CHAPTER 3

MATERIALS AND METHODS

3.1 Vegetable Samples

Table 3.1 lists the 40 plant samples investigated in the present study were randomly choosen. Specimens were purchased from the local market and identified.

3.1.2 Preparation of the Crude Extracts of the Vegetables

One kilogram each of the fresh vegetable was washed in running tap water and dried in hot air oven (Memmert, Germany) at 40°C-60°C for 3-4 days. Dried vegetables were ground into fine powder using a kitchen blender (National). 20.0 g of the dried powdered vegetables were weighed (Mettler, AJ 100) and extracted 24 hours with 200 ml of methanol (Fisher Scientific) and shaken at room temperature (28°C, 250 rpm). Dry methanolic extracts were obtained after removing the solvent by evaporation under reduced pressure using a rotary evaporator (Butchi). The residue was resuspended in 200 ml distilled water and shaken at room temperature (28°C, 250 rpm) overnight. Crude methanol and water extracts were weighed (Mettler AJ 100) and dissolved in dimethysulfoxide (DMSO, Sigma) and water, respectively to form stock solutions at 20 mg/ml. Extracts were and stored at -20°C until use (within two weeks).

Table 3.1 List of selected vegetables being studied

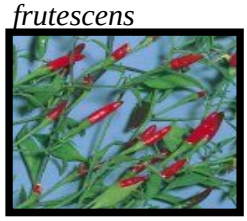
No.	Family name	Scientific name	Vernacular / local name	Common name	Part of vegetable evaluated
1	Liliacea	*Asparagus officinalis* 	asparagus, saparu keras	Asparagas	Whole plant
2	Liliacea	*Allium fistulosum* 	daun bawang	Spring onions	Leaves
3	Liliacea	*Allium tuberosum* 	ku chai	Chinese chive, garlic chives	Whole plant
4	Solanacea	*Capsicum annum* 	chabai, cabai achong, lada merah, chabai seberang, chabai selasih	Pepper/sweet pepper/chili plant	Fruit
5	Solanacea	*Capsicum frutescens* 	Chabai, chabai beruang, chabai rawit, lada merah, lada api, lada kerawit	The bird pepper/little pepper/red pepper/fire peppeer	Fruit

Table 3.1 Continued

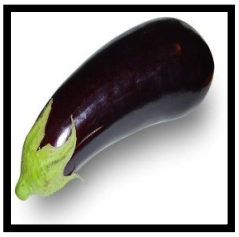
6	Solanacea	*Lycopersicum esculentum* 	tomato	Tomato/love apple	Fruit
7	Solanacea	*Solanum melongena* 	terong	Brinjal	Fruit
8	Solanacea	*Solanum tuberosum* 	ubi benggala, ubi kentang, ubi gendang	The potato	Tubers
9	Cucurbitacea	*Cucumis sativus* 	timun, timun China, mentimun	Cucamber, Chinese timun	Fruit
10	Cucurbitacea	*Cucurbita moschata* 	labu merah, labu perang, ketola, labu manis	Muskmelon, red gourd, chopper gourd, round sweet	Fruit
11	Cucurbitacea	*Lagenaria sceraria* 	labu ayer putih, labu botol	The bottle gourd, calabash cucumber	Fruit

Table 3.1 Continued

12	Cucurbitacea	*Momordica charantia* 	peria, peria laut	Bitter gourd	Fruit
13	Cucurbitacea	*Luffa acutangula* 	petola segi, ketola segi	Loofah	Fruit
14	Compositae	*Cosmos caudatus* 	ulam rajah	Ulam rajah	Leaves

Table 3.1 Continued

15	Compositae	*Lactuca sativa* 	salad, selada	Lettuce	Whole plant
16	Leguminosae	*Archidendron jiringga* 	jering	Black pot	Fruit
17	Leguminosae	*Neptunia prostrata* 	akar kemen hantu, keman ayer, kangkong puteri, tangki, keman gajah	Ghost or wild mimosa, winter mimosa	Leaves
18	Leguminosae	*Pachyrrhizus erosus* 	sengkuwang, bengkuwang, mengkuang, singkong	Yam bean	Tubers
19	Leguminosae	*Psophocarpus tetragonolobus* 	kacang botor, kacang botol, kacang kotor, kacang kelisah, kacang belimbing	Botor bean	Fruit

Table 3.1 Continued

20	Leguminosae	*Parkia speciosa* 	petai, petai papan	Foul-smelling edible seeds	Fruit
21	Leguminosae	*Sesbania grandiflora* 	turi, geti	Turi	Leaves
22	Leguminosae	*Vigna sinensis* 	kacang panjang, kacang belut, kacang perut ayam, kacang putih	Long bean	Fruit
23	Leguminosae	*Pisum sativum* 	kacang puteh	Snow pea, sugar pea	Fruit
24	Leguminosae	*Phaseolus vulgaris* 	kacang buncis	French bean	Fruit

