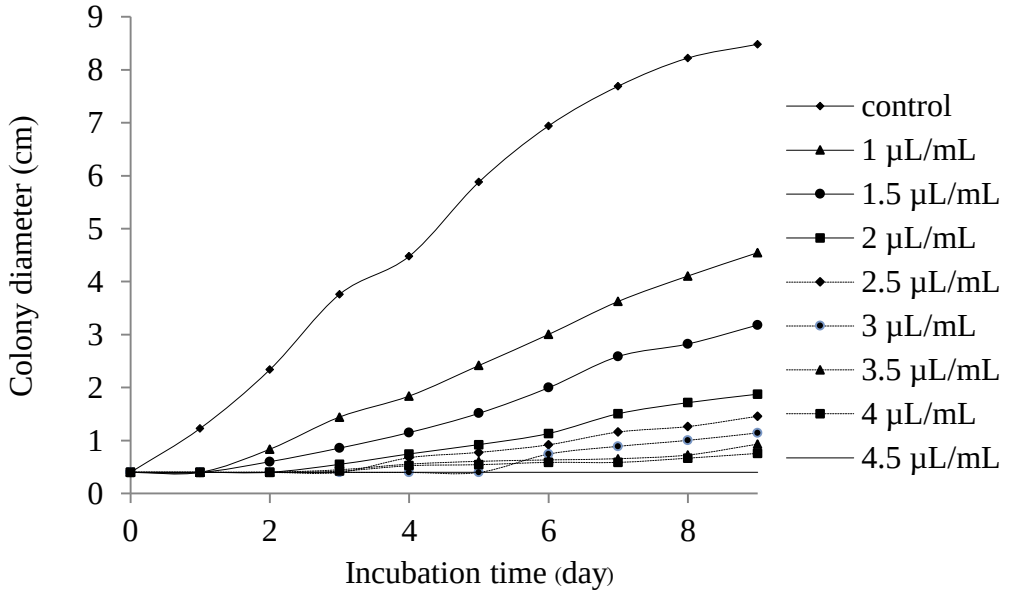


Figure 3.6 Effects of the different concentrations of *Z. piperitum* essential oil on the colony diameter growth of *A. flavus* in PDA medium

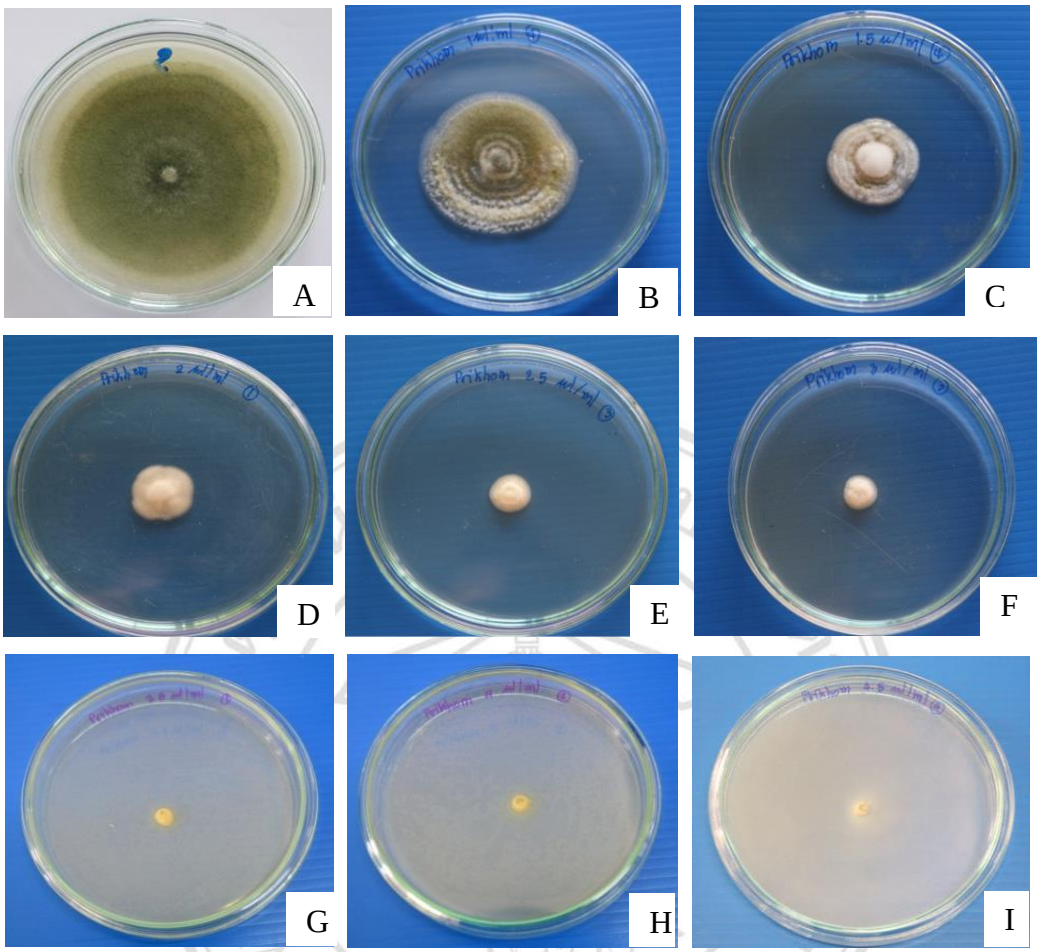


Figure 3.7 *A. flavus* colony grown on PDA control medium (A) and PDA with 1.0 µL/mL (B), 1.5 µL/mL (C), 2.0 µL/mL (D), 2.5 µL/mL (E), 3.0 µL/mL (F), 3.5 µL/mL (G), 4.0 µL/mL (H) and 4.5 µL/mL (I) of *Z. piperitum* oil after incubation at 28±2 ºC for 9 days

3.4 Antifungal activity of essential oils in liquid culture

The antifungal activity of *A. graveolens* and *Z. piperitum* oils against aflatoxigenic fungi was performed in broth medium (2.6.2). The cells were harvested on 9^{th} day and weighed in both wet and dry forms.

3.4.1 Antifungal activity of *A. graveolens* L. oil on mycelial mass

The antifungal activity of *A. graveolens* L. oil on mycelial wet and dry weight was determined in PDB medium (Table 3.5). Five different concentrations of the essential oil were observed to be effective in remarkably inhibiting the biomass production of the fungi (Figure 3.8) and mycelial sizes were smaller when the concentrations of essential oils were increased (Figure 3.9). Mycelial growth at 0.25 µL/mL was decreased to approximately half of that of the control. *A. graveolens* oil was found to be most effective (96.43% inhibition, no mycelial mass observed) at 2.0 µL/mL concentration. The results illustrate that mycelial fungal mass increased with increasing incubation time but gradually decreased when increasing concentration of *A. graveolens* L. oil.

3.4.2 Antifungal activity of *Z. piperitum* oil on mycelial mass

Inhibitory effect of *Z. piperitum* oil on mycelial dry weight was determined in PDB medium (Table 3.6). Eight different concentrations of the essential oil were observed to be gradually effective in inhibiting the biomass production of the fungi (Figure 3.10). High concentrations of essential oil resulted in small size of mycelial fungal mass (Figure 3.11). Mycelial growth in the presence of 2.0 µL/mL *Z. piperitum* oil was decreased to approximately half of that of the control while the most effective concentration (no observed mycelia) was 4.5 µL/mL.

Table 3.5 Mean wet and dry mycelial weight of 9-day old of *A. flavus* in PDB liquid medium supplemented with the essential oil from *A. graveolens* oil at five different concentrations

Concentration of *A. graveolens* oil (µL/mL)	Wet mycelial weight (g)	Dry mycelial weight (g)	% Inhibition*
0	7.1673±0.1604	0.1205±0.0041[a]	-
0.25	3.1120±0.1381	0.0581±0.0110[b]	51.78
0.5	2.5331±0.3644	0.0437±0.0011[c]	63.37
1.0	1.6164±0.0822	0.0350±0.0064[c]	70.95
1.5	1.4228±0.1298	0.0176±0.0065[d]	85.54
2.0	1.0421±0.0129	0.0043±0.0048[e]	96.43

* Calculated from dry weight

Values are mean (n = 3) ± standard deviations. Values followed by the same letter in each column are not significantly different in ANOVA and Duncan Multiple Range Test ($p < 0.05$).

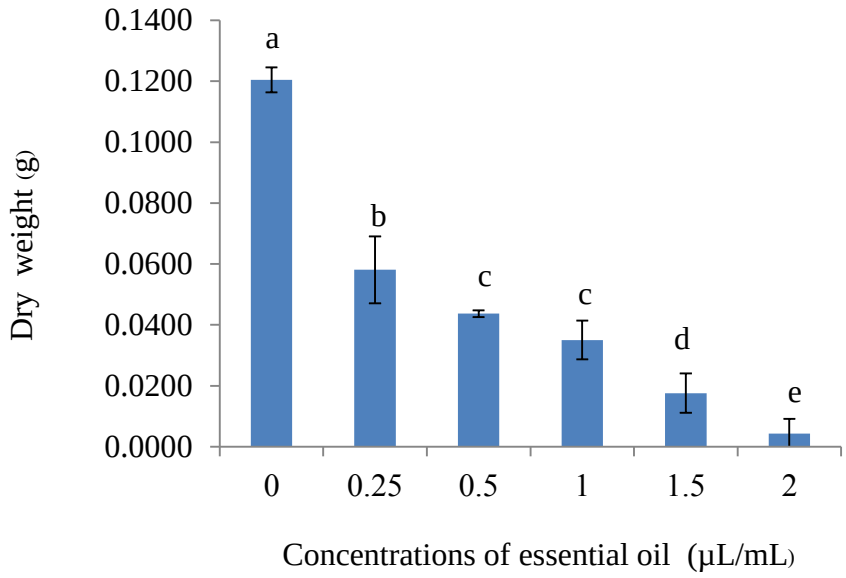


Figure 3.8 Dry weight of mycelial mass of *A. flavus* shaken for 9 days in liquid medium supplemented with the essential oil from *A. graveolens* L. at five different concentrations

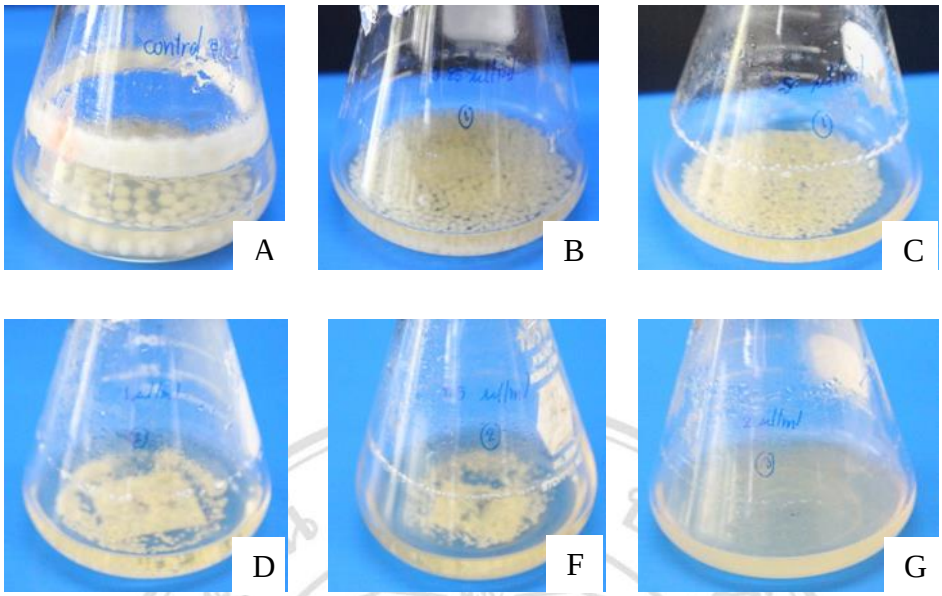


Figure 3.9 Mycelial material of *A. flavus* in control (A), 0.25 µL/mL (B), 0.5 µL/mL (C), 1.0 µL/mL (D), 1.5 µL/mL (E) and 2.0 µL/mL (F) of *A. graveolens* L. essential oil after incubation at 28±2 °C for 9 days

Table 3.6 Mean dry mycelium weight (g) of 9-day old *A. flavus* in liquid medium supplemented with the essential oil from *Z. piperitum* at eight different concentrations

Concentration of *Z. piperitum* oil (µL/mL)	Wet mycelium weight (g)	Dry mycelium weight (g)	% Inhibition*
0	7.6651±0.7791	0.1346±0.0027^{a}	-
1.0	5.9172±0.5735	0.1159±0.0056^{b}	13.89
1.5	4.2699±0.3560	0.0935±0.0117^{c}	30.53
2.0	2.8692±0.2753	0.0656±0.0076^{d}	51.26
2.5	1.7935±0.0481	0.0376±0.0023^{e}	72.06
3.0	1.6609±0.0237	0.0332±0.0011^{e}	75.33
3.5	1.5419±0.1032	0.0296±0.0006^{e,f}	78.00
4.0	1.3728±0.0282	0.0251±0.0026^{f}	81.35
4.5	1.1940±0.1376	0.0047±0.0047^{g}	96.51

* Calculated from dry weight. Values are mean (n = 3) ± standard deviations. Values followed by the same letter in each column are not significantly different in ANOVA and Duncan Multiple Range Test ($p < 0.05$).

