CHAPTER II

MATERIALS AND METHODS

2.1 Propolis sample

Propolis sample was collected from the nest of stingless bee *Trigona laeviceps* located at Roi Et province in the North-East of Thailand.

2.2 Chemicals

Merck's TLC was performed on aluminium sheets precoated with silica gel 60 F254 (20 x 20 cm, layer thickness 0.2 mm) for qualitative analysis and bioautographic assays. Adsorbents such as silica gel (Kieselgel 60, Merck) No.7731 and No.7734 were used for quick column chromatography and open column chromatography, respectively. All organic solvents (hexane (C_6H_{14}), dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc) and methanol (MeOH)) were commercial grade and were redistilled prior to use.

2.3 Equipments

Gas chromatography–mass spectrometry (GC-MS) was carried out on a Hewlett Packard 5890 gas chromatograph coupled to a 5070B Mass Selective Detector. The operation parameters were controlled by a HP series 200 computer with HP59970C Chemstation software. The chromatographic column for the analysis was a fused silica capillary column (length: 29.2 m, diameter: 250.00 um, film thickness: 0.25 um). The carrier gas used was helium at a flow rate of 1 ml/min. All samples were analyzed with the column held initially at 30°C for 2 min and then heated to 250°C at a rate of 11°C /min, and maintained at the upper temperature for up to 43 min. The identification of

the substances was confirmed by comparison of the mass spectra and retention time with those of authentic samples. Mass spectral matches for some compounds (including sugiol) could be found in the Wiley-NBS computer library, version 1994.

2.4 Extraction of propolis for preliminary screening test and separation

Propolis (150 g) (Fig. 2.1) was cut into small pieces and extracted three times with MeOH (3 x 0.5 L) at room temperature for 24 hr. After filtration through a paper filter, the filtrates were combined and the solvent was evaporated *in vacuo* to obtain MeOH crude extract. MeOH extract was dissolved in water and extracted with CH_2Cl_2. The CH_2Cl_2 crude extract was tested for growth inhibitory activity against *Ascosphaera apis*. For a bioassay-guided fractionation and isolation of compounds, the CH_2Cl_2 crude extract was applied in succession to quick column chromatography on silica gel with a hexane/EtOAc/MeOH gradient (0, 20, 30, 40, 60, 80 hexane-EtOAc, 100% EtOAc, 10% MeOH/EtOAc) yielding seven fractions, Scheme 2.1. Each fraction was tested for biological assay.

