CHAPTER III
MATERIALS AND METHODS

3.1 Cell lines

Two cell lines were employed; human amniotic fluid cells (AMC-K46) and human Negroid cervical adenocarcinoma cells (HeLa). All of the cell lines were used in semiconfluent conditions (exponential phase) and maintained in cryopreservative condition in deep freezer (-80 °C).

AMC-K46: The AMC-K46 was established and developed from normal male primary amniotic cells which obtained by amniocentesis. The cells were cultured in RPMI1640, 10%FBS, 5%CO_2, 37°C, 95%RH and used in the experiments at the 14^{th} to 18^{th} passages.

HeLa cells: The HeLa is a continuous cell lines which kindly provided by Assoc. Prof. Dr. Watchara Kasinrerk, Department of Clinical Immunology, Faculty of Associated Medical Sciences, Chiang Mai University. The cells were cultured in MEM, 10% FBS, 5%CO_2, 37°C, 95%RH.

3.2 The plants

The following annonaceous plants were used in this research; *Annona reticulata* L., *Annana squamosa* L., *Cananga odorata* (Lam.) Hook. f. & Thomson var. *odorata*, *Cananga odorata* (Lam.) Hook. f. & Thomson var. *fruticosa* (Craib) J. Sinclair and *Melodorum fruticosum* Lour. The plants were identified by Angkhana Inta and the herbarium samples were deposited to the Ethnobotanical Reseach Section, Department of biology, Faculty of Science, Chiang Mai University.

3.3 Biological study

There are two parts of studied plan in this research:

3.3.1 The basic characteristic and karyotype of the cell lines.

3.3.2 The cytotoxicity of the plant crude extracts to the cell lines.

3.3.1 Cell lines characterization .

3.3.1.1 Morphological characterization:

1. HeLa and AMC-K46 (passage 18th) were subcultured, seeded on 10 cm^2 cultured dish and incubated for 24 hours.
2. The fresh cells were photographed by digital camera (OLYMPUS DP12) on inverted microscope.
3. The medium was removed, and the cells were rinsed with PBS.
4. Added 1 ml. PBS+methanol mixture (1:1), left 10 minutes.
5. Replaced with fresh methanol, left 10 minutes.
6. Discarded the methanol and stained with Giemsa for 2 minutes.
7. Diluted the stain with 4 times volume of water, left 5 minutes and rinsed in deionized water.
8. The stained cells (in the cultured dish) could be stored at room temperature for months before the examination under a compound microscope.

3.3.1.2 Growth pattern:

1. HeLa and AMC-K46 were subcultured and seeded on 25 cm^2 flask at $2x10^4$ cells/ml.
2. The numbers of the cells were counted everyday under the OMG-1/100 on inverted microscope (Figure 3.1).
3. The numbers of the cells were plotted against the numbers of the days after seeding and the pattern of cell growth were determined.

