Materials and Methods

CHAPTER 3

MATERIALS AND METHODS

3.1 Plant Materials

The plants collected for the present study are shown below :

Scientific Name	Date of collection	Source	Plant part(s)
Diospyros graciliflora Hiern.	February, 1994	Botanical Garden (Rimba Ilmu), University of Malaya	stem, stem bark
Diospyros discolor Willd.	February, 1994	Botanical Garden, University of Malaya	stem, stem bark
Diospyros lanceifolia Roxburgh.	July, 1993	Pulau Tioman, Pahang	stem

Five plants from the family of Euphorbiaceae which has been reported for their tumour promoter activity (Norhanom and Yadav, 1995) were also collected on March, 1994 from University of Malaya botanical garden, Rimba Ilmu. They were *Euphorbia hirta* L., *Euphorbia tirucalli* L., *Euphorbia splendes, Jatropha podagrica* Hook. and *Pedilanthus tithymaloides* (L.)Poitt. The description of *D. graciliflora, D. discolor* and *D. lanceifolia* are detailed in Section 2.3.1, 2.3.2 and 2.3.3 respectively.

The plant parts were cut into small pieces and dried for about 7 days at 60 °C in a hot air oven (Memmert). The dried samples were then pulverised using Wiley Laboratory Mill.

3.2 Extraction of Plant Materials

Finely ground dried plant materials of *D. graciliflora*, *D. discolor* and *D. lanceifolia* (50g) were successively extracted with light petroleum ether 35-60 °C (100 ml), chloroform (100 ml) and methanol (100 ml) using Soxhlet extraction apparatus. The solvents were removed at 40 °C under reduced pressure using a rotary evaporator. The concentrates were then dried at room temperature and weighed.

The ground dried plant materials of *E. hirta*, *E. tirucalli*, *E. splendes*, *J. podagrica* and *P. tithymaloides* were subjected to the same extraction procedure using 100 ml light petroleum ether 35-60 °C in order to extract natural phorbol esters from the plants.

3.3 Phytochemical Analysis

Buchi 530 Melting Point Apparatus (uncorrected) was used to determined melting points. Mass spectra were determined on Shimadzu GCMS-QP 2 000A. IR spectra were taken in KBr on Shimadzu IR 470 Spectrophotometer. NMR spectra were recorded on 270 MHz JEOL-JNM-G5×270 FT-NMR Spectrometer in $CDCl_3$ and C_5D_5N. Relative to residual solvent peaks or TMS, chemical shifts were reported.

3.3.1 Chromatographic Methods

In order to separate and purify compound(s) from the various plant extracts, thin layer chromatography (TLC) and column chromatography were used.

(i) Thin-Layer Chromatography (TLC)

Glass plates with 12.5x20 cm and 20x20 cm were used for the study. Initially, the plates were cleaned thoroughly with acetone. Slurry prepared from 60 g silica gel type 60 in 120 ml distilled water was then spread on the plates using a TLC spreading jig. For the purpose of analytical TLC, Kieselgel 60 GF 254 (Merck Art No. 7730) with a nominal thickness of 0.5 mm were used. 1.0 mm thickness Preparative TLC plates were prepared using Kieselgel 60 HF 254 (Merck Art No. 7739). The TLC plates were then dried at room temperature and activated for 30 minutes at 110 °C. Spots of samples were allowed to develop 15 cm from the line of application in saturated condition using appropriate mobile phase.

(ii) Column Chromatography (CC)

A silica gel slurry 120-230 mesh which have been dissolved in a suitable amount of solvent system was poured into an appropriate size of glass column with gentle tapping to remove trapped air bubbles. A known amount of crude extract of sample which was initially dissolved in minimum amount of solvent was then loaded on top of the packed column by means of a pasteur pipette. The appropriate solvent was added slowly into the column in order to elute the extract. The elutions were collected in volumes of 5 ml each and evaporated to dryness.