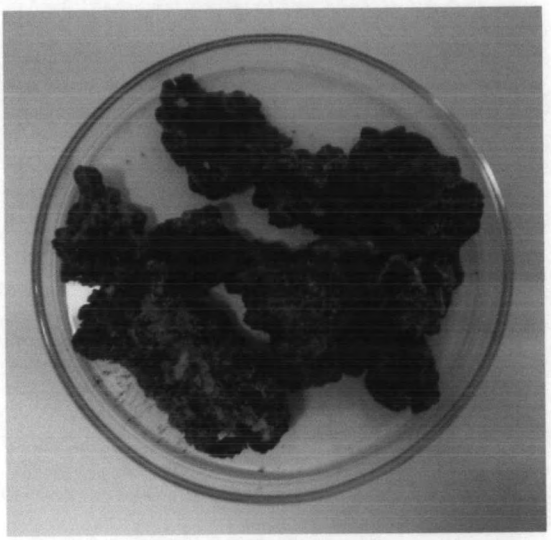
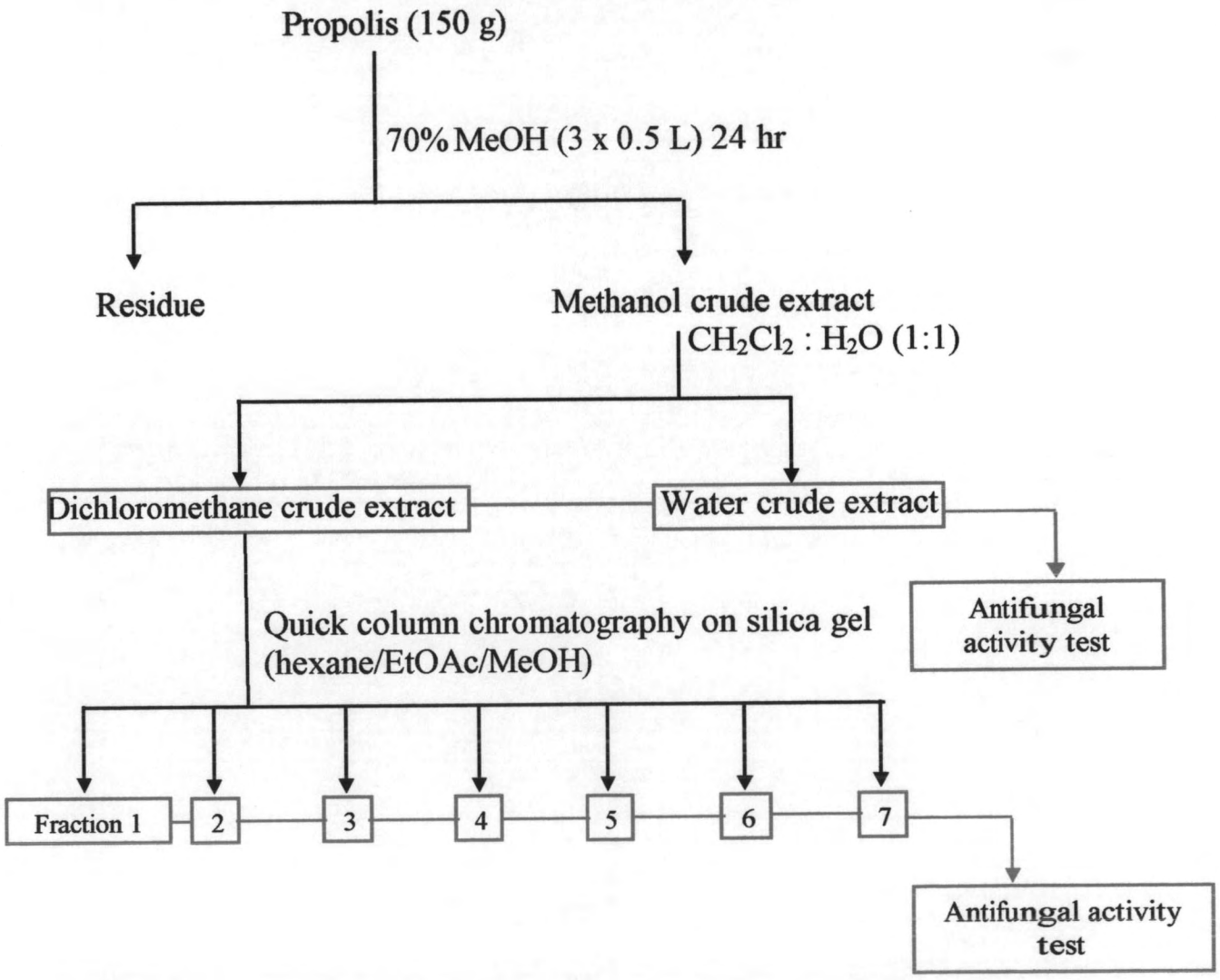


Fig. 2.1 Propolis from the nest of *Trigona laeviceps*.



Scheme 2.1 Extraction and separation for preliminary screening test

2.5 General methods for bioassays

2.5.1 Fungal cultures

An infectious chalkbrood disease causing to *Apis mellifera* isolate of the fungus *Ascosphaera apis* was used as the test microorganism in this experiment. The colony characteristics of the fungi are shown in Fig. 2.2. Fungal cultures were grown on potato dextrose agar (PDA), as formulated in appendix A, at room temperature. Each culture was maintained by subculturing onto a new PDA plate every week.