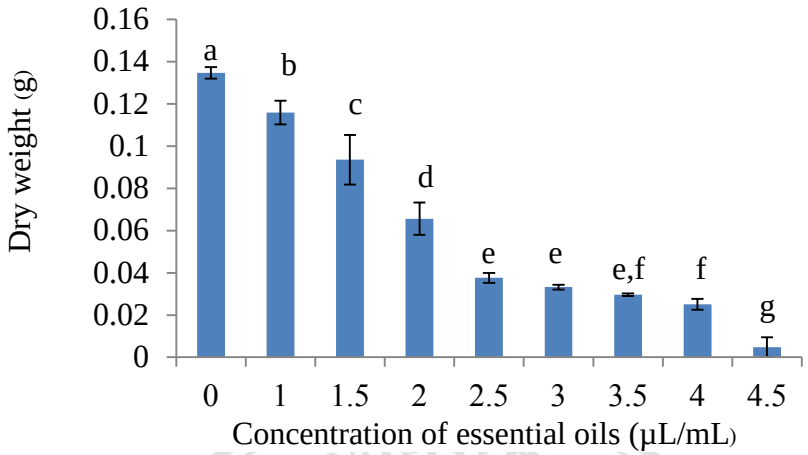


Figure 3.10 Dry weight of mycelial mass of *A. flavus* shaken for 9 days in liquid medium supplemented with the essential oil from *Z. piperitum* at eight different concentrations.

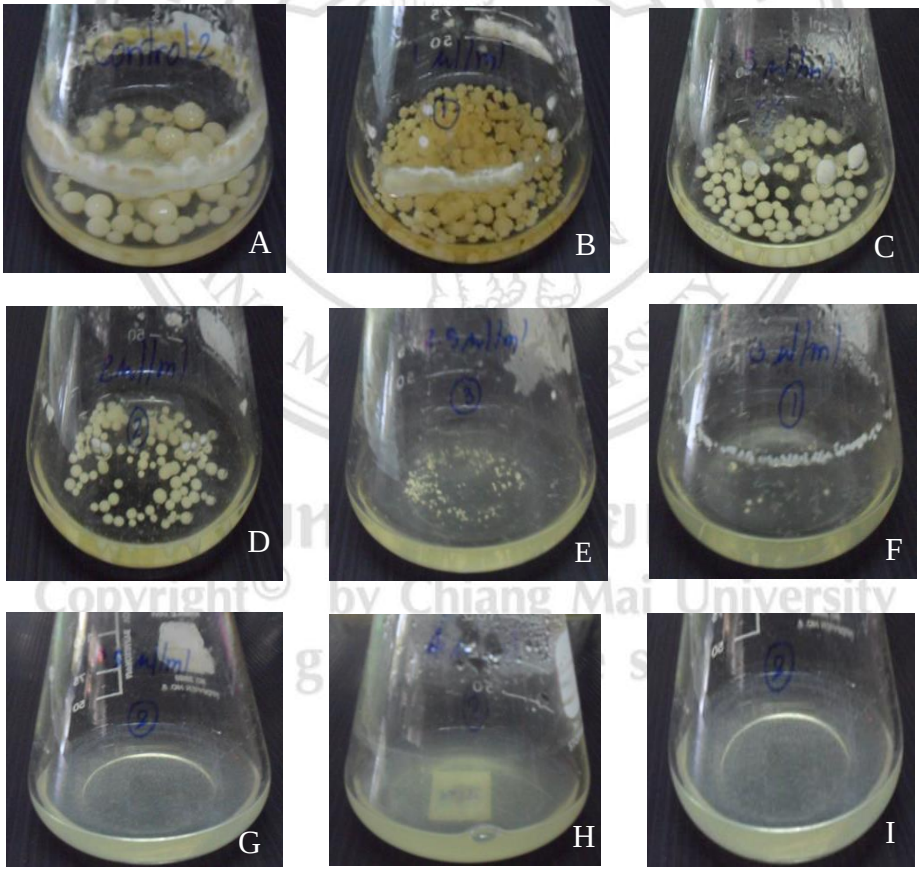


Figure 3.11 Mycelial material of *A. flavus* in control (A), 1.0 µL/mL (B), 1.5 µL/mL (C), 2.0 µL/mL (D), 2.5 µL/mL (E), 3.0 µL/mL (F), 3.5 µL/mL (G), 4.0 µL/mL (H) and 4.5 µL/mL (I) of *Z. piperitum* oil after incubation at 28±2 ^{o}C for 9 days.

3.5 Light microscope examination of the effect of the essential oils on *A. flavus* morphology

Fungal hypha morphology was observed by slide culture. The PDA plates with and without 1.0 µL/mL of *A. graveolens* L. and *Z. piperitum* essential oils, respectively (2.6.3) were examined under a light microscope at 20x and 40x magnification.

3.5.1 The effect of *A. graveolens* L. and *Z. piperitum* oils on morphology of *A. flavus*

The microscopic observation results of fungus treated with 1.0 µL/mL by *A. graveolens* L. and control without oil are shown in Figure 3.12 and 3.13 The control agar plate (Figure 3.12 A) had more conidia and budding conidia spores of *A. flavus* than the ones with 1.0 µL/mL of *A. graveolens* L. and *Z. piperitum* oils (Figure 3.12 B and C) , respectively. The result supported by Figure 3.13 showing morphology degenerative changes in the hyphal and conidial head without and with 1.0 µL/mL for *A. graveolens* L. and *Z. piperitum* oils. Mycelial filament structure without essential oil was regular cell structure homogenous with cylindrical principal axes (Figure 3.13 A), clearly visible conidia sterigmata bearing one a large and radiate conidial heads, and the conidiophore profuse conidiation on conidial heads (Figure 3.13 D). However, The hyphae structures of fungus exposure to oil, appeared spiral coil, thin flat and decreased hyphal diameters due to the lack of cytoplasm (Figure 3.13 B and C). Furthermore, the conidial heads treated with 1 µL/mL of both essential oils were distorted, languish conidial heads structures and flatten conidiophore (Figure 3.13 E and F).

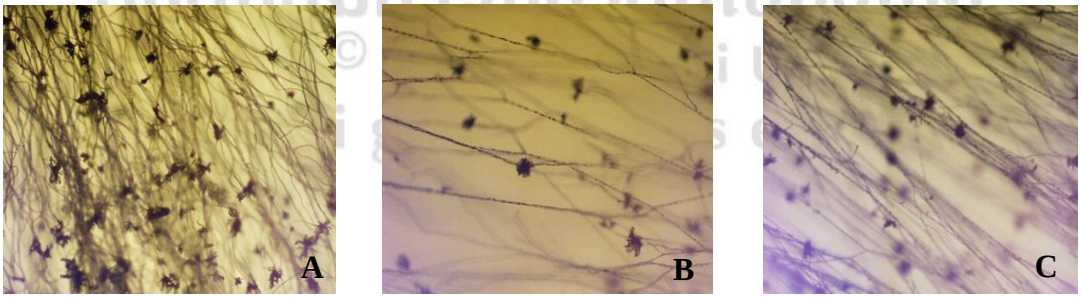


Figure 3.12 Morphology of *A. flavus* under 20X microscope on PDA without (A) and with the essential oils of *A. graveolens* L. (B) and *Z. piperitum* (C) at 1 µL/mL

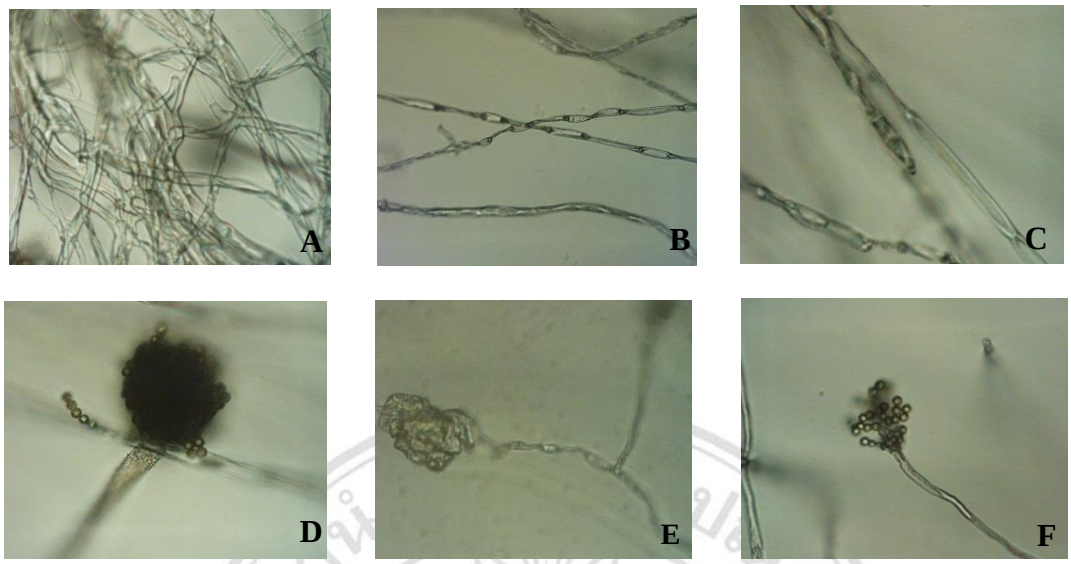


Figure 3.13 Morphology of *A. flavus* under 40X microscope on PDA without (A,D) and with the essential oils of *A. graveolens* L. (B,E) and *Z. piperitum* (C,F) at 1 µL/mL

3.6 Effect of essential oils on ergosterol content in plasma membrane of *A.flavus*

The effect of *A. graveolens* L. and *Z. piperitum* oils on the ergosterol content in the plasma membrane of *A. flavus* was studied. In our study, the ergosterol content was determined by previously described methods by Tian et al., (2012) . This sterol quantitation method is indicative of the ergosterol and 24(28) dehydroergosterol contents based on the exclusive spectral absorption at 230 and 280 nm of extracted sterols (2.6.4).

3.6.1 Effect of ergosterol content in plasma membrane from *A. graveolens* L. and *Z. piperitum* oils

The result indicated that the ergosterol content in the plasma membrane of *A. flavus* was reduced by 0.25 µL/mL of *A. graveolens* L. and 1.0 µL/mL of *Z. piperitum* oils. After 4-day inculation wet mycelial masses in the presence of added *A. graveolens* L. and *Z. piperitum* oils were 4.39 g and 3.72 g, respectively, compared to the control (9.56 g) . After incubated with 0.25 µL/mL for *A. graveolens* L. oil, a reduction of the ergosterol content in the plasma membrane of *A. flavus* was observed at 0.17±0.01 %w/w (32% reduction) of the control. Whereas *Z. piperitum* oil decreased the ergosterol content to 0.18±0.02 %w/w (28% reduction) compared to the control (0.25±0.03 %w/w).

