CHAPTER II

EXPERIMENTAL SECTION

2.1 Plant materials

The dried flowers of *Datura metel* Linn. used in this study were purchased from Cho Kom Per (Plant drug store), Bangkok. This plant was identified by comparison with the herbarium specimen, with vucher specimen No. BK 48905 J.F. Maxwell by a botanist of the Princess Sirindhorn Plant Herbarium building, Ministry of Agriculture and Cooperatives, Bangkok, Thailand.

2.2 Instruments and equipment

The Fourier Transform-Infrared spectrum (FT-IR) was recorded on Nicole impact 410 Spectrophotometer. Spectra of samples were recorded as KBr pellets for solid samples and NaCl cell for liquid samples. The mass spectrum was obtained on Fisson Mass Spectrometer model Trio 2000 at 70 eV. The GC-MS analysis was performed on GC model star 3400Cx and MS model Saturn 4D from Varian. Melting points were determined with a Fisher-John melting point apparatus and are uncorrected. The ^{1}H and ^{13}C-NMR spectra were preformed in deuterated chloroform and otherwise stated with tetramethylsilane (TMS) as an internal reference on Fourier Transform Nuclear Magnetic Resonance Spectrometer of a Bruker, model AC-F200, Avance DPX-400 and Varian, model Mercury-400. The HPLC was performed on RP-18 as stationary phase and MeOH-H_2O, 1:1 was used as mobile phase from Gilson. The chromatotron was conducted on Harrison Research, model 7924T.

Adsorbents used for isolation were silica gel 60 Merck, cat No. 7734, 7749, and 9385 for column chromatography, chromatotron and flash column chromatography, respectively. In addition, Sephadex LH-20/phamacia biotec and Art.1077 Aluminium oxide (Al_2O_3) 90 neutral (70-230 mesh ASTM) were used as adsorbent for column chromatography. Merck's TLC aluminium sheet, silica gel 60

F_{254} precoated was used for qualitative analysis purpose. The spots on the plate were observed under UV light or visualized by spraying with 10% H_2SO_4 in ethanol followed by heating or by dipping with Dragendroff's reagent for determining spot of alkaloid compounds.

2.3 Chemical reagents

All solvents used in this research such as hexane, dichloromethane, ethyl acetate, methanol and ethanol were commercial grade and were purified prior to use by distillation, except for those which were reagent grades.

2.4 Chemical tests

Lieberman-Burchard reaction

This is a general test for steroid or triterpenoid compound. To a solution of the sample to be tested (2-3 mg) in dry chloroform (0.5 mL) was added a few drops of acetic anhydride with shaking, followed by one drop of concentrated sulfuric acid. The color change was observed after a few minutes.

Alkaloid test

Dragendroff's reagent

Add a few drops of Dragendroff's reagent into the sample solution. The orange precipitate suggested the presence of alkaloid nucleus.

Kraut's reagent

The sample solution was added a few drops of Kraut's reagent to yield brown precipitate. This positive test exhibited the presence of alkaloid nucleus.