Table 3.1 Continued

25	Amaranthaceae	*Amaranthus gangeticus* 	bayam kadong, bayam merah	Spinach	Whole plant
26	Amaranthaceae	*Amaranthus viridis* 	bayam putih, bayam itik, bayam pasir, bayam monyet	White bayam, duck's bayam, coast bayam, and monkey bayam	Whole plant
27	Malvaceae	*Hibiscus esculentus* 	kacang bendi, sayur bendi, kacang lendir	Ochro, gombo, ladies fingers	Fruit
28	Oxalidaceae	*Averrhoa carambola* 	belimbing buluh, belimbing asam, belimbing besi	Starfruit	Fruit
29	Euphorbiaceae	*Manihot esculenta* 	ubi kayu, ubi benggala, ubi belanda	The cassava manioc and tapioca plant	Tubers

Table 3.1 Continued

30	Portulacaceae	*Portulaca oleracea* 	gelang pasir, rumput beremi, segan jantan	Beremi	Leaves
31	Convulvulaceae	*Ipomoea batatas* 	keladek, keledek, ubi keladek	Sweet potato	Tubers
32	Convulvulaceae	*Ipomoea reptans* 	kangkong, kangkung	Kangkung	Whole plant
33	Umbeliferae	*Apium graveolens* 	saderi, selderi	Celery	Whole plant
34	Umbeliferae	*Centella Asiatica* 	pegaga	Pegaga	Leaves
35	Umbeliferae	*Daucus carota* 	lobak merah	Carrot	Tubers

Table 3.1 Continued

36	Umbeliferae	*Oenanthe javanica* 	shelum, selom, piopo	Shelum	Leaves
37	Chenopodiaceae	*Beta vulgaris* 	bit	Bitroot	Tubers
38	Chenopodiaceae	*Citrus aurantifolia* 	limau	Citrus	Fruit
39	Cruciferace	*Brassica oleracea* var. *alboglabra* 	kai lan, gailan	Chinese cabbage, Chinese brocoli	Leaves
40	Cruciferae	*Petroselium crispum* 	parsley	Parsley	Leaves

3.2 General procedures

3.2.1 Washing of Apparatus

Stringent washing of all glassware were carried out. All glassware were soaked overnight in 7X detergent (Flow Laboratories), followed by soaking overnight in tap water and then thoroughly rinsed in distilled water before drying in a hot air oven (Memmert) at 60ºC.

3.2.2 Sterilization by Dry Heat

Pipettes were sterilized using the dry heat oven at 180ºC for 2 hours (Memmert, Germany).

3.2.3 Sterilization by Autoclaving

Beakers, bottles, swinnex filter units, conical flasks, centrifuge tubes and eppendorf tips were sterilized by autoclaving at 1000 psi at 120ºC for 20 minutes. Before autoclaving, plastic caps of bottles were loosened, syringe without needle and beakers were wrapped with aluminum foil. After cooling, the loose plastic caps were tightened immediately and Eppendorf tips, centrifuge tubes, conical flask, swinnex filter units and beakers were dried in an oven (Memmert) at 60ºC.

3.3 Cell Culture

3.3.2 Cell Lines

The cell lines used in the present study were cervical cancer-derived, HPV 16-containing cell lines, CaSki (ATCC No.CRL-1550) and cervical cancer-derived, HPV 18-containing cell lines, HeLa (ATCC No.CL-2) and normal human lung fibroblast, MRC-5 (ATCC No.CL171). The cell stocks were stored in liquid nitrogen (-196ºC) prior to use.

3.3.3 Revival of Cells

Cryovial tube was removed from the liquid nitrogen and plunged into a beaker of ice. It was then transferred to a 37ºC water bath (Grand Instrument) for quick thawing. The cells were transferred into 1 ml of 20% supplemented RPMI 1640 medium (Rosewell Park Memorial Institute) or with Earle's BSS 2mM L-glutanine EMEM medium (Eagle's Minimum Essential medium) in a centrifuge tube (Felcon, USA) and spun at 1000 rpm (Clements 2000) for 5 minutes for washing. The supernatant was discarded and the pellet resuspended in 6 ml of 20 % supplemented RPMI 1640 or EMEM and incubated in a 25 ml tissue culture flask (Falcon, USA) at 37ºC in a 5 % CO_2 incubator (Shel Lab).