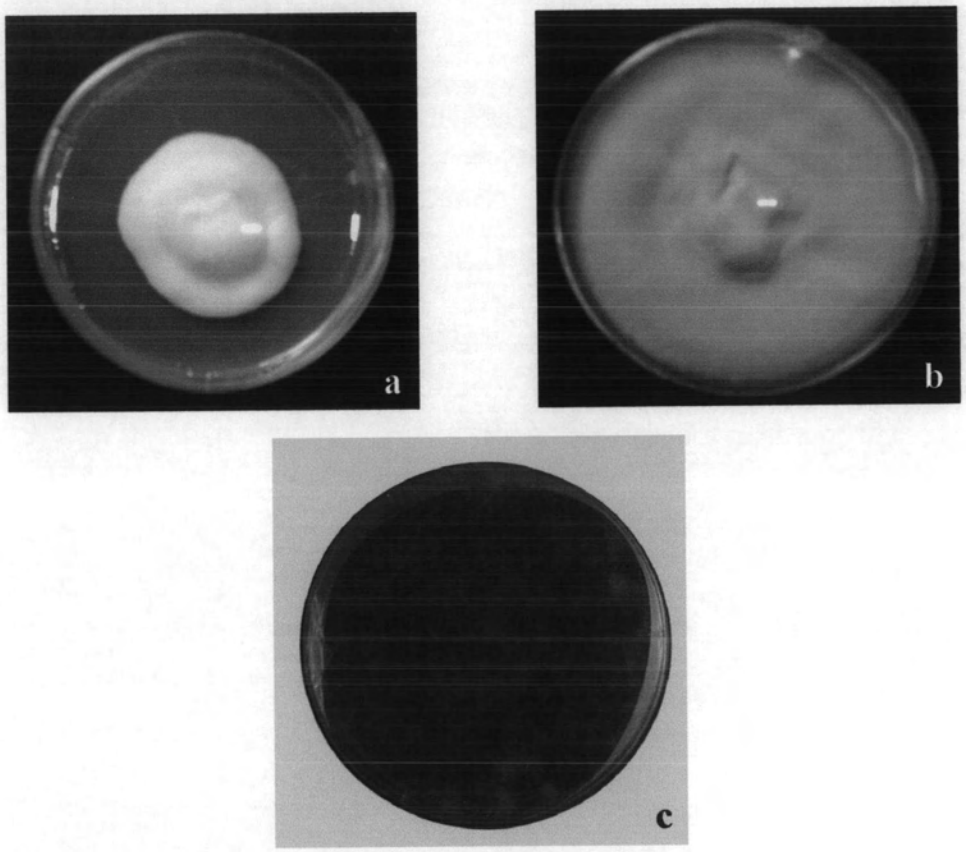


Fig 2.2 The colony characteristics of the *Ascosphaera apis* on PDA used in this research (a, b are characteristics of mycelial at 3 days and 7 days, respectively, and c is characteristic of spore at 7 days)

2.5.2 Conidia suspension preparation

Suspensions of *Ascosphaera apis* conidia were prepared from 7 to 14 day old cultures grown on PDA plates. Plates were flooded with 10 ml of sterile distilled water and the colonies scraped on the agar surface with a glass rod. The suspension was then harvested and filtered through sterile cotton wool to remove mycelia remnants. Conidial concentrations in the suspensions were determined with an improved Neubreumer haemocytometer under phase contrast microscope (400X) (Fig. 2.3) and then adjusted with sterile distilled water to the required concentration prior to conidial germination and TLC bioautographic assays.

