CHAPTER 2

MATERIALS AND METHODS

Materials

1. Devices

Freeze Drier EYELA FD - 1

Balance :CHYO Jupiter SP-200 D and Mettler

ACC Homogenizer AM-77 Nissei

Aspirator A-2S EYELA Tokyo Rikakikai Co., Ltd.

Water Bath SB-35 EYELA Tokyo Rikakikai Co., Ltd.

Neocool Aspirator BP-51 Yamata

Rotary Vacuum Evaporator EYELA Tokyo Rikakikai Co. Ltd.

Filter Paper:Whatman No.2 and Hand Work Kiriyama No.4

Direct Drive Rotary Vacuum Pump Hitachi

Magnetic Stirror Ikemoto PKI

Glass Column for Column Chromatography Vidrex

Glass Plate (20 x 20 cm) Coated with Kieselgel 60 F_{254},

0.25 mm Thick Merck

Tank for TLC

250 ml Flasks Vidrex

300, 100 and 25-ml round Bottom Flasks Vidrex

100-ml Beakers Vidrex

1-ml Automatic Pipette

1, 3, 5, 10, 25-ml Pipettes

Microcapillary

Scissors

2. Chemicals

Methanol, Hexane, Ethyl Acetate, Butanol,

Acetone, Acetic Acid and Distilled Water

Cellulose Powder

Charcoal Powder

Celite

All of these chemicals are guaranteed grade.

3. Plants

E. camaldulensis materials were collected nere Academic Resource Center Chulalongkorn University, Thailand.

M. pigra seeds were obtained from the NWSRI Project in Thailand

O. sativa seeds were obtained from the NWSRI Project in Thailand.

Methods

1. Green Leaf Extraction

Eighty g of *E. camaldulensis* fresh leaves were cut into small pieces and extracted in a homogenizer with cold methanol. The procedure was as follows. The plant material was placed in the homogenizer containing 300 ml of cold absolute methanol. The homogenization was carried out for 10 min at 12 x 1000 rpm. The methanolic extract was separated from the tissue debris by passing the methanolic extract through the Kiriyama No. 4 filter paper in a Kiriyama Rhoto funnel under reduced pressure. The residue remained on the filter paper was then washed with methanol until it was free of green color

indicating that all of the low molecular weight compounds had been extracted (Harborne, 1984).

An equivalent amount of 40 g original material of the combined extract and washing was saved to examine for phytotoxicity (germination and growth) test. The remainder of the methanolic extract was then evaporated in a rotary evaporator at a water bath temperature of 40 ^{o}C. The residue thus obtained was taken up in 120 ml of distilled water, transferred into a separatory funnel and successively extracted with n-hexane (25 ml each, 4 times) and ethyl acetate (25 ml each, 4 times). Each extract obtained as well as the methanolic extract saved was evaporated down to a small volume in a rotary evaporator at a water bath temperature of 40 ^{o}C. From them, an equivalent amount of 0.1, 1 and 5 g fresh material, respectively was taken and used for examining the phytotoxicity towards M. pigra and O. sativa. Tests and controls were triplicated.

2. Fallen Leaf Extraction

To determine whether the fallen leaves of E. camaldulensis contained inhibitory substances, the fallen leaves were weighed, cut into small pieces and a 40 g sample extracted with cold methanol in the same manner as described in the green leaf extraction.

3. Fallen Bark Extraction

To determine whether the fallen bark of E. camaldulensis contained inhibitory substances, the bark were chopped, weighed and a 40 g of finely chopped sample extracted with cold methanol in the same manner as described in the green leaf extraction.

4. Charcoal-Celite Column Chromatography

As mention later, the results of bioassay indicated phytotoxicity in methanolic, aqueous and ethyl acetate extract. Trying to isolate toxic substances from one extract is simpler than from more than one extract. Thus, the methanolic extract was subjected to rough fractionation using charcoal-celite column chromatography. The column was prepared in the following ways.

4.1 Preparation of the Column

After weighing, 5 g charcoal and 10 g celite were mixed thoroughly with 200 ml of acetone for 20 min by means of an electromagnetic stirror, and the resultant mixture was then packed into a column and washed with 50% acetone in distilled water.

4.2 Preparation of the Sample

Twenty g fresh leaf material were extracted with methanol using the procedure described above. The extract was then evaporated to near-dryness in a rotary evaporator at a bath temperature of 40 ^{o}C. The residue was then taken up with the smallest possible amount of acetone, diluted to 50% acetone with distilled water and poured on top of the column packed with charcoal-celite mixture.

Step elution with water/acetone mixture (V/V) was employed from 50/50 to 0/100 at 10% difference. Every 100 ml eluate was collected, reduced to a small volume in a rotary evaporator at 40 ^{o}C. From each concentrate, an equivalent amount of 0.1, 1 and 5 g fresh material, respectively, was taken and checked for growth inhibitory activity towards M. pigra and the most toxic fraction was

further subjected to thin-layer chromatography. Tests and controls were triplicated.