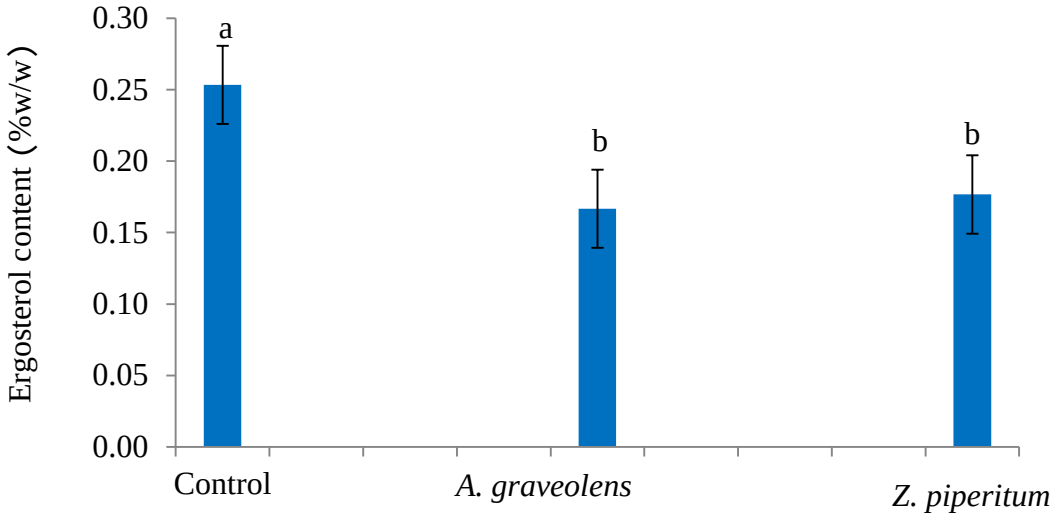


Figure 3.14 Effect of essential oils from *A. graveolens* L. and *Z. piperitum* on ergosterol content in plasma membrane of *A. flavus* in PDB for 4 days. Values are mean (n = 3) ± standard deviations. Values followed by the same letter in each column are not significantly different in ANOVA and Duncan Multiple Range Test ($p < 0.05$).

3.7 Efficacy of essential oils as antifungal coat in dry chili

Dried bird chili, a model product, coated with *A. graveolens* L. and *Z. piperitum* oil were tested for potential to protect against *A. flavus* infection. Pour plate culture at 10^{-5} dilution of infected chili is shown in Figure 3.12, after incubation at 28 ^{o}C for 9 days (2.6.5).

3.7.1 The effect of *A. graveolens* L. and *Z. piperitum* oils on dry chili model

Essential oils from *A. graveolens* L. and *Z. piperitum* were tested for their potential to protect against *A.flavus* infection on dry chili. The results indicated that the dry chili control had more fungal hypha than essential oil coated chili and number of colony decreased when concentration of the essential oil increased. The concentrations of essential oil at 2.0 and 4.0 µL/mL of *A. graveolens* L. oil resulted in reduction of fungal development by 84.00% and 93.32%, respectively (Figure 3.12 B and C) . While *Z. piperitum* oil reduced 45.20 % and 89.32 % (Figure 3.12 D and E) at 4.5 and 9.5 µL/mL respectively, compared with the control (Figure 3.12 A).

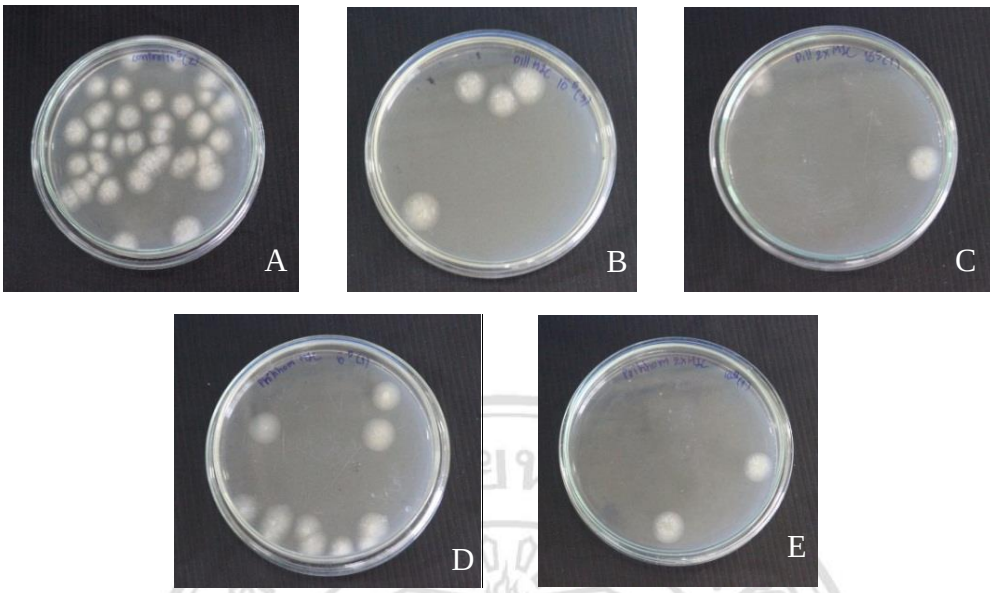


Figure 3.15 Pour plates culture after 9 days of incubation of dried bird chili non-coated (A), coated with 2.0 µL/mL (B), 4.0 µL/mL (C) *A. graveolens* L. oil and with 4.5 µL/mL (D) and 9.0 µL/mL (E) *Z. piperitum* oil.

CHAPTER 4

DISCUSSION

Consumers and food processors demand for new self-preservatives that are biodegradable, toxic residue-free and environmental friendly to control spoilage in food. Essential oils compose of volatile aromatic compounds produced from plant secondary metabolism. They have been used as food flavouring and preservation (Rahman and Kang, 2009). Most of essential oils are classified as generally recognized as safe (GRAS) substances which have low risk on the human health and resistance development in microorganisms (Cardile et al., 2009). Thus, the essential oils from *A. graveolens* and *Z. piperitum* seeds are potentially applied as natural food preservatives.

In this study, Essential oils were extracted from *A. graveolens* and *Z. piperitum* seeds and chemically identified by GC-MS. *A. graveolens* seed oil constituents can be grouped into three-classes with monoterpene hydrocarbon 21.8%, oxygenated monoterpene 40.5% and phenylpropene 33.9%. The major compounds of the essential oil were *trans*-isodillapiole 33.1%, (+) -carvone 26%, (-) -limonene 19.2% and dihydrocarvone 12.3%. A previous study reported that main compound of *A. graveolens* seeds oil was carvone (75.21%), while the content of limonene was 21.56% and dihydrocarvone 3.02% (Radulescu et al., 2010) . Whereas the constituents of *Z. piperitum* seeds oil can be grouped into six sub-classes with monoterpene hydrocarbon 68.6%, oxygenated monoterpene 19.5%, sesquiterpene hydrocarbon 3.2%, oxygenated sesquiterpene 1.0%, phenylpropene 0.2%, and miscellaneous compounds 0.2%. The major components were β-phelladrene 23.2%, sabinene 14.6% and brevifolin 11.9%. Choochote et al., (2007) reported that the major compound of *Z. piperitum* seeds oil were (+) -limonene (37.9%), sabinene (13.3%), and β-myrcene (7.17%). Each element was different depending on species, sub-species and plant varieties, the portion of the plant used, different geographic locations where plants are grown, and weather

conditions during growth and the stage of growth at harvest (Burt, 2004; Nieblas et al., 2011; Silveira et al., 2012) . Essential oils containing terpenes (monoterpenes, sesquiterpenes and oxygenated derivatives) as major compoued were reported to exhibit antimicrobial activity (Cakir et al., 2004). The different compounds in one essential oil may exhibit synergism in antibacterial and antifungal activities (Reginer et al., 2008).

The essential oil of *A. graveolens* seeds showed pronounced antifungal efficacy against *A. flavus*. Mycelial growth curve of the fungus during the 9-day incubation period was shown in Figure 3.4. Mycelial growth was decreased when increasing concentrations of essential oils. While 2.0 µL/mL concentration of oil could completely inhibit mycelial growth of *A. flavus* in agar. Essential oil of *A. graveolens* seeds exhibited excellent performance for inhibition of *Aspergillus* spp growth at 6 µL/mL by poison food techniques (Singh et al., 2005). However, we found that the essential oil reduced mycelial growth with percentage reduction ranging from 71.1% to 92.1% at 0.25-1.5 µL/mL concentrations. Additionally, earlier report showed that *A. graveolens* seed oil inhibited growth of some food born microorganisms (Delaquis et al., 2002; Elgayyar et al., 2001; Fatope et al., 2006; Lopez et al., 2005; Sagdic and Ozcan, 2003). While *Z. piperitum* seed oil completely inhibited *A. flavus* at the MIC value of 4.5 µL/mL. The mycelial growth inhibition percentage was calculated on the 9th day shown in Figure 3.6. The oil significantly reduced fungal growth with inhibition percentage of 46.34, 62.50, 77.83, 82.78, 86.43, 89.03 and 91.04 at 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 µL/mL concentrations, respectively. Prakash et al. (2012) reported that *Z. alatum* oil at 1.25 µL/mL was highly efficient for inhibition of *Aspergillus* spp. growth by poison food techniques. Furthermore, the essential oils from the seeds of *Zanthoxylum* species have been reported in suppressing food spoilage causing microorganisms and oral pathogens (Hurtado et al., 2003; Misra et al., 2013; Park et al., 2008; Tatsadjieu et al., 2003; Yeboa et al., 2005). In addition, effect restraining in the biomass of mycelia fungi was shown remarkably by the essential oil. (Dikbas et al., 2008; Kedia et al., 2014; Prakash et al., 2012; Tatsadjieu et al., 2009; Tian et al., 2012). As shown in Tables 3.5 and 3.6, the result indicated that 2.0 µL/mL of *A. graveolens* oil exhibited the highest inhibitory effect on the fungal biomass whereas *Z. piperitum* oil was shown the most efficiency at 4.5 µL/mL concentration. To sum up, the mycelial growth and the biomass mycelia of the *A. flavus* significantly restricted by the essential oils of *A. graveolens* and

Z. piperitum in a dosage-responsive manner. The effect of *A. graveolens* and *Z. piperitum* oils on *A. flavus* mycelial structure was examined under a light microscope. The microscopic observation results of fungus treated with 1.0 µL/mL by *A. graveolens* and *Z. piperitum* oil and control are shown in Figures 3.12 and 3.13. In vitro light microscope observations of the microstructure of *A. flavus* which is sensitive to essential oils revealed some mechanisms of the *A. graveolens* and *Z. piperitum* oils such as roundly twisted conidial heads and decreased hyphal diameters. The results obtained were similar to those from some previous work in which the microstructure of *Aspergillus* spp. treated with other essential oils was studied (Carmo et al., 2008; Sharma and Tripathi, 2008; Tian et al., 2011; Tolouee et al., 2010). In addition, the components of essentials oil may interfere with the enzymatic synthesis of cell walls (Tripathi et al., 2009). Also, some alteration induced by essential oil may cause a lack of cytoplasm, damage of integrity, the electron transport chain, H^+-ATPase inhibition, and finally cell death as in other essential oils (Burt, 2004).

The effect of *A.graveolens* and *Z. piperitum* oils on the ergosterol content in the plasma membrane of *A. flavus* was studied. Ergosterol, the end-product of the biosynthetic pathway and the main sterol in eukaryotic cells, is responsible for structural membrane character, such as fluidity and permeability same as cholesterol pathway of mammalian cells (Veen and Lang, 2005). Modification in the cell permeability of the plasma membrane led to damage of the normal shape of fungal cell. A previous study has shown the quantity reduction of ergosterol by other essential oils (Kedia et al., 2014; Pinto et al., 2009). In this study, the ergosterol content was determined by Tian et al., (2012) method based on the exclusive spectral absorption at 230 and 282 nm of the extracted sterols (ergosterol and 24(28) dehydroergosterol). The result indicated that the ergosterol content in the plasma membrane of *A. flavus* was reduced by 0.25 µL/mL of *A.graveolens* and 1.0 µL/mL of *Z. piperitum* oils. After incubation of *A. flavus* with 0.25 µL/mL of *A. graveolens* oil, a reduction of the ergosterol content in the plasma membrane was observed at 32% of the control. While 1 µL/mL of *Z. piperitum* oil decreased the ergosterol content by 28%. In previous works, the plasma membrane was the target of essential oils supported by the damage seen under SEM or TEM (Abyaneh et al., 2006; Khan et al., 2011; Nogueira et al., 2010; Tian et al., 2012; Tolouee et al.,

2010). Thus, the plasma membrane is probably an important antifungal target of *A. graveolens* and *Z. piperitum* oils.