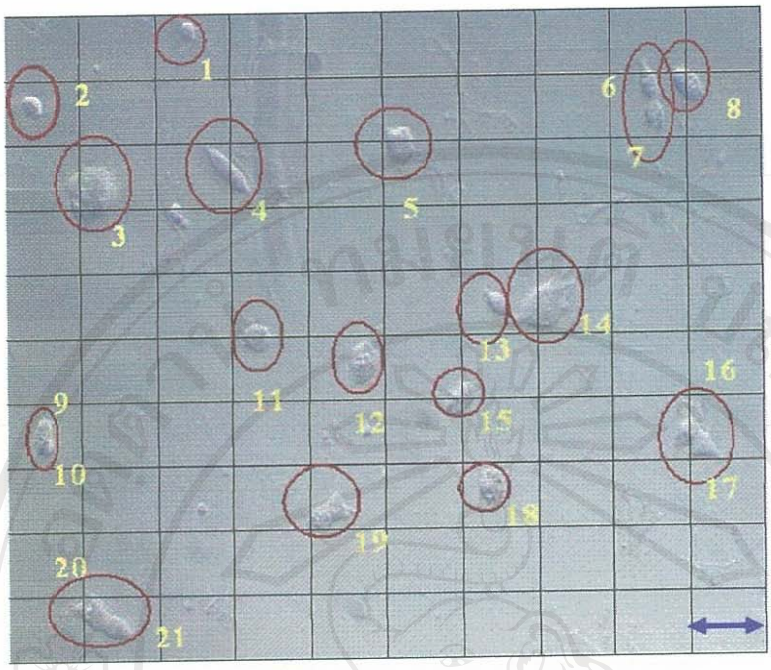

Figure 3.1 The ocular micrometer grid type 1/100(OMG-1/100) as seen from the inverted microscope (Olympus CK40). Twenty-one cells of AMC-K46 were counted as shown in this photograph. (scale bar = 0.1 mm)

3.3.1.3 Karyotype (adapted from Mevatee (1998)):

1. 5 x 10^5 cells AMC-K46 were seeded on 25 cm^2 cultured flask containing 4 ml. cultured medium and incubated for 48 hours.
2. Colcemid (0.05 µg/ml.) was added to the flask and incubated for 30 minutes.
3. The medium was gently removed and kept in a 15 ml centrifuge tube and added 2 ml of 0.05% trypsin to the flask.
4. After 5 minutes of incubation at room temperature, the cells (trypsin-medium mixture) were centrifuged at 1,000 rpm. for 10 minutes. The speed and duration of centrifugation was used along in this method, except for any other specification stated.
5. Added the pellet with 5 ml PBS, resuspended, centrifuged, and then discarded the supernatant.
6. The pellet was resuspended in 5 ml 0.075 KCl (hypotonic solution) then centrifuged, discarded supernatant.

7. Resuspended the pellet in 5 ml hypotonic solution and centrifuged.
8. Added 5 ml freshly prepared ice cold acetic acid-methanol mixture (1:3), then centrifuged at 1,300 rpm for 10 minutes and discarded supernatant.
9. Added 5 ml freshly prepared ice cold acetic acid methanol (1:2) centrifuged and discarded supernatant.
10. Repeated step 8 once again.
11. Discarded supernatant, resuspended and aspirated (avoid bubbles) the pellet in (1:3) acetic methanol to get finely dispersed cell suspension.
12. Drop the cells suspension from approximately 3 inches distance above a pre-cleaned slide by using Pasteur pipette (one drop per slide).
13. Stained the metaphase chromosome with G-banding technique (Mevatee, 1998); briefly, the dried spreading chromosome on a slide was soaked with 0.25% trypsin in normal saline for 30-45 seconds then stained with 10% Giemsa in Weise phosphate buffer for 20 minutes, and photomicrographed under the microscope.
14. The photomicrographs of G-banding chromosome were analysed by the Photoshop program.

3.3.2 The cytotoxicity of the plant crude extracts to the cell lines.

3.3.2.1 Preparation of the annonaceous crude extracts

Bark, leaves and young fruits of the plants were collected (Table 3.1) and dried in a hot air oven at 50°C until the stability of the weight was observed. The dried materials were ground to powder and extracted with 80% ethanol overnight. The ethanolic extracts were filtered, evaporated by rotary evaporators and lyophilized. Dried residues were weighed and stored at -20°C until needed. The crude extracts preparation was summarized as a diagram shown in Figure 3.2.

plants	bark	leaves	young fruits
A. reticulata	*	*	*
A. squamosa		*	*
C. odorata		*	
C. odorata var. *fruticosa*		*	
M. fruticosum		*	

Table 3.1 The plants with collected parts (*) which were extracted and available in this research.

