CHAPTER II
MATERIAL AND METHODS

2.1 Plant Materials

Plant materials used in this research are :

- *Ageratum conyzoides* Linn. collected from field of Bangkhen Rice Experiment Station, Bangkok.

- *Sphaeranthus africanus* Linn. collected from paddy field of Bangkhen Rice Experiment Station, Bangkok.

- *Eupatorium odoratum* Linn. collected from roadside, Ayutthaya.

- *Eupatorium adenophorum* Spreng. collected from mountainous area in Chiangmai, Thailand by the Weed Science Subdivision, Botany and Weed Science Division, Department of Agriculture, Ministry of Agriculture and Cooperatives, Bangkok, Thailand. The specimen of *E. adenophorum* Spreng. was compared with voucher number SN 013144 by a botanist of the Royal Forest Department, Ministry of Agriculture and Cooperatives, Bangkok, Thailand.

The above mentioned ground parts of each species were collected from field, dried in shed and kept at room temperature until used.

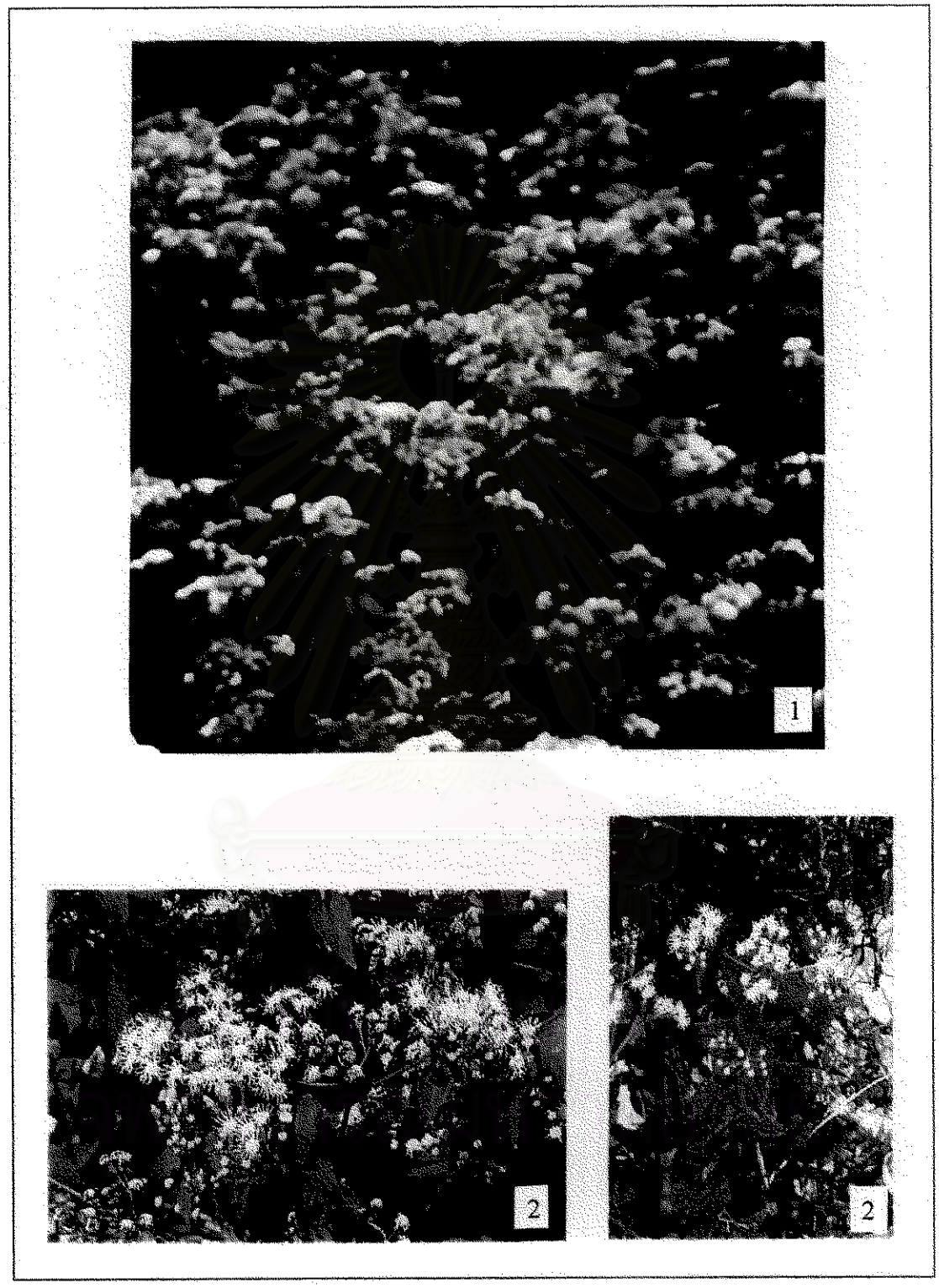


Fig. 2.1 Plant materials

1. *Eupatorium adenophorum* Spreng.

2. *Eupatorium odoratum* Linn.