The action mechanism of essential oils has not fully understood, but it is regarded as involving membrane destruction due to its hydrophilic or lipophilic characteristic (Cowan, 1999). However, *A. graveolens* L. oil exhibited higher antifungal activities than *Z. piperitum* oil, which may be attributed to the high contents of (-)-limonene, dihydro-carvone, (+)-carvone and *trans*-isodillapiole, high oxygenate monoterpenes and high phenylpropene. A recent report has shown that limonene and carvone were the most potent antifungal compounds against the variety of microorganisms that cause food spoilage such as *A. flavus* and *A. niger* (Aggarwal et al., 2001; Marei et al., 2012). Dillapiole, the main component of *Piper aduncum* oil showed antifungal activity against *Clinipellis perniciosa* (witches' broom) (Almeida et al., 2009). In other reports, oxygenated monoterpenes exhibited antimicrobial activity against plant pathogenic microorganisms (Mahmoud, 1994; Pauli, 2001 and Regnier et al., 2008). Furthermore, Zambonelli et al., (1996) reported that the oxygenated monoterpenes acted against pathogens because they prevented enzymatic reactions during synthesis of cell wall. A recent report has shown that some essential oil high phenylpropene such as *Foeniculum vulgare* Mill. ssp. vulgare var.azoricum (Mill.) Thell] leaves (rich in anethole) exhibited antimicrobial activity against Gram-positive bacteria (Senatore et al., 2013).

The main functional groups of *A. graveolens* L. oil were ketone and methoxy groups while hydrocarbons were the major functional group of *Z. piperitum* oils. The antifungal activity of essential oils is generally depending on lipophilicity of hydrocarbons structure and hydrophilicity of functional groups. The antimicrobial activity rank of essential oils is as follows: phenols> aldehydes> ketone> alcohol> ether> hydrocarbons (Kalemba and Kunicka, 2003). The mechanism action of ketone group (dihydro-carvone, (+)-carvone) and methoxy groups (*trans*-isodillapiole) may be disruption of the plasma membrane, increased permeability, extensive loss of the intracellular proteins and finally resulting in cell death similar to other phenylpropene (eugenol) (Devi et al., 2010) or ketone and methoxy groups may be able to form hydrogen bonds with the active sites of enzyme active center similar to phenol group (Bluma et al., 2008). Monoterpenes hydrocarbons which had low antimicrobial activity

may result from free functional group causing limitation of hydrogen bonding capacity or water solubility (Victório et al., 2008). However, it does not mean that monoterpene hydrocarbons has no antifungal activity, as it has been indicated that some of essential oils such as *Schinus molle* fruit (rich in α-phellandrene, β-pinene, β-phellandrene) exhibited antifungal activity against *Botrytis cinerea* (Ibrahim and Naser, 2014) and *S. terebinthifolius* fresh leaf (rich in sabinene and α-pinene) exhibited antibacterial and antifungal activities against pathogen (Gundidza et al., 2009). Moreover, Glisic et al., (2009) reported that fraction of α-pinene and mixture of α-pinene and sabinene from *Juniperus communis* L. showed the highest antimicrobial activity against bacteria, yeast and fungi. Furthermore, a previous study has shown that essential oils have a greater antimicrobial activity than the pure major components of essential oil (Gill et al., 2002; Morillon et al., 2002). Additionally, that minor components may also have important synergistic effect (Burt, 2004; Tripathi et al., 2009). However, the essential oil may be infiltrated into the lipid rich portion of the cell membrane to destroy or disrupt cell membrane by cross linkage reactions, damage of electrolytes, cytoplasm coagulation, damage the membrane protein, increased permeability leading to leakage of the plasma membrane, and reduction in level of amino acids, sugars and decreased the proton motive force, intracellular ATP synthesis and finally resulting in the death of the cell because essential oil, which has low molecular weight and highly lipophilic components passes simply through cell membranes and organization (Chao et al., 2005; Inouye et al., 2000; Nazzaro et al., 2013).

In the last experiment, we studied antifungal activity of essential oils applied on product surface. The potential application of essential oils to control spoilage fungi on agriculture products was previously reported (Kumar and Nambisan, 2013; Prakash et al., 2012; Tian et al., 2011; Tzortzakis, 2009). Dried bird chili, a model product, coated with *A. graveolens and Z. piperitum* oils was tested for potential to protect against *A. flavus* infection. Pour plate culture at 10^{-5} dilution of infected chili is shown in Figure 3.12. After incubation at 28 ^{o}C for 9 days, the concentrations of essential oil at 2.0 and 4.0 µL/mL of *A. graveolens* L. oil resulted in reduction of fungal development by 84.00% and 93.32% respectively. While *Z. piperitum* oil reduced 45.20% and 89.32 % at 4.5 and 9.5 µL/mL, respectively compared with the control. Thus, the essential oils of *A. graveolens* L. and *Z. piperitum* are likely to be developed into food preservatives.

The model product experiment showed the susceptibility to fungal infection because humidity conditions and nutrition in food favor spoilage fungi growth more than in laboratory media (Tzortzakis, 2009) . Thus, the model products are required higher concentrations of essential oils than in laboratory media in order to completely inhibit fungal growth. The use of essential oils can safely inhibit microorganisms in food without residues after storage. However, the essential oils have limitation of use because they have strong flavors and have applicability only in products with compatible flavor. In addition, the use of essential oil in food has to meet the standard safety limit values and sensory characteristics such as color, aroma, and firmness (Tian et al., 2011).

CHAPTER 5

CONCLUSION

1. The constituents of *A. graveolens* essential oil could be grouped into three sub-classes with monoterpene hydrocarbon 21.8%, oxygenated monoterpene 40.5% and phenylpropene 33.9%. The major compounds of the essential oil were *trans*-isodillapiole 33.1%, (+)-carvone 26%, (-)-limonene 19.2% and dihydro-carvone 12.3%.

2. The constituents of *Z. piperitum* essential oil could be grouped into six sub-classes with monoterpene hydrocarbon 68.6%, oxygenated monoterpene 19.5%, sesquiterpene hydrocarbon 3.2%, oxygenated sesquiterpene 1.0%, phenylpropene 0.2%, and miscellaneous compounds 0.2%. The major components were sabinene 14.6%, β-phelladrene 23.2% and brevifolin 11.9%.

3. The essential oil of *A. graveolens* reduced mycelial growth with percentage reduction ranging from 71.1% to 92.1% at concentrations of 0.25-1.5 µL/mL and 2.0 µL/mL of oil could completely inhibit mycelial growth *A. flavus* in agar.

4. *Z. piperitum* oil completely inhibited *A. flavus* growth in agar, as observed at the MIC value of 4.5 µL/mL and the oil significantly reduced fungal growth with inhibition percentage of 46.34, 62.50, 77.83, 82.78, 86.43, 89.03 and 91.04 at 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 µL/mL, respectively.

5. *A. graveolens* L. oil at 0.25 µL/mL was able to decrease *A. flavus* growth in broth medium by approximately half of the control. *A. graveolens* oil was found most effective (96.43% inhibition, no mycelial mass observed) at 2.0 µL/mL.

6. Inhibitory effect of 2.0 µL/mL *Z. piperitum* oil against *A. flavus* in broth medium was shown as decreased fungal mass to approximately half of the control and the most effective concentration (no observed mycelia) was 4.5 µL/mL.

7. *A. graveolens* L. and *Z. piperitum* oils at 1 µL/mL had deteriorated effects on morphology of *A. flavus* such that the conidial heads were distorted, languish conidial heads structures and flatten conidiophore. While, the hyphae structures were appeared spiral coil, thin flat and decreased hyphal diameter.

8. The result indicated that the ergosterol content in the plasma membrane of *A. flavus* was reduced by 0.25 µL/mL of *A. graveolens* L. oil and 1.0 µL/mL of *Z. piperitum* oil. The ergosterol content in the plasma membrane of *A. flavus* treated with *A. graveolens* L. oil was 0.17±0.01%w/w (32% reduction of the control). Whereas *Z. piperitum* oil decreased the ergosterol content to 0.18±0.02%w/w (28% reduction) compared to the control (0.25±0.03%w/w).

9. Essential oils from *A. graveolens* L. and *Z. piperitum* were tested for their potential to protect against *A. flavus* infection on surface of dry chili. 2.0 and 4.0 µL/mL of *A. graveolens* L. oil resulted in reduction of fungal development by 84.00% and 93.32%, respectively. While *Z. piperitum* oil reduced to 45.20% and 89.32% at 4.5 and 9.5 µL/mL, respectively compared with the control.

10. The essential oils of *A. graveolens* L. and *Z. piperitum* are likely to be developed into food preservatives.

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APPENDIX A

PREPARATION OF THE CHEMICAL REAGENTS

A.1 Fungal media preparation

(i) PDA (Potato Dextrose Agar)

39 grams of PDA powder was suspended into 1000 mL distilled water. The liquid medium was stirred until thoroughly mixed and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

(ii) PDB (Potato Dextrose Broth)

26.5 grams of PDB powder medium were suspended into 1000 mL distilled water. The liquid medium was stirred until thoroughly mixed and sterilized at 121 ^{o}C 15 lbs pressure for 15 min.

A. 2 Preparation of Tween 20 solution for pour plate dilution series

5 mL of Tween 20 was dissolved into 100 mL distilled water. Nine mL of 5% v/v Tween 20 solution was pipetted into test tube. Each test tube was sterilized at 121 ^{o}C 15 lbs pressure for 15 min.

A. 3 Preparation fungal spore suspensions solution

Three-day old *A. flavus* spores from the culture grown in potato dextrose agar slant were aseptically transferred into screw-capped vials containing 10 mL of sterile 5%v/v of Tween 20. They were gradually mixed and count number spore by haemocytometer before used further.

A. 4 Reagent preparation for extraction ergosterol assay

(i) 25% alcoholic potassium hydroxide solution

25 grams of potassium hydroxide were dissolved into 35 mL distilled water and brought to 100 mL with absolute ethanol. It was stored in dark glass bottle at room temperature.

APPENDIX B

FUNGAL COUNTING

B.1 Standard plate count

The standard plate count method consists of diluting a sample with 5%v/v Tween 20 diluent until the fungi are dilute enough to count accurately. That is, the final plates in the series should have between 30 and 300 colonies. Fewer than 30 colonies are not acceptable for statistical reasons (too few may not be representative of the sample), and more than 300 colonies on a plate are likely to produce colonies too closed to each other to be distinguished as distinct colony-forming units (CFUs). The assumption is that each viable fungal cell is separated from all others and will develop into a single discrete colony (CFU). Thus, the number of colonies should give the number of fungi that can grow under the incubation conditions employed. A wide series of dilutions (e.g., 10^{-4} to 10^{-8}) is normally plated because the exact number of fungi is usually unknown.

The procedures:

(i) Label the bottom of six petri plates 1-15. Label five tubes of 5%v/v Tween 20 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}.

(ii) Using aseptic technique, the initial dilution is made by transferring 1 mL of *A. flavus* spore solution sample to a 9 mL sterile 5%v/v Tween 20 blank (Figure B.1. This is a 1/10 or 10^{-1} dilution).

(iii) Immediately after the 10^{-1} dilution has been shaken, uncap it and aseptically transfer 1ml to a second 9 mL 5%v/v Tween 20 blank. Since this is a 10^{-1} dilution, this second blank represents a 10^{-2} dilution of the original sample.

(iv) Shake the 10^{-2} dilution vigorously and transfer 1 mL to the third 9 mL blank. This third dilution represents a 10^{-3} dilution of the original sample.

(v) Repeat the Shake the 10^{-3} dilution again and aseptically transfer 1.0 mL to one petri plate.