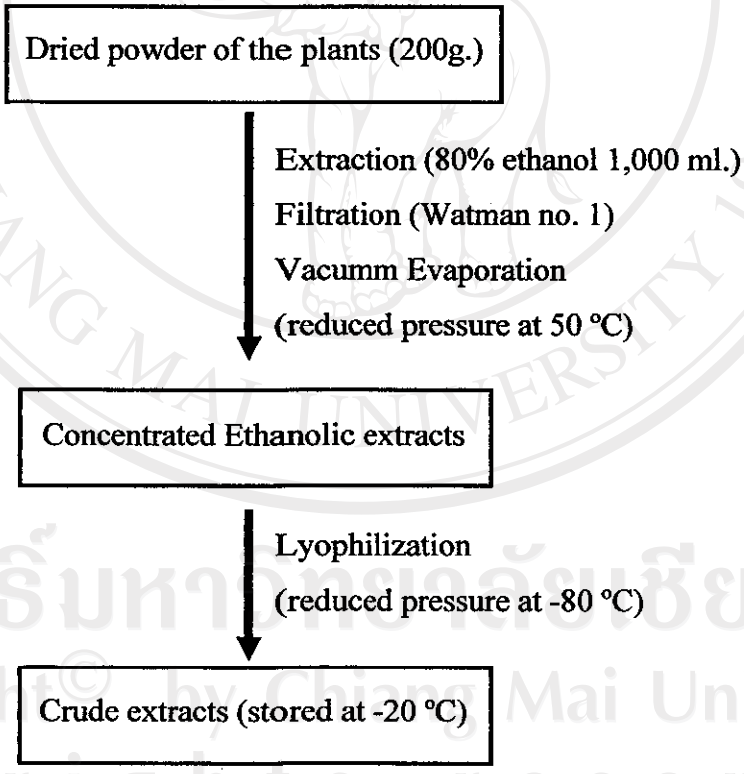

Figure 3.2 Summarized diagram of the crude extract preparation from annonaceous plants.

3.3.2.2 The preparation of the crude extracts for the cytotoxicity assay to the cells.

The dried crude was brought to room temperature and weighted at a µg-base before dissolved in DMSO. The crude-DMSO mixture (CDM) was then diluted in the cell growth medium at a desired concentration prior to used as a freshly crude-working solution (CWS).

3.3.2.3 MTT assay conditions studied:

(1) The dividing and non-dividing density of the cells:

1. AMC-K46 and HeLa were subcultured and seeded in 96 well-plate in 20 innoculum sizes ranging from 500 to $2x10^5$ cells/well. (Figure 3.3).
2. After 24 hours, the suitable cell densities were determined under the inverted microscope for dividing or non-dividing by the criteria of optimum space between the cells for cell division and the contact inhibition of confluent condition, respectively.
3. The plate was then carried on through the MTT assay to obtain the standard curve of the absorbance against the cell densities following Freshney (2000), as briefly summarized below.
   - The 20 µl of MTT dye (10µg/ml) was added to each well and incubated in humidified incubator at 37 °C for 4 hours.
   - Removed the medium and the MTT dye from the wells and added 200 µl DMSO to each well (to dissolve the remaining MTT-formazan crystals), incubated for 10 minutes at room temperature.
   - The absorbance (OD) at 570 nm was recorded with a reference wavelength of 640 nm by the microplate reader (Anthos 2010).

(2) The preparation of the cells on a multiwell plate.

The densities of the cells determined as dividing and non-dividing were prepared and seeded each onto each half of a 96 well-plate (Figure 3.4). The plate was incubated in standard condition (see 3.1) for 24 hours before the MTT assay.

	1	2	3	4	5	6	7	8	9	10	11	12
A	X	X	X	X	X	X	X	X	X	X	X	X
B	X	500	2,000	4,000	6,000	8,000	10,000	30,000	50,000	70,000	100,000	X
C	X	500	2,000	4,000	6,000	8,000	10,000	30,000	50,000	70,000	100,000	X
D	X	500	2,000	4,000	6,000	8,000	10,000	30,000	50,000	70,000	100,000	X
E	X	1,000	3,000	5,000	7,000	9,000	20,000	40,000	60,000	80,000	200,000	X
F	X	1,000	3,000	5,000	7,000	9,000	20,000	40,000	60,000	80,000	200,000	X
G	X	1,000	3,000	5,000	7,000	9,000	20,000	40,000	60,000	80,000	200,000	X
H	X	X	X	X	X	X	X	X	X	X	X	X

Figure 3.3 Diagram representation of a 96 well-plate seeded with triplicate of each of 20 innoculun sizes ranging from 500 to $2x10^5$ cells/well. The PBS was filled in the well with "X", to eliminate the marginal effects.

