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.

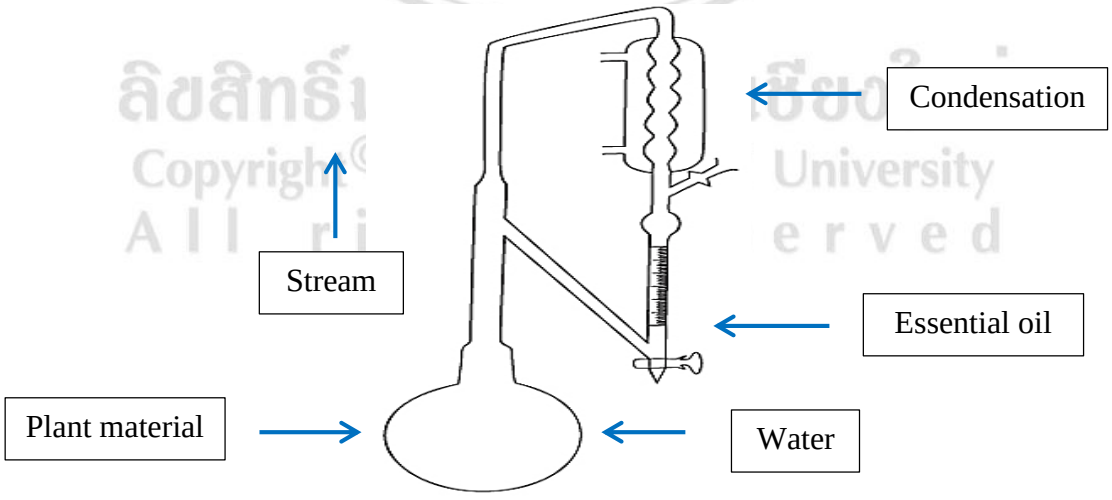

Figure 2.3 Clevenger-type apparatus

(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.

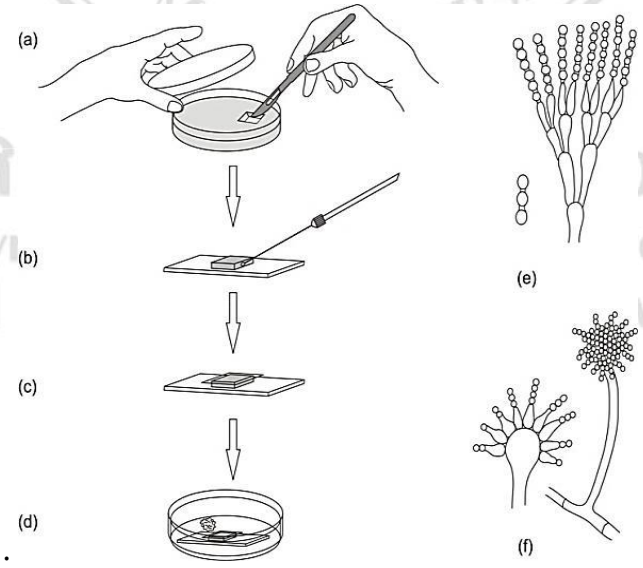

Figure 2.5 Slide Culture Method

(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).