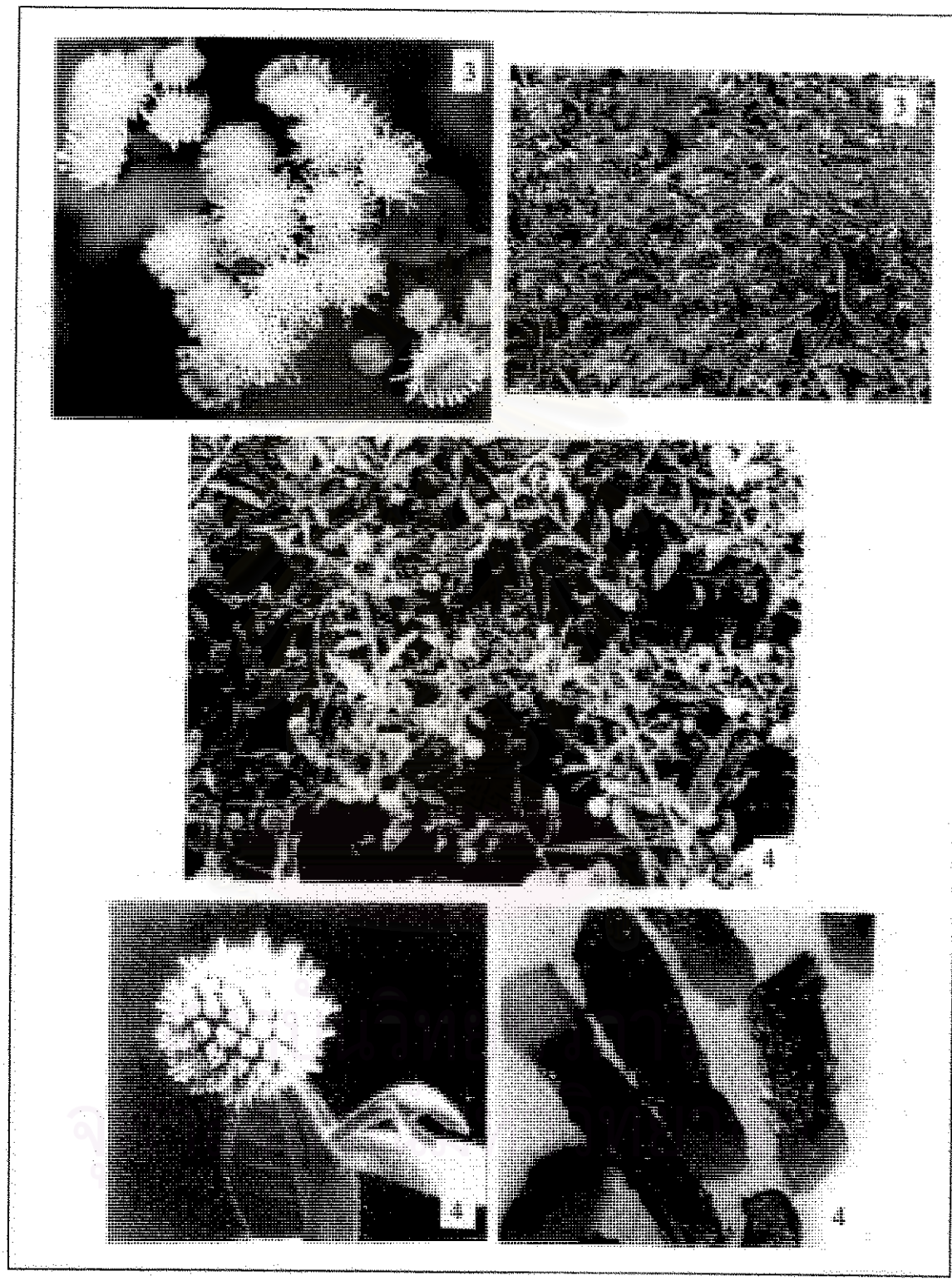


Fig. 2.1 (cont.)

3. *Ageratum conyzoides* Linn.

4. *Sphearanthus africanus* Linn.

2.2 General Procedures

Melting points were determined with a Fisher-Johns melting point apparatus and are uncorrected. Chromatotron equipment, Harrison Research Model 7924 T, was used for certain separation (Raksilp, 1995). Thin layer chromatography (TLC) was performed on aluminium sheets precoated with silica gel (Merck Kieselgel 60 PF_{254}) (Randerath, 1996). Column chromatography was performed on silica gel (Merck Kieselgel 60 G) (Fessenden and Fessenden, 1983).

The FT-IR spectra were recorded on a Fourier Transformed Infrared Spectrophotometer model Impact 410: solid samples were incorporated to potassium bromide to form a pellet. The ^{1}H-NMR and ^{13}C-NMR spectra were obtained from a Bruker model AC-F 200 spectrometer and a Jeol, model JNMR-A500 which operated at 200.13 MHz for ^{1}H and 50.32 MHz for ^{13}C-nuclei. The GC-MS analysis was performed by a Fisson Gas-Liquid Chromatography Model GC 8000-Fisson Mass Spectrometer Model Trio 2000.