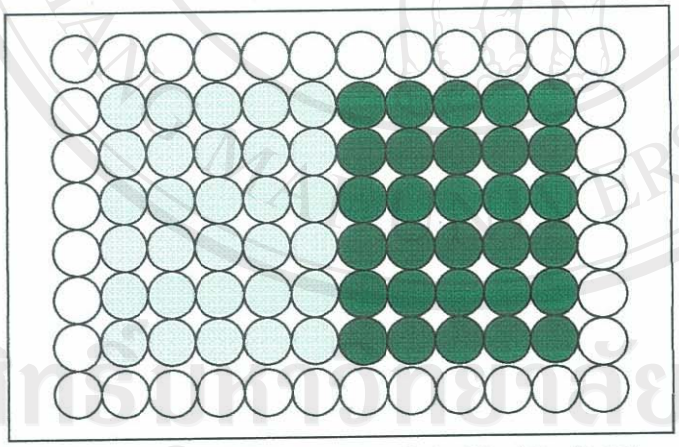

Figure 3.4 A 96 well-plate containing on each half with dividing and non-dividing density of the cells. (= dividing density; = non-dividing density; = PBS for the elimination of marginal effect).

(3) The cytotoxicity assay of SDS:

The SDS (Sodium dodecyl sulfate) was employed in this research as a standard positive control agent. This experiment aimed to determine the MTT_{50} of SDS to the cell lines. The following protocol was applied to the two cell lines as appropriated.

1. A 96 well–plate with the dividing and non-dividing cell densities on each half was prepared as desired previously (Figure 3.4).
2. After 24 hours of incubation, added 200 µl of 50, 70, 100, 120, 130, 150, 170, 200, and 250 µg/ml SDS-medium solutions, to each well with a triplicate of each of the SDS concentration.
3. After 24 hours of incubation, replaced the SDS-medium solution with 200 µl of the fresh medium.
4. Added 20 µl MTT dye (10µg/ml) and incubated for 4 hours.
5. Absorbance (OD) at 570 nm with a reference wavelength of 640 nm was recorded by the microplate reader (Anthos 2010).
6. The % survived cells was determined by the equation below (Pintha, 2003);

$$\% \text{ survived cells} = \frac{\text{mean OD in each group} \times 100}{\text{mean OD in control group}}$$

7. MTT_{50} (the 50% survived cells determined by the OD from the MTT assay) was analyzed by Probit analysis (Priprobit 1.63) with significant value at $p < 0.05$ (Pearson Chi-square Test).

3.3.2.4 The effect of the plant crude extracts

The effects of the plant crude extracts to the cells were evaluated in 3 aspects; the proliferation of the cells, the chromosomal aberration and the cell morphological effect. The proliferation effect or antitumor activity assay of each crude was determined by the MTT assay.

(1) MTT base cytotoxicity measurement:

1. The cells was subcultured and seeded to the 96 well-plate as described in 3.3.2.3 (2) (Figure 3.4) and incubated for 24 hours.
2. Seven concentrations of CWS were desired as appropriated and each of which was added on a triplicated-base treatments to the plate. The SDS at MTT_{50} concentration (see 3.3.2.3 (2)), the 1% DMSO in the cells growth medium and the cell growth medium, was added to the cells as the positive control, the solvent control and the medium control, respectively (Figure 3.5).
3. After 24 hours of incubation, all of the solutions were removed gently and 200µl of the freshed growth medium was added into each well.
4. The plate was then carried on for MTT assay as described in step 3 of 3.3.2.3 (1).