5. Thin-Layer Chromatography

As mention later, among the six fractions from charcoal-celite column chromatography, the water/acetone (50/50) fraction showed the strongest inhibitory activity on the growth of *M. pigra* seedlings. This fraction was further subjected to purification using thin-layer chromatography. The procedure was as follows. Acetone of the fraction was completely removed under reduced pressure at 40 ^{o}C using a rotary evaporator. The aqueous concentrate thus obtained was then sublimed in a freeze-drier, and the non-sublimable material was left in a dried state. The dried material was taken up in 10 ml of absolute methanol. A half ml of the solution (equivalent to 1 g fresh leaf material) was taken and applied as a band on a silica gel TLC plate. The plate was then developed using butanol/water/acetic acid mixture (80/15/5, V/V/V) as a developing solvent. After developing for more than 10 cm, the plate was dried, then studied under short wave (254 nm) and long wave (366 nm) UV-light. Compounds with fluorescence/absorbance were marked on the plate. Difference zones on the chromatogram were scraped off directly into the vials to study the phytotoxicity towards *M. pigra* seedlings.

Effects of substances from the chromatogram showing toxicity towards *M. pigra* on rice seedlings were also determined.

6. Identification of Toxic Substance (s)

Toxic substance(s) from the thin-layer chromatogram showing toxicity towards *M. pigra* were identified by simultaneous

chromatography with five chemicals known to be allelochemicals in E. camaldulensis leaf litter: gallic, ferulic, p-coumaric, chlorogenic and caffeic acids. After developing for more than 10 cm, the plate was dried and studied under UV-light.

7. Effects of 13 Phenolic Compounds on Growth of M. pigra and O. sativa Seedlings.

This experiment was designed to determine the effects of 13 phenolic compounds on seedling growth of M. pigra and rice. Five concentrations were used (0.1, 1, 10, 100 and 1000 ppm). Each treatment was replicated two times. The procedure was as follows.

After weighing, each sample except gallic acid was dissolved in MeOH in an amount required to reach the desired concentration after transferring to a vial containing 1.5 g of cellulose powder with 6 ml of distilled water. In the case of gallic acid it was directly dissolved in distilled water because gallic acid reacts with MeOH at room temperature (Fukui & Koshimizu, 1981). After drying out in a vacuum chamber, 6 ml of distilled water were added and the vials were left without mechanical disturbance. After mass of the cellulose powder in a vial congealed, uniformly germinated Oryza sativa cv. RD 23 or Mimosa pigra seeds were placed in group of six. The vials were then covered with vinyl film and placed in a growth chamber with a temperature of 30 °C under continuous fluorescence light (ca. 3,000 lux) for 1 week before harvest.

8. Bioassay.

8.1 Germination Test.

8.1.1 Petri Dish Preparation. First, 2 circles of Whatman No. 2 filter paper were placed on the bottom of a glass petri dish with tight-fitting cover. Next, known amount of aliquot of the test solution was pipetted onto each dish. After removing the solvent in the petri dish by evaporation in a hood, 6 ml of distilled water were dropped onto the dried filter papers. The dishes were kept overnight at room temperature befor sowing the test seeds.

8.1.2 Test Seed Preparation.

8.1.2.1 O. sativa Seeds. O sativa seeds were washed with tap water, only seeds sunk in the water were used.

8.1.2.2 M. pigra Seeds. Only a small percentage of M. pigra seeds germinate under normal conditions, thus the seeds were subjected to moist heat activation by plunging them into boiling water for 1 min before allowing them to germinate. In this way, the germination percentage is raised to about 90%.

Twenty seeds of M. pigra or O. sativa prepared as described above were sown on the filter paper in the Petri dish so as to be immersed in the aqueous solution. The dishes were then placed in a growth chamber. Incubation was carried out at 30 ^{o}C under continuous fluorescence light (ca. 3,000 lux). The dishes were removed only for germination counts performed after 24, 48 and 72 hr for M. pigra and 48, 72 and 96 hr for O. sativa. Tests and controls were triplicated.

8.2 Growth Test.

8.2.1 Vial Preparation. To facilitate the collection of roots during harvest, all of the seedlings were grown in cellulose powder. Known amount of aliquot of the test solution was pipetted into each vial containing 1.5 g of cellulose powder. After drying out in a vacuum chamber, 6 ml of distilled water were added and the vials were then left without mechanical disturbance. After mass of the cellulose powder congealed, the vials were ready to be subjected to bioassay.

8.2.2 Test Seedling Preparation.

8.2.2.1 O. sativa Seedlings. O sativa seeds were soaked in a Petri dish containing tap water for 2 days in a growth chamber with a temperature of 30 ^{o}C and a light intensity of 3,000 lux. After that, uniformly germinated seeds were collected for growth test.

8.2.2.2 M. pigra Seedlings. M. pigra seeds were plunged into boiling water for 1 min, then allowed to germinate in a growth chamber with a temperature of 30 ^{o}C and a light intensity Of 3,000 lux. Next day, uniformly germinated seeds were collected for growth test.

After mass of the cellulose powder in a vial congealed, uniformly germinated O. sativa or M. pigra seeds were placed in group of six. The vials were then covered with vinyl film and placed in a growth chamber with a temperature of 30 ^{o}C and a light intensity of 3,000 lux at plant level. Tests and controls were triplicated. Roots and shoots were harvested after 1 week. Methods of measuring the test plants were as follows.

8.3 Measure Method

8.3.1 *M. pigra* Seedlings   Hypocotyl and longest root length were determined and compared with control.

8.3.2 *O. sativa* Seedlings   The second leaf sheath and longest root length were determined and compared with control.