2.3 Chemicals

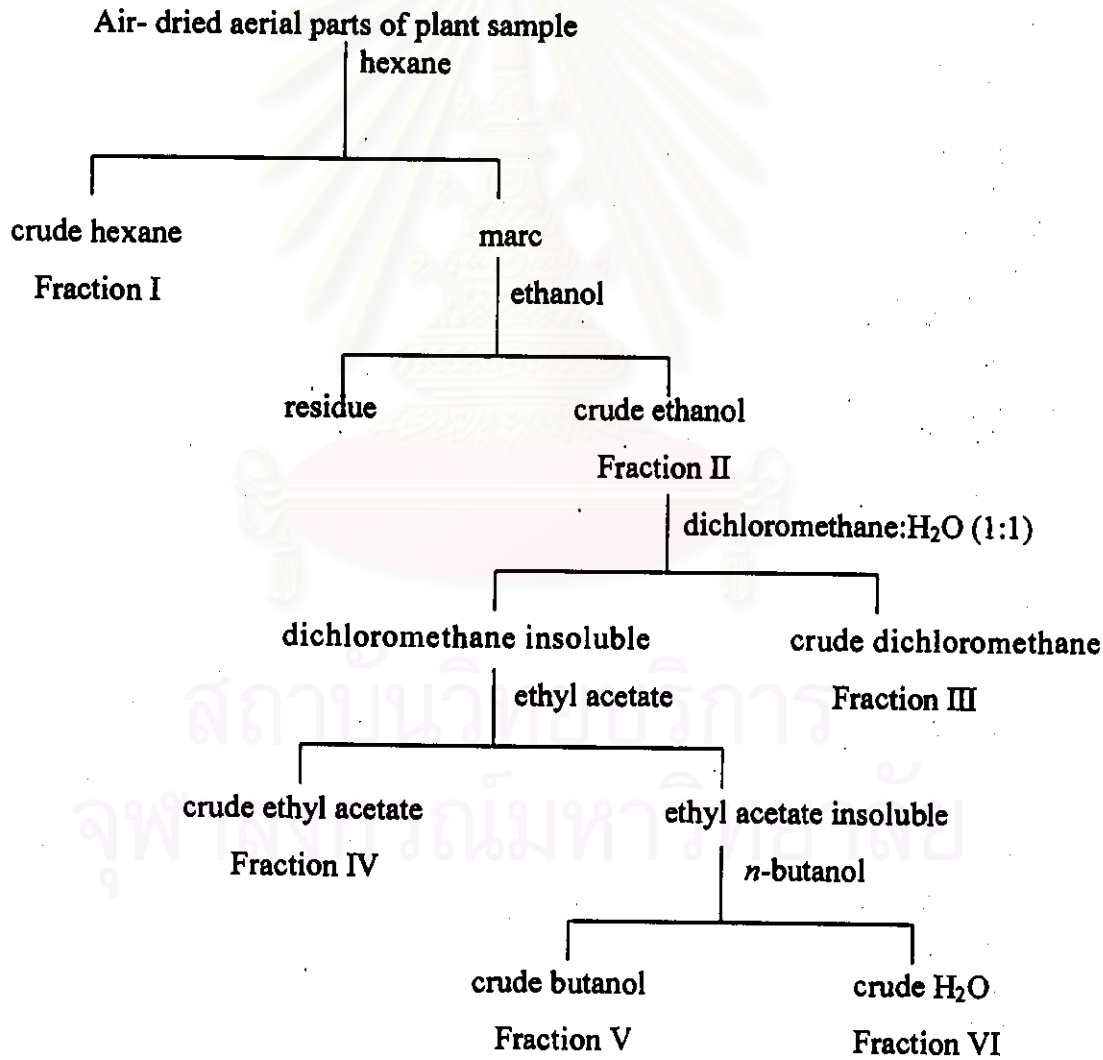
Solvent: All solvents used in this research were purified prior to use by standard methodology except for those which were reagent grades.

Other substances: Merck' s silica gel 60 Art.7734 1000 (70-230 mesh ASTM) was used as adsorbents for column chromatography; No.7731 for quick column chromatography and No. 7749 for chromatotron, respectively. Other chemicals, unless otherwise stated, were of the highest purity available purchasing from Fluka Chemical Company and were used without further purification.

2.4 General Extraction for Preliminary Study

Plant samples were air-dried and milled. Each specimen was soaked in hexane for 3 days at room temperature. The solution was filtered and evaporated by rotatory vacuum evaporator to remove the solvent. The hexane soaking procedure was

repeated three times. The residue was re-extracted with 95 % ethanol for 5 days at room temperature. The ethanol soaking procedure was repeated three more times or until the solution was colorless. Using rotatory vacuum evaporator to almost dryness evaporated the ethanolic extract. The ethanolic crude extract was further partitioned between dichloromethane and water in ratio 1:1 to give a dichloromethane fraction and water-soluble fraction. The water-soluble fraction was extracted with ethyl acetate, then the left of ethyl acetate crude extract was extracted with *n*-butanol to afford the *n*-butanol extract. The extraction procedure for preliminary study of plant samples is shown in Scheme 2.1.



Scheme 2.1 The extraction and fractionation for preliminary study of plant samples

2.5 Bioassay Experiments

As mentioned earlier, one of the objectives was to search for active principles from Compositae weeds that gave excellent bioassay results and could possibly use for agricultural and medicinal purposes ; particularly as anticancer agents. Plant growth inhibition method was used as main bioassay to follow the bioactive compounds. The bioassay species for plant growth inhibition are *Oryza sativa* cv RD 23, *Mimosa pigra* Linn., *Echinochoae crusgalli* Beauv. Detailed bioassay experiments performed were as followed:

2.5.1 Plant Growth Inhibition

(i) For crude extract

Crude extracts of 0.1, 0.5 and 1.0 g equivalent to fresh materials were taken 3 mL and poured into glass tube (30 *mm*ϕ × 120 *mm* H) containing 1.5 g of cellulose powder (Toyo, type D), and 3 tubes were used for each concentration. The same amount of the same solvent of crude extract was added instead of extracts as control. All the tubes were dried up in vacuo oven at 50°C overnight. Then cellulose powder was stirred well-mixed, 4.5 mL of distilled water was added to each tube. Six uniformly germinated seedlings (1-2 mm length of radical) were transplanted in each tube. Then, the tubes were covered with transparent vinyl film and kept in growth chamber at 30°C, 24 h daylight. After seven days, measured the longest root and length of the secondary leaf sheath. Performed each experiment in three replications and six units each replication.

$$\% \text{ Inhibition} = [(A-B) / A] \times 100$$

where A is the mean root length / the height of secondary leaf sheath in control set and B is the mean root length / the height of leaf sheath in treatment set.

(ii) For pure compounds and fractions

The solutions of 10, 100 and 1000 ppm for pure compounds and 10, 100, 1000 and 10000 ppm for fractions were prepared. Three seedlings were transplanted in each tube. Each experiments was performed in three replications and

three units for each replication. The procedure is the same as (i) (Zungsontiporn, 1995).

2.5.2 Brine Shrimp (*Artemia salina* Linn.) Cytotoxicity Test

Brine shrimp eggs were hatched in artificial seawater prepared from dissolved NaCl 38 g in 1 L distilled water. A plastic divider with several 2 mm holes was clamped in the dish to make two unequal compartments. The eggs were sprinkled into the darken larger compartment and illuminated the smaller compartment. Incubated at 22-29°C for 24 h, the phototropic nauplii were deshelled and were collected with micropipette from the illuminated side.

Serial dilution of samples (10, 100 and 1,000 μg/mL) was made in the well of 24-well microplates with 100 μL atificial seawater. Control wells contained DMSO were included in each experiment. A suspension of nauplii containing 5 organisms per 100 μL was added in each well and then covered plate incubated at 22-29°C with illumination for 24 h. Plates were then examined under binocular microscope to determine the acute LC_{50}. The numbers of dead (non-motile) nauplii in each well were counted after 6 h. Counting for the chronic LC_{50} begins at 24 h after initiation of the test. LC_{50} values were calculated by Probit analysis (Meyer *et al*, 1982) (Solis *et al*, 1992).

2.5.3 The Inhibitory Effect for Tumor Cell Lines

Preliminary screening of crude extracts of aerial parts of *E. adenophorum, E. odoratum, A. conyzoides* and *S. africanus* has been carried out by workers at Beijing Medical University, Beijing, China. The use of MTT assay to study the inhibitory effect on tumor cell lines was reported. Seven cell lines were used : Human Bladder Carcinoma (BIU), Human Leukemia Carcinoma (HL-60), Human Nasopharyngeal Carcinoma (KB), Human Gastric Carcinoma (BGC-823), Human Erythroleukemia Carcinoma (K-562), Colon Carcinoma (HCT-8) and Hepatocellular Carcinoma (Bel-7402).

Cell lines were cultured under conventional conditions: 37°C, 5%CO_2 + 95%Air, 100% relative humidity, in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum, Penicillin 100 IU·mL^{-1} and Streptomycin 100 μg·mL^{-1}.

The tetrazolium dye (MTT = 3,4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide) assay is based on a reduction of MTT formazan by living cells. The reduced formazan can be measured with a microplate spectrophotometer.

MTT assay: Cell lines were seeded in 96 well microlitre plates in well with 5×10^4 cells. A stock solution of extract was added to each well and 8 replicate wells without extract were severed as controls. The plates were incubated for 72 h. After the incubation, 20 μL of PBS solution with MTT 5 mg·mL^{-1} were added to each well and the plates were reincubated for a further 4 h. The formazan crystals formed were dissolved in 200 μL of acid-isopropanol. The plates were read on a Model 450 Microplate reader at 570 nm (Wiboonpun, 1996).