3.3.2 Preparation of Reagents for Detection of Compounds

(i) 10% Vanillin in Concentrated Sulphuric Acid

Ethanol and concentrated sulphuric acid were mixed in a ratio of 1:4 respectively. 0.5 g of vanillin powder was added to 100 ml of the above mixture and stirred. This solution was freshly prepared before use.

(ii) Dragendorff's Reagent

Solution A : 1.7 g bismuth nitrate in 100 ml water : acetic acid (80 : 20)

Solution B : 40 g potassium iodide in 100 ml of distilled water

Solution C : 20 ml acetic acid in 70 ml distilled water

Before use, 5 ml of solution A and 5 ml of solution B were added to solution C.

3.3.3 Detection of Compounds

Detection of secondary metabolites of alkaloids, terpenes and saponins were done by means of suitable spraying reagents.

(i) Alkaloids

Dragendorff's reagent was sprayed onto the dried chromatographed TLC plates. Red, orange or yellow spots would indicate the presence of alkaloids (Harbone, 1984).

(ii) Terpenoids

Ten percent of vanillin in concentrated sulphuric acid was sprayed onto the dried TLC plates. The plates were then heated in the oven at 120 °C for about 10 minutes. Coloured spots of either purple, black, blue, red or pink would indicate the presence of terpenoids (Harbone,1984).

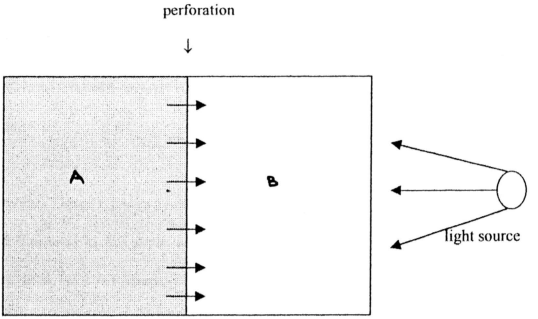
3.4 Bioassays

3.4.1 Bioassay for Bioactive Compounds : Brine Shrimp Lethality

Sea water was prepared and filtered (38 g sea salt per litre of water). The sea water was then poured into a small tank. The set up of the tank is shown in Figure 3.1. Shrimp eggs were added to the covered side (Side A) of the divided tank and were allowed to hatch and matured as nauplii after two days. The hatched shrimps would be attracted through perforations in the tank by light on the other side of the tank (Side B).

Stock solutions of plant extracts were prepared in dimethyl sulphoxide (DMSO) at a concentration of 10 µg/ml. The plant extracts were tested at concentrations of 10, 100 and 1 000 ppm in three replicates. Ten shrimps were added to each vial containing 5 ml sea water. The number of surviving shrimps were counted after 24 hours. The data collected was then analysed with a Finney computer programme to determine the LC_{50} values and 95% confidence intervals for statistically significant comparison of potencies. The cut-off value of LC_{50} was 200 ppm (McLaughlin, 1990). Extracts with LC_{50} values of less than 200 ppm were considered to be bioactive.

Figure 3.1 : Set-up of a tank for brine shrimp lethality bioassay



Note :

⟶ movement of shrimps

A covered side of tank/ hatching of shrimp eggs

B uncovered side of tank

3.4.2 Anti-Tumour Promoter Activity Bioassay : Inhibition of EBV EA Expression in Raji Cells

(i) Preparation of Plant Extracts

Plant extracts which were positive for brine shrimp lethality were weighed and dissolved in dimethyl sulphoxide (DMSO) (Sigma) as a stock solution of 20 mg/ml and kept at -20 °C until use.