(vi) Do the same for the 10^{-5}, 10^{-6}, 10^{-7} and the 10^{-8} dilutions.

(vii) Remove one agar pour tube from the approximate 48 ºC water bath. Carefully remove the cover from the 10^{-4} petri plate and aseptically pour the agar into it.

(viii) The agar and sample are immediately mixed by gently moving the plate as in a Figure B.1.

(ix) Repeat this process for the remaining three plates.

(x) After the pour plates have cooled and the agar has hardened, they are inverted and incubated at 28±2 ºC for 2-3 days.

(xi) At the end of the incubation period, select all of the petri plates containing between 30 and 300 colonies. Plates with more than 300 colonies cannot be counted and are designated too many to count. Plates with fewer than 30 colonies are designated too few to count.

(xii) Count the colonies on each plate.

(xiii) Calculate the number of bacteria (CFU) per milliliter or gram of sample by dividing the number of colonies by the dilution factor multiplied by the amount of specimen added to liquefied agar.

$$\frac{\text{Number of colonies (CFUs)}}{\text{Dilution X amount plated}} = \text{fungi/ml}$$

Example;

The number of fungi (CFU) per milliliter that were counted from the *A. flavus* fungal colonies were 54, 48, 52 colonies forming units by plating triplicated at 10^{-6} dilution.

$$= \frac{54+56+52 \text{ CFUs}}{10^{-6} \times 3} = 54\times10^{6} \text{ CFU/mL}$$

Therefore; The number of fungi from *A. flavus* culture was about 5.4×10^{7} CFU/mL

(xiv) Process once more to produce a 10^{-4} dilution.

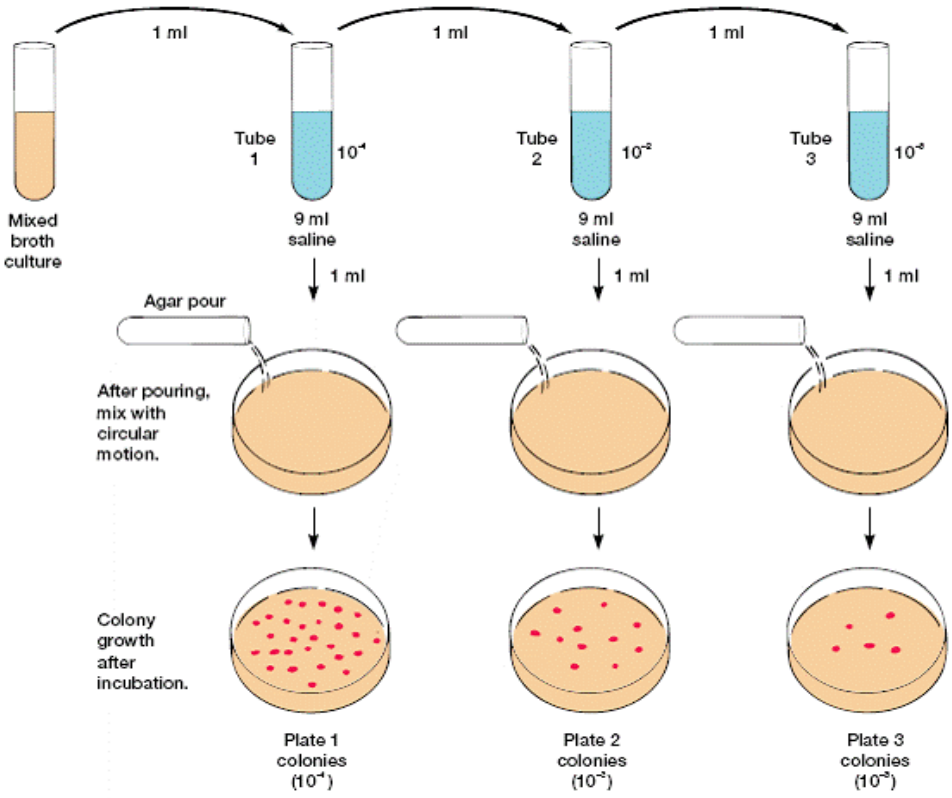


Figure B.1 Procedure of pour plate counting

(http://www.agro.kmutnb.ac.th/e-learning/521302/1_clip_image016.gif)

(Date: November 18, 2014)

B.2 Direct microscope method

Petroff-Hausser counting chambers can be used as a direct method to determine the number of fungal cells in a culture or liquid medium. In this procedure, the number of cells in a given volume of culture liquid is counted directly in 10-20 microscope fields. The average number of cells per field is calculated and the number of fungal cells mL^{-1} of original sample can then be computed. A major advantage of direct counts is the speed at which results are obtained. The grid is divided into 9 large squares, each 1 mm x 1 mm, by triple lines. Each large square is divided into 25 medium squares, each 0.23 mm on a side, and each medium square is further divided into 16 small squares, each 0.05 mm on a side (Figure B.2).

The procedures:

(i) Mix spore solution well.

(ii) Add 10 µL of spore solution to each side of the hemocytometer.

(iii) Count number of spores in zones A, B, C and D on both sides of the hemocytometer, record them, and calculate the average of the two sides.

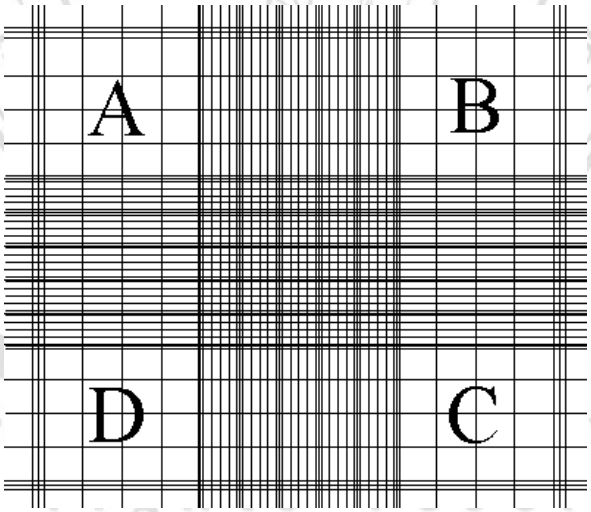


Figure B.2 Petroff-Hausser counting chamber

(http://home.cc.umanitoba.ca/~adam/lab/images/Hemato.gif)

(Date: November 18, 2014)

Where; A = Total number of cell or spore inside 9 large squares (cell)

B = Volume required of spore suspension (µL)

The surface in 1 small square = 1/400 mm^2, depth = 1/10 mm

Therefore in 1 small square has a volume (1/400 mm^2) × (1/10 mm) = 1/4000 mm^3

Since 16 small squares equal 1 large square

Therefore in 1 small square has a volume $16 \times 1/4000\ mm^3 = 16/4000\ mm^3$

And 9 large squares have a volume $= 9 \times (16/4000\ mm^3)$

Or referred that 9 large squares have a volume $= 9 \times (16/4000)\ \mu L$

If in 9 large squares, the counted number of cells = A cells

Therefore in the volume of $9 \times (16/4000)\ \mu L$ has cells = A cells

If the volume of spore suspension = B µL

$$\text{Therefore in volume of B µL there are} = \frac{A \times B\ (\times \text{dilution})}{9 \times (16/4000)}\ \text{cells}$$

Example;

Determination of spore suspension concentration of inoculated *A. flavus* was calculated as shown in Figure B. 3

7			2
4			5

3			6
4			5

Figure B.3 Spore numbers inside 4 zone squares of counting chamber in 2 times

Average of total spore number inside 4 zone squares as (18+18)/2 = 18 cells

At dilution 10^{-2}; the volume of 1000 µL of spore suspension has spores

$$= \frac{18 \times 1000 \times 10^{-2}}{9 \times (16/4000)} = 5 \times 10^3\ \text{cells}$$

Therefor; the concentration of spore suspension = 5×10^3 cells/mL

APPENDIX C

Another *Zanthoxylum* species found in Thailand

C. *Zanthozylum limonella* Alston

C. 1 Scientific classification

Kingdom: Plantae

Order: Sapindales

Family: Rutaceae

Genus: *Zanthoxylum*

Species: *Z. limonella* Alston

Common name: Makhan

Local name: Makhan (มะแขว่น, มะแข่น), Bakhan (บ่าแขว่น, บ่าแข่น)

C. 2 Botanical characteristics of plant

Zanthozylum limonella Alston (Rutaceae), locally called ‘‘Makhan’’, is an evergreen shrub distributed in the northern part of Thailand. *Z. limonella* Alston is a perennial plant 5-25 m high, spiny trunk, glossy leaves and red leaf stalk. The flowers are greenish white, in small corymbs and fruits are green and brown ripe with shiny black seeds. Characteristics of *Zanthoxylum limonella* Alston plant is shown in Figure C. 1.

Figure C.1 Characteristics of *Zanthoxylum limonella* Alston: Stem (A), Flowers (B), Leaves (C), Fruits (D), Dry fruits (E)
(file:///C:/Users/Administrator/Downloads/Makwan_CS2.pdf) (A-C)
(http://www.pralanna.com/img/id/2dsc06044.jpg) (D)
(http://4.bp.blogspot.com/24vFSw1fQ18/T7OJulAoNaI/AAAAAAAAALw/zCqal7_6UiU/s1600/Rutaceae.JPG) (E)
(Date: November 13, 2014)

C. 3 Composition of essential oil

Itthipanichpong et al. (2002) reported that limonene (31.09%), terpin-4-ol (13.94%) and sabinene (9.13%) were major components of *Z. limonella* Alston dried fruit.

C. 4 Benefits

Z. limonella Alston plants have been traditionally used in food; especially ripe fruits have been commercialized in local markets as a popular spice (Tangjitjaroenkun et al., 2012). Its roots, stem-barks, stems and fruits are used for treating stomachache and toothache (Tangjitjaroenkun et al., 2012). The essential oil from fruits affects the gastrointestinal system, exhibit stimulation effect on different smooth muscles by non-specific mechanism and antioxidant activity (Itthipanichpong et al., 2002; Tangjitjaroenkun et al., 2012). Charoenying et al., (2008) reported that the crude chloroform extract exhibited antimalarial activity against *Plasmodium falciparu*m and antituberculous activity against *Mycobacterium tuberculosis* H37 Ra.

APPENDIX D

TABLES OF RAW DATA

Table D.1 Diameter (cm) of *Aspergillus flavus* colony grown in agar medium supplemented with the essential oil from *Anethum graveolens* L. at five different concentrations*

Concentration		Day								
		1	2	3	4	5	6	7	8	9
0	1	1.23	2.33	3.76	4.46	5.90	6.94	7.70	8.36	8.44
	2	1.23	2.34	3.75	4.43	5.85	6.94	7.70	8.20	8.50
	3	1.23	2.35	3.77	4.54	5.88	6.94	7.67	8.10	8.50
	av	1.23±0.00	2.33±0.01	3.76±0.01	4.48±0.06	5.88±0.03	6.94±0.00	7.69±0.02	8.22±0.13	8.48±0.03
0.25	1	0.64	1.10	1.79	2.17	2.20	2.32	2.32	2.32	2.35
	2	0.42	0.76	1.35	1.74	2.31	2.42	2.43	2.45	2.45
	3	0.59	1.00	1.65	2.00	2.48	2.50	2.5	2.51	2.51
	av	0.55±0.12	0.95±0.17	1.60±0.22	1.97±0.22	2.33±0.14	2.41±0.09	2.42±0.09	2.43±0.01	2.45±0.08

