CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

3.1.1 Plants

Traditional plants expected to have an ability against fungal which grow on shallot and onion were used in this experiment. Garlic (*Allium sarivum* L.), galangal (*Alpinia nigra* B.L.), ginger (*Zingiber officinale*), lemon grass (*Cymbopogon citratus*) and onion (*Allium cepa* L.) were purchased from local market in Chiang Mai province. Freshly harvested shallot (*Allium ascalonicum*) was purchased from Lamphun province.

The investigation in both *in vitro* and *in vivo* test, aqueous extraction used fresh plant, in the case of ethanolic extraction and methanolic extraction used dry plant.

3.1.2 Culture medium

Potato dextrose agar (PDA) medium containing potato 200 g, dextrose 20 g, agar 15 g and distilled water 1,000 mL (Paterson and Bridge, 1994) was used in this experiment.

3.1.3 Media and chemical reagents

Name of chemical reagents	Production company
Agar	-
Clorox	-
95% Ethanol	MERCK
Glucose	MERCK

Name of chemical reagents	Production company
Methanol	MERCK
Tween 80	LABCHEM

3.1.4 Equipments

Name of equipments	Production company
Analytical balance (2 digit)	OERTING
Autoclave Model ACV-3167	IWAKI
Autopipette	GIBTHAI
Blender EM-11	SHARP
Centrifunge Model Harmonic series	GEMMY
Filter holder	SARTORIOUS
Haemacytometer Precicolor	HBG GERMANY
Hood	TOPLAB
Hot air oven	MEMMERT
Laminar flow cabinet	LABCONCO
Membrane (0.2 µm pore size)	SARTORIUS AG
Membrane (No.1 and No.4)	WHATMAN
Microscope	OLYMPUS
Refrigerated centrifuge Model Super T21	SORVELL
Rotary Evaporater	EYELA
Vortex Mixer-2 Genie	BOHEMIA
Water bath Model 1225 PC	SHEL-LAB

3.2 Methods

3.2.1 Screening of pathogenic fungi from shallot and onion

In order to screen plant pathogenic fungi from sheath of rotting shallot and onion, the following method were done.

The sheath of shallot and onion was shelled and soaked in the Clorox solution for 1 min. After that, it was put on PDA medium plate. The plate was then incubated at room temperature (28-32ºC) for 3 days. The fungi grow on PDA plate were isolated and purified by point method. The isolated fungal were kept on PDA slant at 4ºC for further studies.

3.2.2 Preparation of spore suspension

The isolated fungal were cultured on PDA plate and incubated at room temperature for 3-5 days until sporulation. Ten mL of 0.1% Tween 80 was added for spore collection. Spore were harvested by centrifugation at 1000×*g* for 15 min and then washed with 10 mL sterile distilled water. This step was repeated three times. After that, spores were suspended in sterile distilled water and stored at 4ºC. Finally, spore counting was done by using a haemacytometer and used for further experiments.

3.2.3 Preparation of plant extracts

Traditional plants including garlic, galangal, ginger, lemon grass, shallot and onion were selected to test activity against fungal isolated from sheath of rotting shallot and onion as mention above. In this experiment distilled water, 95% ethanol and methanol were used as a solvent and the extraction were done as following method.

3.2.3.1 Aqueous fresh plant extraction

One hundred gram of each fresh plant was blended with 100 mL distilled water in a blender. After blending, the mixture was filtered by a cotton cloth. Then, the solution was centrifuged at 5000×*g* for 15 min at 30ºC. Supernatant was collected and sterilized with the cellulose acetate filter (0.2 µm pore size). The crude extract was kept at 4ºC as a stock solution.

3.2.3.2 Ethanolic dry plant extraction

Extraction method was modified from the procedure described by Hsieh *et al.* (2001). Ten grams of each dry plant was extracted with 10 mL of 95% ethanol in a water bath at 50ºC for 1 h and filtered by a cotton cloth. Then, the residue was re-extracted. The combined filtrate was centrifuged at 5000×*g* for 15 min at 30ºC. After that, the supernatant was concentrated on a rotary evaporater at 45ºC. Finally, volume was made up to 10 mL with 0.1% Tween 80 solution and sterilized with the cellulose acetate filter (0.2 µm pore size). The crude extract was kept at 4ºC as a stock solution.