(ii) Cell Lines and Tissue Culture Medium

The Raji cell line used in this study was obtained from the National Cancer Institute, Bethesda, Maryland, (U.S.A.) (courtesy of Dr. D.V. Ablashi). The cell line, comprising of EBV genome-carrying human lymphoblastoid cells, were cultivated in a culture medium consisted of RPMI 1640 medium (Flow, Irvine, UK) supplemented with L-glutamine (0.2 g/L), inactivated fetal calf serum (10%), streptomycin (100 µg/ml), penicillin (100 IU/ml) and fungizone (50 µg/ml). Cell cultures were mainted at 37 °C in a humidified atmosphere of 5% CO_2 in air. The culture medium was changed every 3 days.

(iii) Inhibition of EBV EA Induction in Raji Cells

Rapidly dividing cells at a density of $1x10^6$ cells/ml were incubated with the plant extracts at various concentrations of 0.02, 10, 20, 40, 80 and 160 µg/ml in the presence of 4 mM sodium n-butyrate (Sigma); each experiment was carried out in duplicate. The cells were incubated for 72 hours in a humidified incubator at 37 °C with 5% CO_2 in air.

The Raji cells were harvested on the third day by centrifugation at 800 rpm for 5 minutes, washed 3X in phospate buffer saline (PBS, pH 7.2), placed on a multi-well teflon-coated slide, dried under cold air, and then fixed in cold acetone (-20 °C) for 10 minutes and used in indirect immunofluoresence assay (Norhanom *et al.*, 1987; Goh *et al.*, 1995; Norhanom and Hazra, 1997).

The fixed Raji cells were overlaid with 20 µl of EBV-positive serum (EA titer 1:20) obtained from nasopharyngeal carcinoma (NPC) patients at Kuala Lumpur General Hospital. The slides were incubated in a humidified chamber at 37 °C for 45 minutes after which they were rinsed with PBS for 10 minutes. The slides were further overlaid with 20 µl of anti-human IgG conjugated to fluorescein-isothiocynate (1:15 dilution), incubated at 37 °C for 45 minutes and again rinsed with PBS. After washing with PBS, the slides were dried under cold air and mounted in glycerol-PBS (9:1) buffer.

(iv) Reading of Slides

The fluoresence (number of positive cells) was counted under the microscope equipped with epi-ultraviolet source, BG 12 exciter filter, BG 38 supressor filter and 530 barrier ocular filter. Cells with intensely brilliant intracellular fluoresence were classified as positive, while cells with dull, diffuse or no fluoresence were scored as negative. The proportion of positive cells were recorded as a percentage of total cells counted. The percentage of inhibition was calculated base on the consideration that the expression (30%) of EBV EA by Raji upon induction with TPA is considered as 0% inhibition.

Negative Control (TPA) = 100% Promotion, 0% Inhibition

Expression % = $\frac{\text{No. of fluorescing cells}}{\text{No. of fluorescing cells in negative control}} \times 100$

Inhibition % = 100% - Expression%

3.4.3 Cytotoxicity

Ideally, a bioassay detecting cell viability should be sufficiently rapid to make handling of large sample size feasible and specific to quantitatively detect viable cells in a mixture of dead and living cells. Viable cell count was done by means of a hemacytometer.

The counting chamber and coverslip of a Neubauer hemacytometer were cleaned with water and alcohol. 0.1 ml of a well mixed Raji cell suspension, already treated with the plant extracts at concentrations of 0, 0.02, 10, 20, 40, 80 and 160 µg/ml and incubated for 72 hours were transferred to tubes with 0.9 ml of 0.4% trypan blue .The chamber of the hemacytometer was then filled with the cells and the cells were examined through a light microscope with a 10X objective. The unstained viable cells were counted in four large squares (1 by 1 mm). Dead cells were stained blue and hence not counted.

The number of living cells in 1 ml of the culture media was calculated using the following formula :

$$\text{Cells per ml} = \frac{\text{No. of cells counted}}{\text{No. of squares}} \times 10^4 \times 10 \times 2$$

10^4: Counting chamber conversion factor

10: Dilution factor with trypan blue

2: volume of original cell suspension in PBS