2.5.4 Antifungal Activities with *Candida albicans.*

Thin layer chromatography. Silica gel G60 F_{254} glass baked plates were used for agar overlay bioautographic assays with *C. albicans.* Silica gel G60 F_{254} aluminum sheets were used for other TLC bioassays. The TLC were developed with following solvent systems; 65:65:5 of chloroform : methanol : water for polar crude extract, 1: 1 of ethyl acetate : ligroine for all lipophilic extracts. All TLC plates were run in duplicate, one of them being used as the reference chromatogram. UV active spots were detected at 254 and 366 nm. The reference chromatograms were strained with Godin reagent.

Microorganisms and culture media. Strains of *Candida albicans* were obtained from clinical isolates of the Service de Dermatologie, Center Hospitalier Universitaire Vaudois (CHUV), Lausanne, Switzerland. Sabouraud liquid medium (diagnostic Pasteur, Marnes La Coquette, France) was used for broth culture of *C. albicans*. All media were autoclaved at 120°C for 20 min. In order to obtain an exponential growth phase of *C. albicans*, the Sabouraud broth (20 mL) was inoculated for 5-6 h before testing.

Inoculum for assay. Malt agar (Oxoid) for *C. albicans* were used as the solid media for the overlays. The molten media were maintained in water bath at 45°C. The final concentration in the solid medium was approximately 105 cells/mL. The suspension was prepared immediately before carrying out the tests.

Bioautography. Chromatograms were placed on hot plate maintained at 35°C. Approximately 10 mL of the inoculum was rapidly distributed over the TLC plate (10×20 cm) with a sterile pipette. After solidification of the medium, TLC plates were incubated overnight at 30°C in polyethylene boxes lined with moist chromatography paper. The bioautograms were sprayed with an aqueous solution (2.5mg/mL) of thiazolyl blue (methylthiaazolytetrazolium chloride; MTT) (Fluka), and incubated for 4 h at 30°C. Clear inhibition zones were observed against a purple background (Rahalison *et al*, 1991).

2.5.5 Antioxidant Activity

(i) Reduction of 2,2-diphenyl-1-pidrylhydrazyl {=2,2-Diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl; DPPH} radical. TLC autographs assay: after developing and drying, TLC plates were sprayed with a 0.2% DPPH solution in MeOH. The plates were examined at 30 min after spraying. Active compounds appearred as yellow spots against a purple background.

(ii) Bleaching of β-carotene. TLC autographic assay: after developing and drying, TLC plates were sprayed with a β-carotene solution in chloroform (0.2 mg/mL). The plates were exposed to 254 nm UV light for 20 min before examination. β-carotene undergoes bleaching except in places where antioxidative substances prevented the degradation. Active compounds appearred as orange spots against a white background (Hostettmann *et al*, 1997).

2.5.6 Larvicidal Activity

The extracts were dissolved in dimethyl sulfoxide (DMSO) with maximum concentration of 50 mg/mL and 500 ppm for final test solutions. Five geometrical dilutions were prepared in DMSO to obtain identical solvent concentration of all samples. The DMSO solutions (100 mL) were added to 9.9 mL of tap water (final DMSO concentration 1%) in graduated test tubes. *Aedes aegypti* eggs were incubated for 24 h in tap water. Approximately 20 second instar larvae were introduced into the test solutions and mortality were evaluated visually after 30 min and 24 h (Bergeron *et al*, 1996).

2.6 Extraction

Air-dried aerial parts of *E. adenophorum* Spreng. were minced to coarse powder. The plant initially extracted with *n*-hexane by soaking for 4-5 days at room temperature. The residue was re-extracted three times. The solution was filtered and the solvent was evaporated yielding hexane crude extract (Fraction I). The plant residue left after hexane extraction was soaked in ethanol and the solution removed. Repeat until the solution was colorless. Filter the solution and evaporate. The ethanolic crude extract (Fraction II) was further partitioned between dichloromethane and water in ratio 1:1 to yield dichloromethane fraction (Fraction III) and water-soluble fraction. The water-soluble fraction was extracted with ethyl acetate and yielded ethyl acetate crude extract (Fraction IV). The latter was then extracted with *n*-butanol to afford *n*-butanol extract (Fraction V).