Concentration		Day								
		1	2	3	4	5	6	7	8	9
0.50	1	0.40	0.64	1.15	1.42	1.82	1.90	1.99	2.01	2.08
	2	0.40	0.61	1.10	1.44	1.53	1.53	1.59	1.59	1.59
	3	0.40	0.83	1.45	1.95	2.44	2.50	2.58	2.58	2.60
	av	0.40±0.00	0.69±0.12	1.23±0.19	1.60±0.30	1.93±0.46	1.98±0.49	2.05±0.50	2.06±0.50	2.09±0.51
1.0	1	0.40	0.40	0.40	0.54	0.73	0.85	0.96	1.07	1.05
	2	0.40	0.40	0.40	0.57	0.67	0.82	0.94	1.00	1.08
	3	0.40	0.40	0.40	0.57	0.73	0.84	0.91	1.02	1.21
	av	0.40±0.00	0.40±0.00	0.40±0.00	0.56±0.02	0.71±0.03	0.84±0.02	0.94±0.03	1.03±0.03	1.11±0.09
1.5	1	0.40	0.40	0.40	0.40	0.40	0.40	0.55	0.66	0.71
	2	0.40	0.40	0.40	0.40	0.40	0.40	0.63	0.65	0.68
	3	0.40	0.40	0.40	0.40	0.40	0.40	0.53	0.58	0.64
	av	0.40±0.00	0.40±0.00	0.40±0.00	0.40±0.00	0.40±0.00	0.40±0.00	0.57±0.05	0.63±0.04	0.68±0.04
2.0	1	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
	2	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
	3	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
	av	0.40±0.00	0.40±0.00	0.40±0.00	0.40±0.00	0.40±0.00	0.40±0.00	0.40±0.00	0.40±0.00	0.40±0.00

*Result presented in 3.3.1

Table D.2 Diameter (cm) of *Aspergillus flavus* colony grown in agar medium supplemented with the essential oil from *Zanthoxylum piperitum* at eight different concentrations*

Concentration (µL/mL)		Day								
		1	2	3	4	5	6	7	8	9
0	1	1.23	2.33	3.76	4.46	5.90	6.94	7.70	8.36	8.44
	2	1.23	2.34	3.75	4.43	5.85	6.94	7.70	8.20	8.50
	3	1.23	2.35	3.77	4.54	5.88	6.94	7.67	8.10	8.50
	av	1.23±0.00	2.33±0.01	3.76±0.01	4.48±0.06	5.88±0.03	6.94±0.00	7.69±0.02	8.22±0.13	8.48±0.03
1.0	1	0.40	0.85	1.44	1.83	2.39	3.0	3.65	3.90	4.45
	2	0.40	0.83	1.44	1.84	2.38	2.98	3.63	4.16	4.64
	3	0.40	0.83	1.45	1.85	2.48	3.03	3.60	4.26	4.55
	av	0.40±0.00	0.84±0.01	1.44±0.01	1.84±0.01	2.42±0.06	3.00±0.03	3.63±0.03	4.11±0.19	4.55±0.01
1.5	1	0.40	0.60	0.89	1.00	1.60	2.04	2.65	3.00	3.36
	2	0.40	0.64	0.87	1.19	1.55	2.01	2.68	2.94	3.33
	3	0.40	0.57	0.82	1.07	1.40	1.95	2.44	2.54	2.89
	av	0.40±0.00	0.60±0.04	0.86±0.04	1.15±0.07	1.52±0.10	2.00±0.05	2.59±0.13	2.83±0.25	3.18±0.26

Concentration (µL/mL)		Day								
		1	2	3	4	5	6	7	8	9
2.0	1	0.40	0.40	0.51	0.75	0.95	1.18	1.53	1.82	1.87
	2	0.40	0.40	0.54	0.71	0.82	0.99	1.38	1.51	1.74
	3	0.40	0.40	0.60	0.78	1.00	1.23	1.61	1.82	2.02
	av	0.40±0.00	0.40±0.00	0.55±0.04	0.75±0.05	0.92±0.09	1.13±0.13	1.51±0.12	1.72±0.18	1.88±0.14
2.5	1	0.40	0.40	0.40	0.68	0.71	0.83	1.07	1.13	1.32
	2	0.40	0.40	0.40	0.67	0.80	0.91	1.17	1.30	1.52
	3	0.40	0.40	0.40	0.68	0.82	1.02	1.24	1.36	1.53
	av	0.40±0.00	0.40±0.00	0.40±0.00	0.68±0.01	0.78±0.06	0.92±0.10	1.16±0.09	1.26±0.12	1.46±0.12
3.0	1	0.40	0.40	0.40	0.40	0.40	0.76	0.89	0.98	1.09
	2	0.40	0.40	0.40	0.40	0.40	0.77	0.81	1.06	1.21
	3	0.40	0.40	0.40	0.40	0.40	0.71	0.88	0.98	1.14
	av	0.40±0.00	0.40±0.00	0.40±0.00	0.40±0.00	0.40±0.00	0.75±0.03	0.89±0.02	1.01±0.05	1.15±0.06
3.5	1	0.40	0.40	0.48	0.56	0.59	0.60	0.62	0.63	0.73
	2	0.40	0.40	0.42	0.54	0.63	0.70	0.71	0.86	1.05
	3	0.40	0.40	0.44	0.56	0.60	0.60	0.64	0.89	1.02
	av	0.40±0.00	0.40±0.00	0.45±0.03	0.55±0.01	0.62±0.02	0.65±0.05	0.68±0.04	0.73±0.12	0.93±0.18

Concentration (µL/mL)		Day								
		1	2	3	4	5	6	7	8	9
4.0	1	0.40	0.40	0.42	0.52	0.53	0.55	0.55	0.64	0.69
	2	0.40	0.40	0.44	0.53	0.54	0.60	0.60	0.71	0.82
	3	0.40	0.40	0.41	0.53	0.57	0.62	0.62	0.66	0.77
	av	0.40±0.00	0.40±0.00	0.42±0.02	0.53±0.01	0.55±0.02	0.59±0.04	0.59±0.03	0.67±0.04	0.76±0.07
4.5	1	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
	2	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
	3	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
	av	0.40±0.00	0.40±0.00	0.40±0.00	0.40±0.00	0.40±0.00	0.40±0.00	0.40±0.00	0.40±0.00	0.40±0.00

*Result presented in 3.3.2

Table D.3 Effect of concentrations of *Anethum graveolens* L. at 0.25 µL/mL and *Zanthoxylum piperitum* at 1 µL/mL on ergosterol content in plasma membrane of *A.flavus* in PDB for 4 days

Sample	Filter paper	Filter paper + Pellet	wet	A_{230}	A_{282}**	% Ergosterol content
Control 1	0.5571	10.0781	9.5210	1.0414	1.0250	0.25 ± 0.03^{a}
Control 2	0.5613	11.0498	10.4885	1.0631	1.0562	
Control 3	0.5562	9.2227	8.6665	0.9056	1.0315	
A. graveolens 1	0.5818	5.3261	4.7443	0.5776	0.6304	0.17 ± 0.01^{b}
A. graveolens 2	0.5534	4.3283	3.7749	0.5730	0.5102	
A. graveolens 3	0.5488	5.1947	4.6459	0.5734	0.6346	
Z. piperitum 1	0.5504	4.6113	4.0609	0.4705	0.4775	0.18 ± 0.02^{b}
Z. piperitum 2	0.5691	4.1480	3.5789	0.6119	0.5122	
Z. piperitum 3	0.5521	4.0624	3.5103	0.5223	0.5131	

*Result presented in 3.6.1

**Control dilution $=10^{6}$, *A. graveolens* and *Z. piperitum* dilution $=10^{4}$

Values are mean (n = 3) ± standard deviations. Values followed by the same letter in each column are not significantly different in ANOVA and Duncan Multiple Range Test ($p < 0.05$).

Table D.4 Efficacy of essential oil in the conservation of Bird Chilli, a model product after 9 days of incubation*

Sample	Number Spore				% Inhibition
	1	2	3	av	
Control	37	21	17	2.50×10^{6a}	-
A. graveolens MIC	4	6	2	4.00×10^{5b}	84.00
A. graveolens 2XMIC	2	1	2	1.67×10^{5b}	93.32
Z. piperitum MIC	10	10	19	1.30×10^{6a}	48.00
Z. piperitum 2XMIC	4	2	2	2.67×10^{5b}	89.32

*Result presented in 3.7.1

Values are mean (n = 3) ± standard deviations. Values followed by the same letter in each column are not significantly different in ANOVA and Duncan Multiple Range Test ($p < 0.05$).

APPENDIX E

Mass spectra and the structures of main components of the essential oils

The mass spectra of pure componds are presentd in order to be compared with the result in section 3.2. Their structures are also documented [W8N05ST (Wiley ver.8.0)].