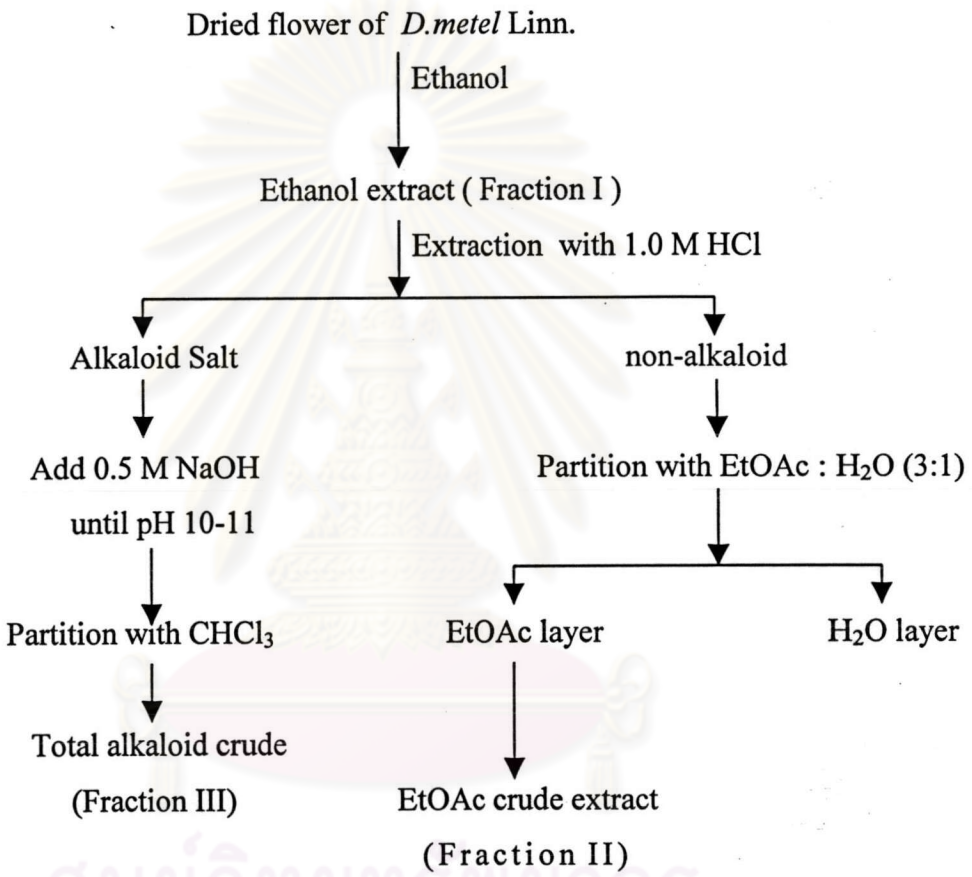
Marme's reagent

The sample solution was added a few drops of Marme's reagent. The brown precipitate indicated the occurrence of alkaloid compounds.

2.5 Extraction procedure

Dried flowers of *D. metel* Linn. (10 kg) were extracted with ethanol by soaking at room temperature for three days. The extraction was repeated for several times until the color of the extract was clear. The solution was filtered and solvent was removed under vacuum to furnish ethanolic extract (Fraction I). The ethanolic crude extract was further extracted with 1.0 M HCl and filtered. Alkaloids were liberated around pH 10-11 from the filtrate by the addition of an aqueous solution of

0.5 M sodium hydroxide and extracted with chloroform. The latter extract was dried over anhydrous sodium sulfate to yield the total alkaloids (Fraction III), which were quantified by gravimetry (31.08 g on average, 0.31% relative to dried flowers).[48] The residue (non-alkaloid fraction) was extracted by partition between ethyl acetate and water in ratio of 3:1 to gain an ethyl acetate soluble fraction (Fraction II) and water soluble fraction. The general scheme for the extraction is shown in Scheme 2.1.



Scheme 2.1 The general extraction procedure

2.6 Chemical reaction

Hydrolysis of Mixture **1**

A solution of 10% ethanolic KOH (3 mL) was added to Mixture **1** (156 mg). The reaction mixture was heated under reflux on a water bath for 4 hours. Evaporation of ethanol gave a solid, which was further extracted with diethyl ether three times. The combined diethyl ether was dried over anhydrous calcium chloride. Evaporation of the solvent furnished a solid with pale yellow solution. After recrystallization this solid with a mixture of chloroform and methanol, bright needle

designated as Mixture **1a** (64.2 mg), m.p. 117-118°C was obtained. The remained solid was dissolved in water and acidified with diethyl ether to afford another impure solid. This component was recrystallized for several times with a mixture of chloroform and methanol to yield Mixture **1b** (50.5 mg), m.p. 41-42 °C.

2.7 Biological screening assay

Brine shrimp cytotoxic lethality test[49]

This method is rapid, simple and inexpensive screening test for cytotoxicity. Its advantage of requiring small amounts of compound and the employment of microplate technology facilitates the testing of large number of samples and dilutions.