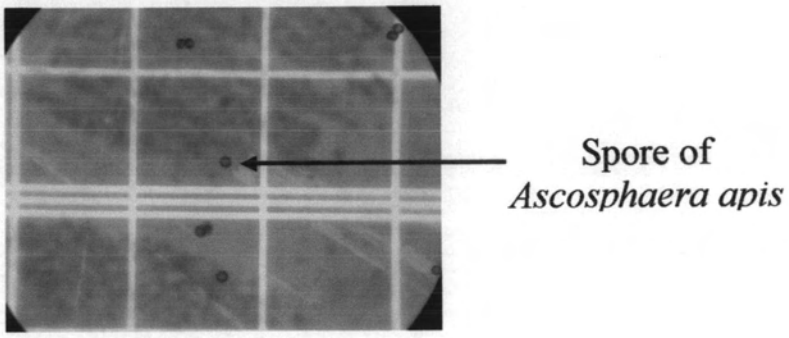


Fig 2.3 The spores of *Ascosphaera. apis* were determined with an improved Neubreumer haemocytometer under phase contrast microscope (400X)

2.6 Bioassays for antifungal activity

2.6.1 Effect of dichloromethane crude extract for antifungal (mycelial) activity

The bioassay was determined by the modified method from agar medium assay (Bhasabutra, 1997) with *Ascosphaera apis*. A testing

sample was dissolved in DMSO which was directly added to PDA at the final concentration: 150, 170, 190, 200, 230, 250, 500 and 1,000 ppm. Control plates contained only PDA. After sterilization at 15 psi and 121°C, the medium was poured into petri plates. A disc (10 mm diameter) from pure culture of *Ascosphaera apis* was aseptically transferred to the center of the petri plate. The plates were incubated at room temperature. Radial measurements of colony size were taken for all plates when the fungi reached the edge of the control plate, and calculated for percent inhibition. All treatments were replicated three times.

$$\text{Percentage to inhibition} = \frac{(C - T) \times 100}{C}$$

C : colony diameter of control plate (cm)

T : colony diameter of treatment (cm)

2.6.2 Effect of dichloromethane crude extract for antifungal (spore) activity

Ascosphaera apis grown on PDA plates for 7 days were used to prepare the conidial suspension and the spore density estimated as described above. Conidia were diluted to approximately 10^3 conidia/ml. Conidia of *Ascosphaera apis* which suspended in potato dextrose broth (PDB), with added crude extract for preliminary antifungal test was dissolved in DMSO at the final concentration of 1,000 ppm. Control tubes contained conidial suspension and PDB. The tubes were incubated at room temperature for 0, 1 and 24 hr, respectively. Then the suspensions were poured into PDA petri plates (10 mm diameter). The plates were incubated at room temperature. After 24 hr colony of spore germination for each plate was observed.

2.6.3 Bioautographic assay

Bioactivity testing on TLC was performed by a modification of standard bioautographic assays (Beom *et al.*, 2000, Wedge *et al.*, 2000). Direct bioautography was used for screening the extract. *Ascosphaera apis* grown on PDA plates for 7 days were used to prepare the conidial suspension and the spore density estimated as described above. Conidia were diluted to approximately 10^3 conidia/ml. Three pieces of TLC were prepared in this experiment. On each TLC, each fraction to be tested was applied and then developed with the appropriate solvent system. After the solvent was completely evaporated, the plate was marked after visualizing the spot position by UV. Conidia of *Ascosphaera apis* which suspended in PDB was sprayed directly onto each piece of the previously developed plate and a fungi suspension was applied to the other piece of the developed plate. Then 10% (v/v) sulfuric acid in ethanol was sprayed, followed by heating to detect those compounds with no UV absorption. After incubation in a moisture chamber for 3 days the plate was stained with 1% (v/v) lactophenol blue in 5% (v/v) acetic acid for 5 minutes and then destained with 5% (v/v) acetic acid for 10 minutes. A clearly visible growth inhibition zone was observed against a blue background to display antifungal activity. All treatments were replicated three times.

2.7 Statistical analysis of data

The concentrations at which the tested compounds caused an inhibition of 50% (IC_{50}) were determined using the following general procedure. The different percent efficacies were transformed into probit. The linear regression was traced by representing the probit in relation to the logarithm of the antifungal concentration. This probit transformation allows

the best correlation for linear regression to be obtained (Chan *et al.*, 1989). From this equation, IC_{50} for fungal activity was calculated (Garrigues *et al.*, 1994).