E 1. The major component of *Anethum graveolens* L. essential oil

1.1 (-)-Limonene

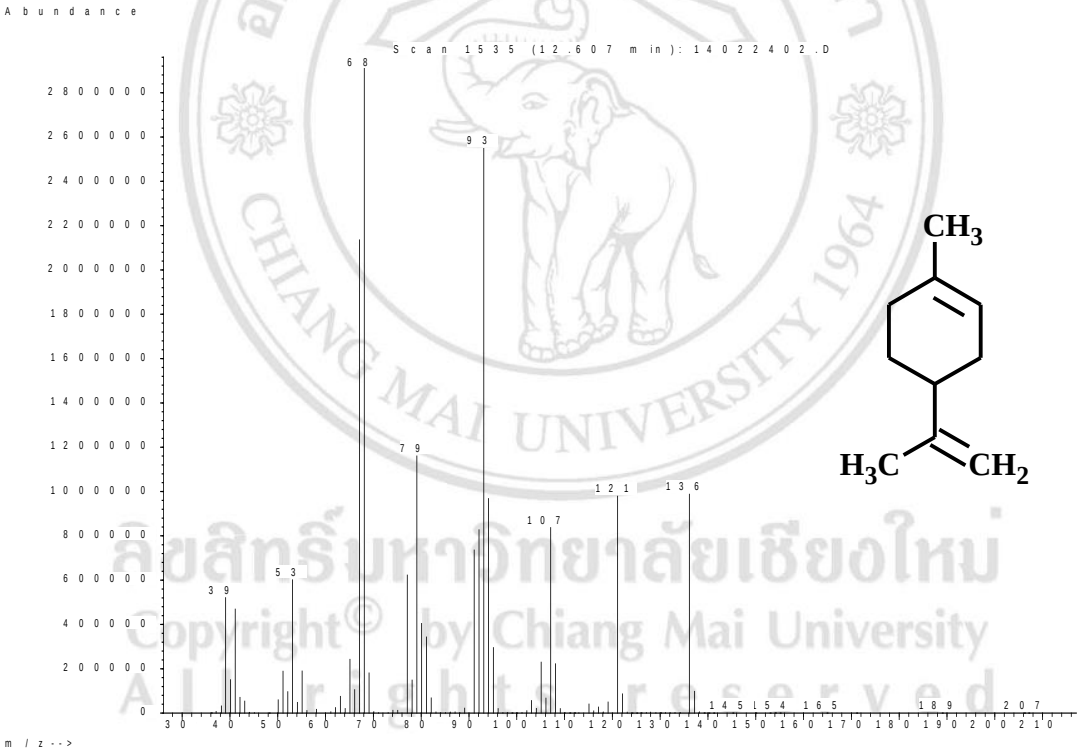


Figure E.1.1 The mass spectrum and structure of (-)-limonene

1.2 Dihydrocarvone

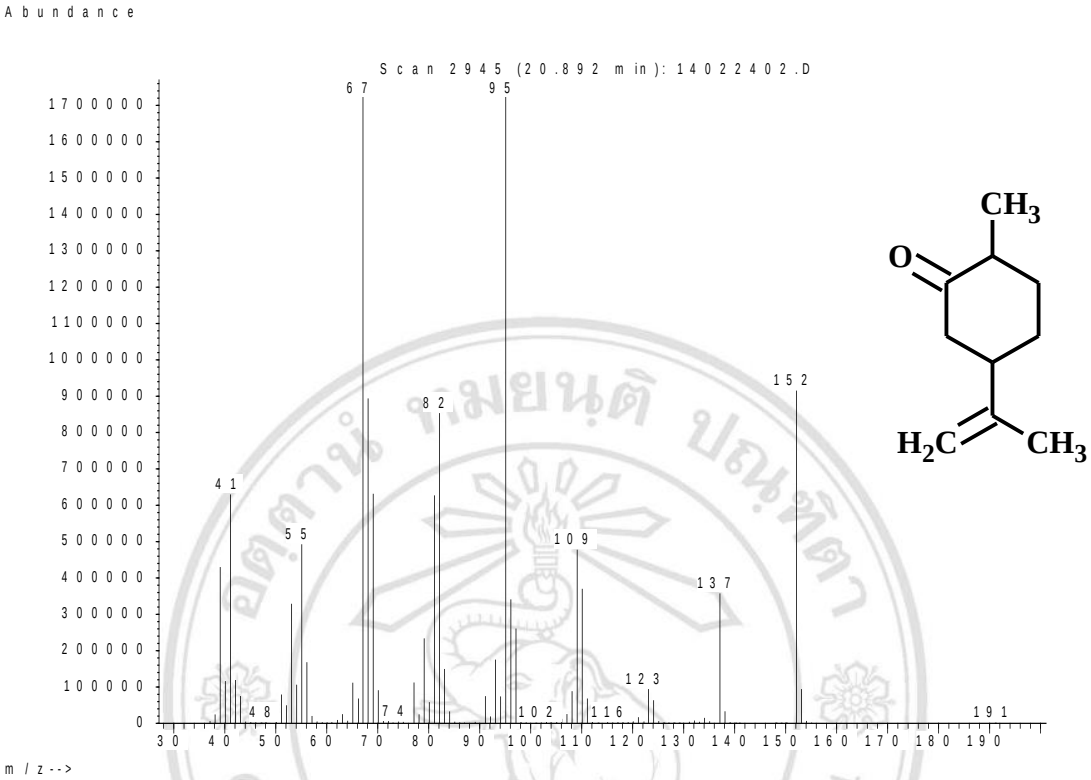


Figure E.1.2 The mass spectrum and structure of dihydrocarvone

1.3 (+)-Carvone

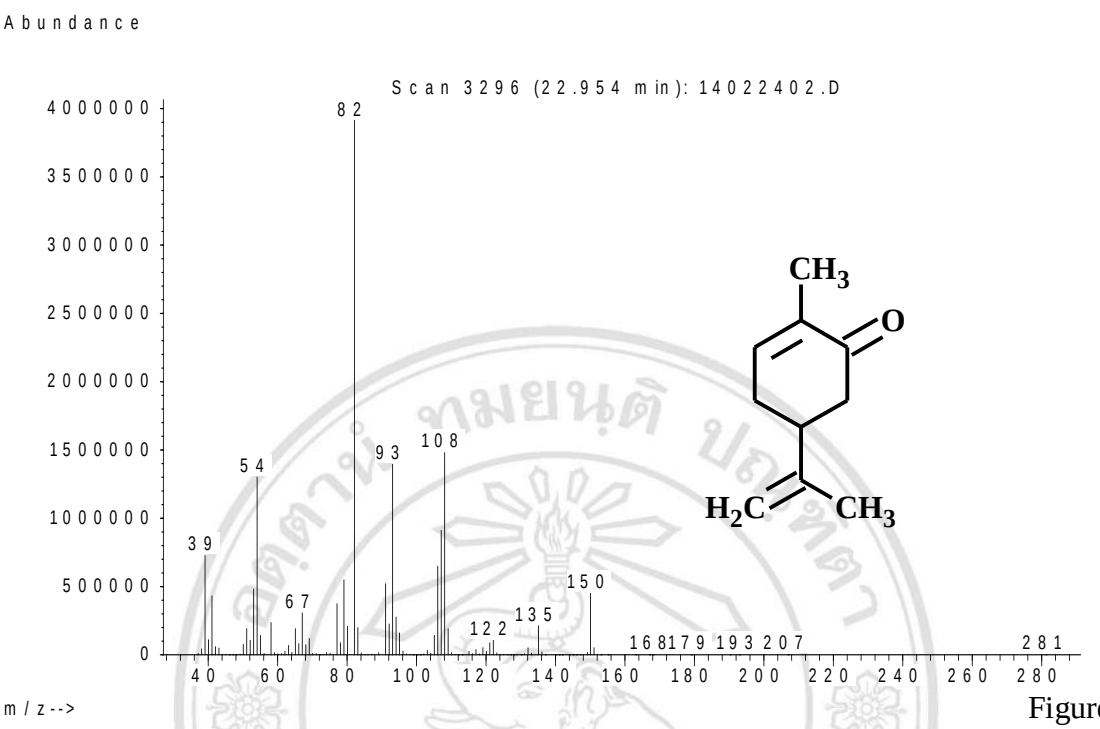


Figure E.1.3 The mass spectrum and structure of (+)-carvone

1.4 *trans*-Isodillapiole

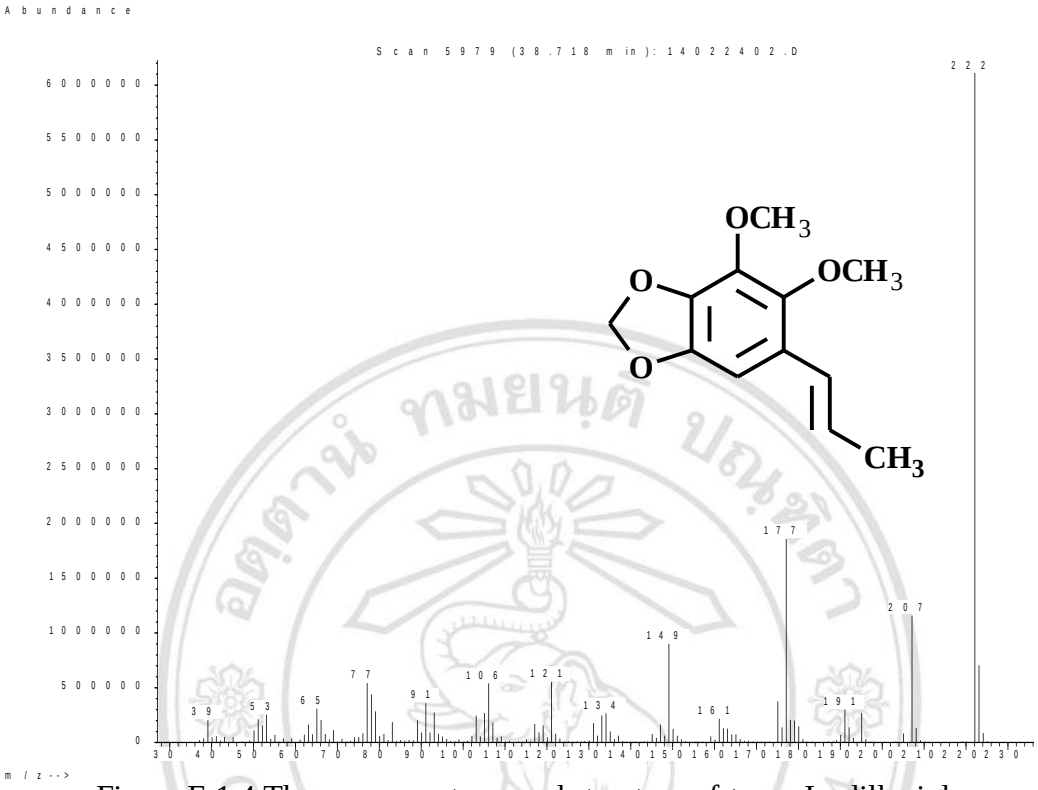


Figure E.1.4 The mass spectrum and structure of *trans*-Isodillapiole

E 2. The major component of *Zanthoxylum piperitum* essential oil

2.1 (+)-Sabinene

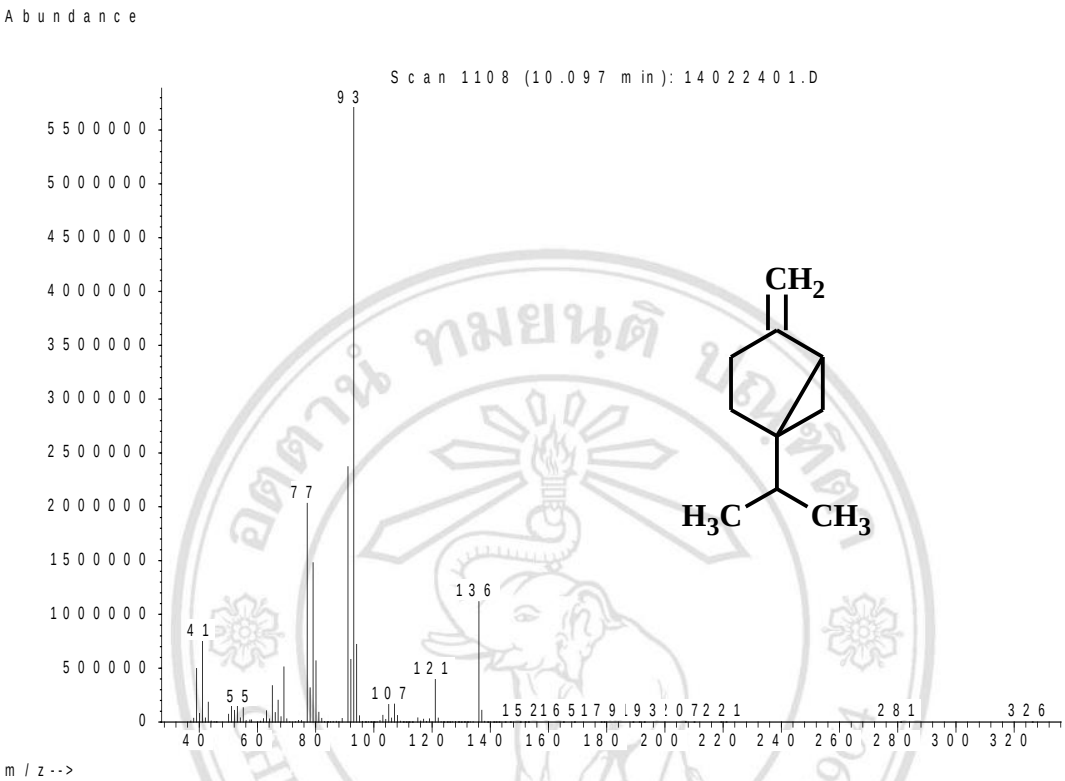


Figure E. 2.1 The mass spectrum and structure of (+)-sabinene

2.2 β-Phellandrene

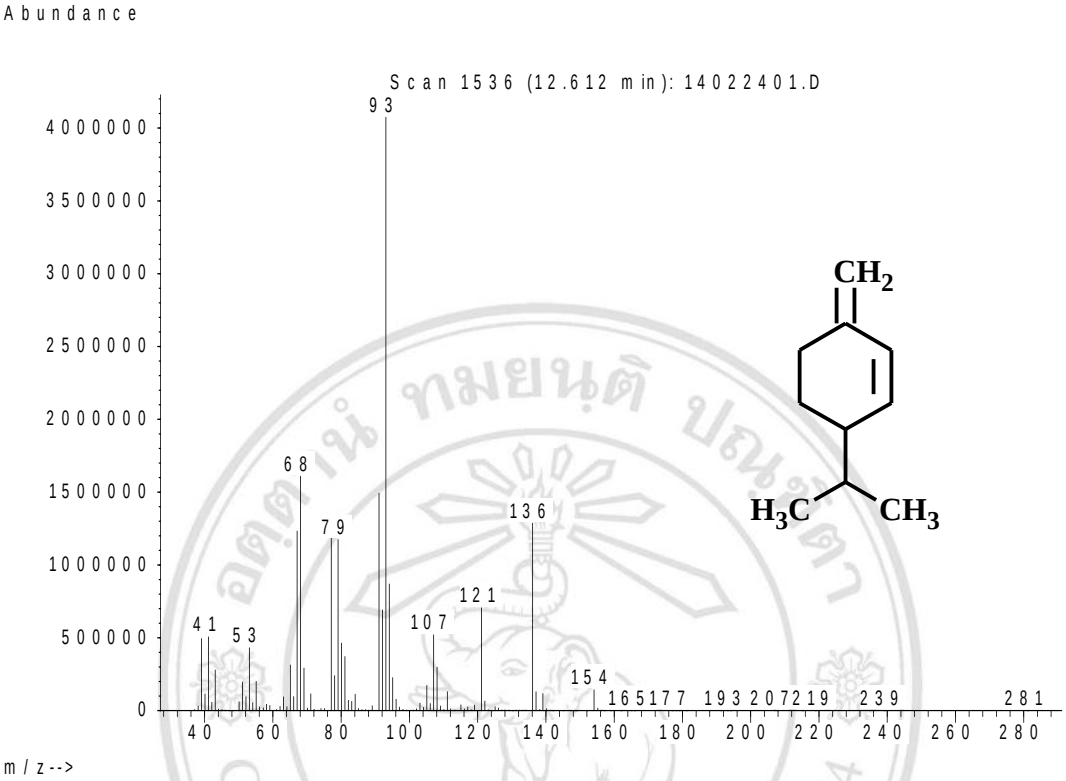


Figure E. 2.2 The mass spectrum and structure of β-phellandrene

2.3 Brevifolin

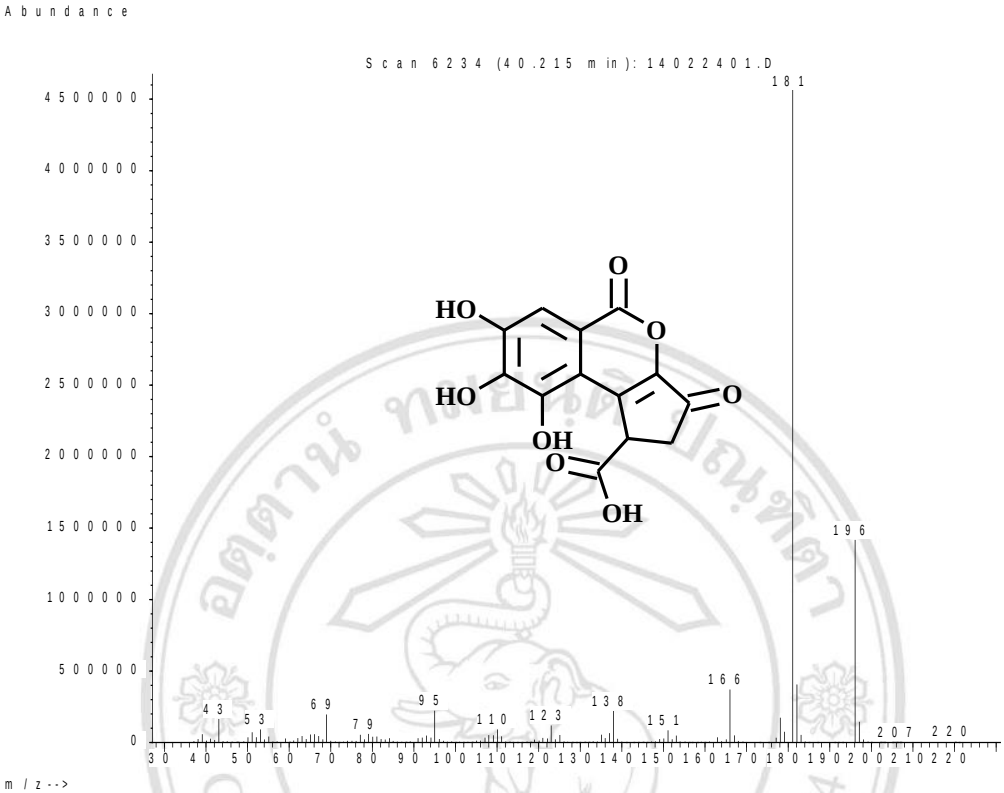
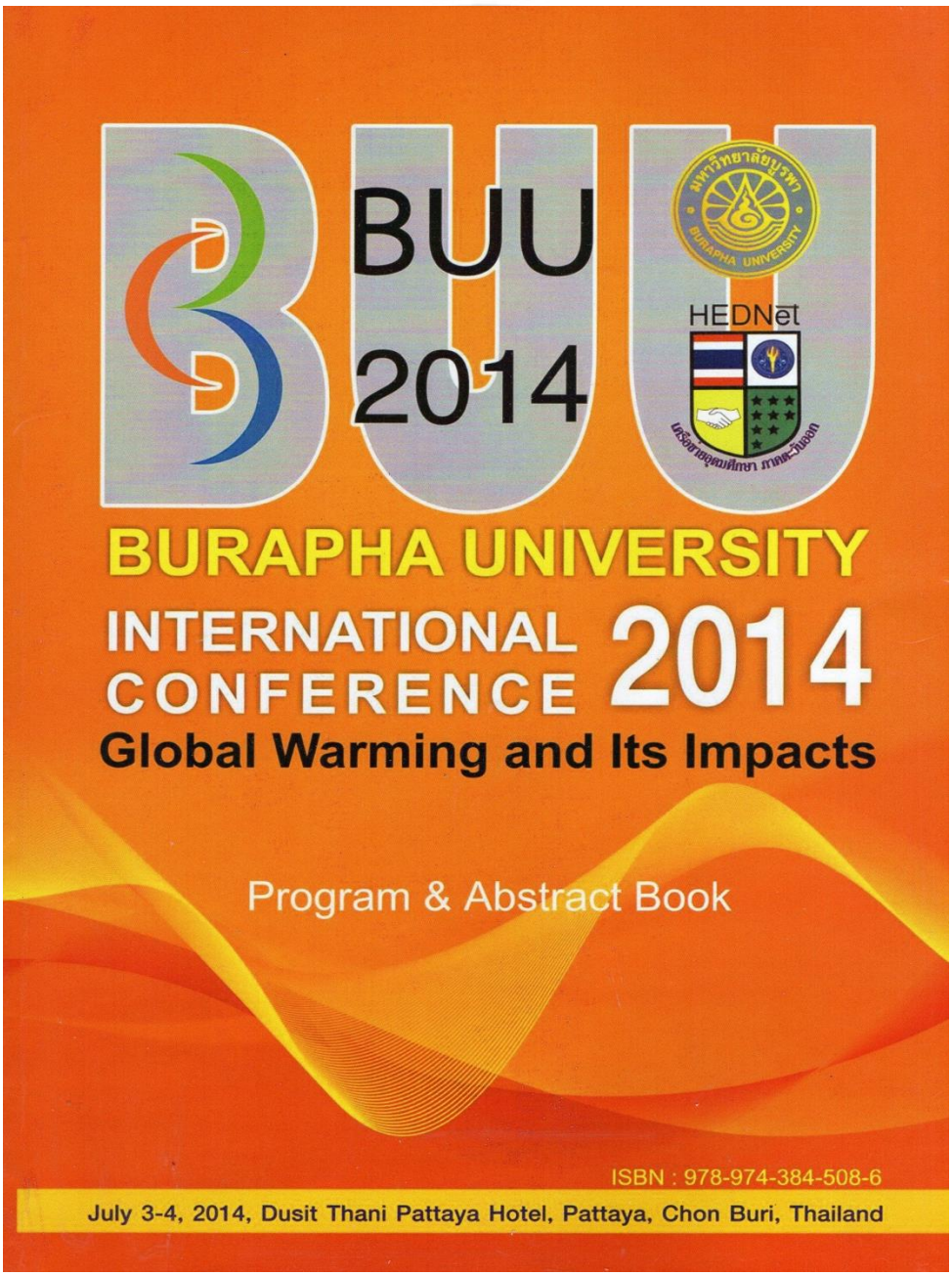


Figure E. 2.3 The mass spectrum and structure of brevifolin

PUBLICATION

Phuangsri C., Nuntawan N., Niamsup H. Antifungal Activity of Essential oils from Some Spices Against *Aspergillus flavus*. *Burapha University International Conference 2014*. 2014; 424-429.



Burapha University International Conference 2014
Burapha University, Thailand
July 3-4, 2014

STP652-16

Antifungal Activity of Essential oils from Some Spices Against *Aspergillus flavus*

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ABSTRACT

The antifungal activities of essential oil from four spices, *Anethum graveolens* L. (Dill), *Foeniculum vulgare* Mill. (Fennel), *Coriandrum sativum* (Coriander), *Zanthoxylum* spp. (Prikhom), against *Aspergillus flavus* isolated from bird chili powder were investigated. The preliminary study on antifungal activity was evaluated at a concentration of 1µl/ml by poisoned food technique in solid culture. *A. graveolens* L. oil exhibited highly effective antifungal activity, thus *A. graveolens* L. was selected to determine the minimal inhibitory concentration (MIC) using the concentrations of the essential oil between 0.25 and 2 µl/ml. The wet and dry mycelial weights of the treated fungus were also determined in a liquid culture. The minimum inhibitory concentration of *A. graveolens* L. for the tested fungus was found to be 2.0 µl/ml on 9th day of the mycelial growth. Thus, the essential oil of *A. graveolens* L. could be potentially used as an alternative food preservative against spoilage caused by the fungus.

Keywords: *Anethum graveolens* L., Antifungal, Essential oil, Minimal Inhibitory Concentration (MIC), Spices

INTRODUCTION

Contamination of spoilage microorganism in food has been a serious problem in food industries and consumers. Food is easily attacked by various microorganisms such as *Aspergillus* species leading to significant economic loss. Some species introduce a very serious risk for consumers because of the dangerous secondary metabolites they produce in crops (Tian et al., 2012). In particular, *Aspergillus flavus* is mainly responsible for spoilage in many foods and production of aflatoxins (Beyki et al., 2014). Aflatoxins are widely known for carcinogenic, teratogenic, hepatotoxic, mutagenic, and