Test methodology

Samples for testing (4 mg) were dissolved in 80 μL DMSO prior to add artificial seawater to make 4000 μL and allowed to shakewell to afford solution A (1000 ppm). Serial dilutions of solution A was made up to 100 and 10 ppm in artificial seawater (solution B and C, respectively). The control solution was prepared by dissolving 80 μL DMSO in 3920 μL artificial seawater. A suspension of nauplii containing 10 organisms of *A. salina* (100 μL) was added to each well of 24-well microplates by micropipette. The assay at each concentration was conducted for six replicates. The microwells were incubated at 22-29°C for 24 hours. The numbers of dead nauplii in each well after 6 hours (acute toxicity) and 24 hours (chronic toxicity) were counted under binocular microscope. LC_{50} values were calculated by Probit analysis program.

Plant growth inhibition on *Lactuca sativa* L[50]

The following procedure was assayed for plant growth inhibition screening test. The advantage of this assay is rapid, easy and cheap.

The procedure for crude extracts

Tested crude extract was dissolved in a proper solvent at concentration of 1, 0.5 and 0.1 g of dried plant. The 3 mL of crude extract solution was poured into a plate (diameter 90 mm) which contained a filter paper. The controlled plate was prepared using the same methodology with no tested sample. All plates were dried up overnight. After overnight, the 3.0 mL of distilled water was added to each plate. Eight seeds of *L. sativa* L. were transplanted in each plate, 3 plates for each

concentration. All plates were covered and left at room temperature. The germinated seed numbers were recorded at 7 days after application. Five seedlings were randomly selected to measure the root and shoot length.

The procedure for pure compound

Tested compound was dissolved in a proper solvent at concentration of 1000, 100 and 10 ppm. The 1.0 mL of each concentration of sample was poured in a plate (diameter 50 mm) containing a filter paper. The controlled plate was prepared using the same methodology with no tested compound and the test procedure was followed that described above. The inhibitory effect of the substance on germination was calculated by

$$\% \text{ Germination Inhibition} = \{1-(T/C)\}x100\ \%$$

Where

"T" is the germination number of treated seedlings and "C" is the germination number of controlled set.

Germination inhibition 100 % means completely inhibitory effect.

Moluscicidal activity against *P. canaliculata*

This method was modified from the procedure described by WHO.[51-52]

Preparation of P. canaliculata L. or Golden Apple snail

The unitform size of *P. canaliculata* (diameter of shell ~3.5 cm) was used. For each test, three snails were placed in 1000 mL of dechlorinated water overnight at room temperature.

Preparation of sample for testing

Tested crude extract was dissolved in a proper solvent at concentration of 10, 1 and 0.1 g equivalent of dried plant. For tested compound was made concentration of 1000, 100, 10 ppm. 3 mL of each concentration was poured into a plate (diameter 90 mm) containing a filter paper. The controlled plate was prepared using the same methodology with no tested sample. All plates were dried up overnight. After overnight the dried filter paper was cut to small pieces and put in each tested beaker, 3 beakers for each concentration. All beakers were covered and left at room temperature. The dead numbers of snail were recorded at 24 hours for 3 days after application. The percentage mortality of snail was calculated by

$$\% \text{ mortality} = \{(O-C)/T\}x100$$

where;

"O" is mortality number of snail in tested set

"C" is mortality number of snail in controlled set

"T" is total number of tested snail

LC_{50} values were calculated by Probit analysis program.

Insecticidal activity by vial test contact toxicity[53-54]

This test utilized the dry film or vial method test to screen for the potential compound with insecticidal activity. A polyphagous insect, the common cutworm, *Spodoptera litura* was used as a model for meanwhile investigation.

The general procedure

The samples were prepared by dissolving 4 mg of test substance in 4 mL of acetone to provide 1000 ppm solution. Serial dilution of this stock solution was made to obtain 500, 100, 50, and 10 ppm, respectively. Then 1 mL of each concentration solution was poured into the glass vial (3 replication). Following, each vial that contained 1 mL of sample was evaporated, and the treated vial was placed in an open space for a few minutes to ensure complete removal of acetone. The test substances have already coated on the wall and bottom of vial. Common cutworms were reared on an artificial diet in a controlled environment. The 15 of first instar larvae were placed in the vial to free movement for 6 hrs, then the larvae were transferred into the new vial containing artificial diet and were kept at 25°C for 5 days. After the fifth day, died cutworms were counted and converted to percentage of died larvae of *S. litura*. Finally, the LD_{50} in ppm was calculated by Probit analysis program.