3.2.3.3 Methanolic dry plant extraction

Methanolic plant extraction was done according to Nair *et al*. (2005). Ten grams of each dry plant was placed in 100 mL of methanol in flask and plugged with cotton. The mixture was shaken at 200 rpm for 24 h. Then, it was filtered through a cotton cloth and centrifuged at 5000×*g*, 30ºC for 15 min. The supernatant was collected, and the solution was evaporated on a rotary evaporater at 45ºC to make the final volume one-fourth of the original volume. The solution was sterilized with the cellulose acetate filter (0.2 µm pore size). Finally, the crude extract was kept at 4ºC as a stock solution.

3.2.4 Antifungal activity test

The agar well method was modified from the procedure described by Valsaraj *et al*. (1997). One hundred microliters of the spore suspension from each isolate (approximately 10^6 spores/mL) was spreaded on PDA plate and incubated at room temperature for 30 min. The center of each PDA plate was then bored by a sterile cork borer (7 mm diameter). After that, 100 µL of each crude extract was added to the well. For the control, sterile distilled water, and methanol were used to substitute aqueous plant extract, methanolic plant extract, respectively. Ethanol and 0.1% Tween 80 solution were used as control for ethanolic plant extract. The plate was

incubated at room temperature for 5 days. Antifungal activity was daily observed by measuring diameter of inhibition zone. The plate which had inhibition zone was incubated for a month to monitor the stability of plant extract. The value was recorded as the mean diameter of triplicates.

3.2.5 Study of plant extract concentration and antifungal activity

The plant extract from each extraction method which showed highest antifungal activity (aqueous fresh garlic extract, ethanolic dry galangal extract, methanolic dry galangal and methanolic dry ginger extract) were selected for testing of their ability in different dilution against fungal growth. Plant extracts were diluted to 2, 5 and 10 fold with sterile water, ethanol and methanol, separately before using for antifungal activity test by well method as described in 3.2.4.

3.2.6 Effect of plant extracts on fungal inoculated shallot and onion

In the *in vitro* study on the effect of plant extract concentration on antifungal activity, the crude plant extract showed highest antifungal activity. In this experiment, aqueous fresh garlic extract, ethanolic dry galangal extract, methanolic dry galangal and methanolic dry ginger extract were investigated their ability against fungal growth in *in vivo* test.

In vivo test of the extracts on disease controlling was modified from the procedure described by Obagwu and Korsten (2003). The freshly harvested shallot was purchased from Lamphun province and onion was purchased from a local market in Chiang Mai province. Leave and root were cut from shallot and onion then they were sterilized by soaking in 95% ethanol for 30 second. Shallot and onion (0.5 kg per group) were inoculated by immerse needle in 10^6 spores/mL spore suspension of each isolated and wounding on the surface (in three positions with 1 mm width and 2 mm depth). After that, they were incubated at room temperature for 2 hours. Each group of shallot and onion were dipped in each plant extract for 30 second and allowed to dry. Distilled water, ethanol and methanol were used as the control. Finally, they were incubated at room temperature. The bulb disease assessment (BDA)

was determined by counting the number of bulbs which showed fungal growth (Ke-Qiang and Bruggen, 2001). The inhibition effect represent in the term of percentage of inhibition was calculated as the following equation:

$$\text{Percentage of inhibition (\%)} = \frac{\text{No. of BDA (control) - No. of BDA (treatment)}}{\text{No. of BDA (control)}} \times 100$$

Weight loss and color change were also investigated. Percentage of weight loss was calculated by the following equation:

$$\text{Percentage of weight loss (\%)} = \frac{\text{weight loss}}{\text{initial weight}} \times 100$$

3.2.7 Statistical analysis

Data were analyzed by ANOVA (analysis of variance) appropriate for the completely randomized design (CRD). Differences between treatment means were assessed by least significant difference test. Statistical analyses were conducted using the Statistix version 7 (Analytical Software, Inc., Tallahassee, FL, USA). Significance level for the separation of the group means was set at $P<0.05$.