immunosuppressive properties, and can inhibit several metabolic systems (Joseph et al., 2005). High moisture content and high temperature are the major factors for the growth of pathogenic fungi and the production of the toxin in many countries, especially in tropical and subtropical regions (Chun et al., 2006). The use of synthetic preservatives or antimicrobial agents to control fungal spoilage of food has led to a number of environmental and health problems. Consumers and food processors concern about using synthetic preservative and require new self-preservative that are biodegradable and environmental friendly. Various plant products have been recognized and used for food preservation and in medicine because of their antifungal properties. Spices are the tropical

Burapha University International Conference 2014 (BUU2014)
July 3-4, 2014, Dusit Thani Pattaya, Pattaya, Thailand

ACCEPTANCE LETTER

June 3, 2014

Dear Chorpaka Phuangsri,

On behalf of the organizing committee, I am glad to inform that your manuscript entitled **"Antifungal Activity of Essential oils from Some Spices Against *Aspergillus flavus*"** has been accepted for **poster** presentation at the Burapha University International Conference held in Dusit Thani Pattaya, Pattaya, Thailand, on July 3-4, 2014. Your manuscript will be published in peer-reviewed proceedings. Acceptance of your contribution carries with it the obligation for at least one of the authors to actually present it at the meeting. Details on registration form, methods of payment, author guidelines, important dates and other practical issues can be found on the conference website www.buu2014.buu.ac.th.

You must access the http://www.buu2014.buu.ac.th/?q=node/27 and attach the "Scanned Bank Transfer Slip" together as soon as possible. Please note that if you did not attach the slip at the same time as you fill in the registration form, please fill out the new registration form again.

Make sure to register for the conference as soon as possible to confirm your participation. Registration must be completed before June 10, 2014.

Your contribution will be of great value to the success of this event. We are looking forward to seeing you at the conference.

Kind regards,

Assist. Prof. Somtawin Jaritkhuan, Ph.D.
General Co-chair
Email: buu2014@buu.ac.th

Burapha University International Conference
"Global Warming and Its Impacts"
July 3-4, 2014, Pattaya, Thailand

Best Poster Award

to

Chorpaka Phuangsri

for the paper entitled

Antifungal Activity of Essential oils from Some Spices Against Aspergillus flavus

Professor Sompol Pongthai, FRTCOG, MPH, LLB
Acting President of Burapha University
Chairman of the Universities in Higher Education Development Eastern Network

บัณฑิตวิทยาลัย มหาวิทยาลัยเชียงใหม่

เกียรติบัตรฉบับนี้ให้ไว้เพื่อแสดงว่า

นางสาวช่อผกา พวงศรี

สหสาขาวิชาเทคโนโลยีชีวภาพ

เป็นผู้สร้างชื่อเสียงและเป็นแบบอย่างที่ดีงามแก่นักศึกษาสหสาขาวิชา ประจำปีการศึกษา ๒๕๕๕-๒๕๕๖

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(รองศาสตราจารย์ ดร.เอกชัย แสงอินทร์)
คณบดีบัณฑิตวิทยาลัย มหาวิทยาลัยเชียงใหม่

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Academic Performance

I received best poster presentation award in Burapha University International Conference 2014.