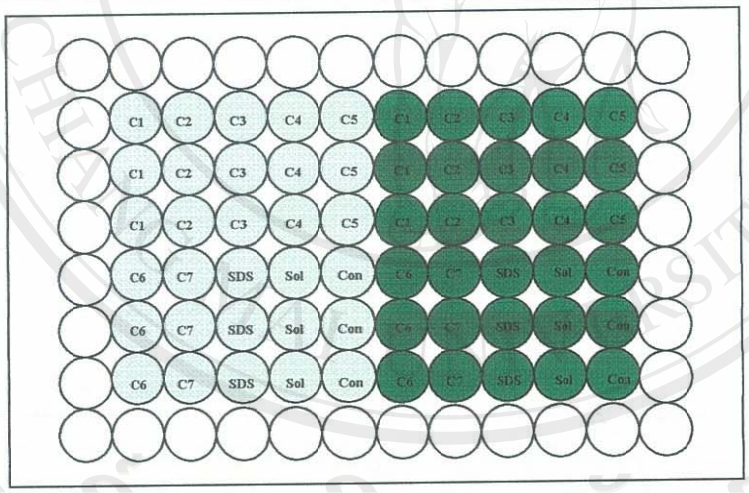

Figure 3.5 A desired 96 well-plate for the MTT assay: ◯ = dividing density; ● = non-dividing density; ◯ = PBS (for elimination of the marginal effect); C1-C7 = the desired concentrations of crude extract; SDS = MTT_{50} of SDS (see 3.3.2.3 (3)); Sol = solvent control; Con = growth medium control.

(2) Chromosome aberration assay:

The chromosome aberration was studied only in the AMC-K46. Three of the most highly active crude extracts to the cell lines were chosen to be used in this experiment (see 4.5.2.2) including: *A. reticulata* (young fruit), *A. squamosa* (young fruit) and *M. fruticosum* (leaves).

1. Eighteen cultured dishes (size of 10 cm^2) were each seeded with $1x10^5$ cells of AMC-K46.
2. After 24 hours of incubation, the dishes were added triplicately with CWS and solvent controls as indicated below;
   (i) Added 1 ml medium contained MTT_{20} concentration of crude extracts of young fruits of *A. reticulata*.
   (ii) Added 1 ml medium contained MTT_{20} concentration of crude extracts of young fruits of *A. squamosa*.
   (iii) Added 1 ml medium contained MTT_{20} concentration of crude extracts of leaves of *M. fruticosum*.
   (iv) Added 1 ml medium contained DMSO 1% (solvent control)
   (v) Added 1 ml completed growth medium (medium control).
   (vi) Added 1 ml medium contained Mitomycin C (MMC) 0.5 µg./ml (positive control)(Klangsinsirikul, 2003)
3. After 24 hours of exposure, the chromosome was harvested as described in 3.3.1.3 (step 3-12).
4. The chromosome aberrations was studied following the methods described by Klangsinsirikul (2003); briefly, the spreading metaphase chromosome were stained by non-banding technique with 10% Giemsa in Weise phosphate buffer for 10 minutes. The Mitotic Index and chromosome aberration were evaluated as followed:

   4.1 Mitotic Index (M.I)

   - The numbers of metaphase cells in 1,000 cells were counted and calculated using the equation below;

$$\text{M.I.} = \frac{\text{metaphase cells in 1,000 cells}}{1{,}000} \times 100$$

$$\text{Decreasing M.I} = \text{M.I.}_{\text{control}} - \text{M.I.}_{\text{treatment}}$$

$$\text{Percentage of decreasing (P-D.M.I.)} = \frac{M.I._{control} - M.I._{treatment}}{M.I._{control}} \times 100$$

4.2 Chromosome aberration screening

- At least 50 spreading metaphases were scored with the following observation terms:
  - the number of chromosome,
  - the percentage of aberration and
  - the type of aberration observed on chromosome and chromatid (deletion, breaking, interchange, fragment, dicentric and ring chromosome).

(3) Morphological studied:

1. HeLa and AMC-K46 was subcultured and seeded in $10cm^2$ culture dishes for 24 hours.
2. Added 1 ml medium containing the MTT_{50} concentration of the effective dose of the crude extracts, 1% DMSO (solvent control) and the complete growth medium (medium control).
3. After 24 hours of exposure followed steps 2-8 of 3.3.1.1.

3.4 Statistical analysis

Mitotic index, the decreasing of mitotic index, the percentage of the decreasing of mitotic index and the number of chromosome aberration in each treatment was analyzed in one-way ANOVA by SPSS statistical program with significant value at $p<0.05$.