3.3.4 Maintenance of Cells

CaSki and HeLa were maintained in RPMI 1640 medium while MRC5 cells were maintained in EMEM medium in 25 ml tissue culture flask. The cells were incubated in a 5 %

CO_2 incubator at 37ºC. The medium was changed at 3-day intervals or when the colour of the medium changed from red to yellow colour. The culture was routinely checked under an inverted microscope (IMT-2 Olympus, Japan) for fungus, mycoplasm and bacteria contamination. The culture was passaged weekly, and the culture medium was changed once every 3 days.

3.3.5 Subculturing of Cells

Cultured CaSki and HeLa and MRC5 cells attached and formed a single layer in culture flasks. Before subculturing, the medium was removed and the cells were washed twice using phosphate buffer saline (PBS) (pH 7.2). The cells were detached from the flask by incubating cells with 1 ml of 0.25% trypsin-EDTA and 3 ml of PBS (pH 7.2) for 5 minutes after which the flask was sharply tapped to detach the cells. The detached cells were transferred into a centrifuge tube and centrifuged for 5 minutes at 1000 rpm. The supernatant was removed and 3 ml of 10 % supplemented medium was added to resuspend the pellet. The cell suspension was split and transferred into 3 tissue culture flasks each containing 6 ml culture media. The cell cultures were then further incubated in a 5% CO_2 incubator at 37ºC.

3.3.6 Cryopreservation of Cells

For the best survival on thawing, cells should be subconfluent and fed with fresh culture medium (0-40 % RPMI 1640 or EMEM medium) 24 to 48 hours before freezing. The cell suspension was centrifuged at 1000 rpm for 5 minutes using a bench centrifuge (Clements, 2000) and the supernatant was discarded. The cell pellet was resuspended in 3 ml of freezing medium

and the mixture was stored in 3 cryovial tubes (Nunc) and each tube contain 1cm of the mixtured cell pellet. The type of cells, date and subculture number were recorded on the labeled of cryovial tubes using a permanent maker. The vials were then stored in a -70ºC freezer overnight before being transferred into liquid a nitrogen tank (-196ºC).

3.4 Analysis for Cytotoxic Activity of Vegetable Extracts

3.4.2 Cell Line

The cell lines used in the cytotoxic assay were CaSki cells, HeLa, and MRC5.

3.4.3 Serial Dilution of Plant Stock Solution

Aliquots of 10 µl from the vegetables stock solution (20 mg/ml) was diluted in 90 µl of sterile distilled water to produce a stock concentration of 2,000 µl/ml. It was then further diluted with 10% DMSO to provide final concentrations of 100 µg/ml and 10 µg/ml.

3.4.4 Viable Cell Counting/Cell Enumeration

For cell enumeration, cells with confluent monolayer were detached from the flask using 0.25% trypsin-EDTA. The cell pellet**,** obtained by centrifugation at 1000 rpm for 5 minutes, was resupended in 1 ml of 10% RPMI 1640 medium to produce a stock cell suspension. 100 µl of the stock cell suspension was then transferred into a provial with 900 µl of 0.4% of trypan blue and mixed well. 20 µl of suspension with dye was subsequently loaded exclusion onto a

haemocytometer (Scherf). The heamocytometer was examined under a microscope and the unstained living cells were counted. The dead cells were stained with trypan blue while the living cells were not stained (Appolinary, 2000).

3.4.4 Treatment of Cell Lines with Plant Extracts

Briefly, a cell concentration of 30,000 cells/ml were plated in 96 well microtitre plates in a volume of 190 µl. The plates were incubated in a CO_2 incubator at 37°C for 24 hours to allow the cells to adhere and achieve 70% confluence at the time of the addition of the vegetable extracts. After 24 hours, the crude extracts were then added to the wells at varying concentrations (1, 10, 25, 50, 100 µg/ml). Doxorubicin was used as the positive control and the negative control were wells with untreated cells (without extracts). The plates were further incubated for 72 hours.

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

$$\text{Cells per ml} = \frac{\text{No.of viable cells counted}}{\text{No.of square}} \times 10^4 \times 1.0 \times 0.9$$

Counting chamber Conversion factor

Volume of original cell suspension

Dilution factor with Trypan Blue

After 72 hours of incubation, the growth medium was removed and replaced with 200 µl of medium containing 50 µg/ml neutral red before leaving to recover for 3 hours to allow for dye

uptake into the lysosomes of viable and uninjured cells. The media was removed after 3 hours and cells were rapidly washed with the neutral red washing solution. The dye was eluted from the cells by adding 200 µl of neutral red resorb solution and incubation for 30 minutes at room temperature with rapid agitation on a microtitre plate (LT BioMax 500) for 15 minutes. The absorbance (OD) was measured at 540 nm using ELISA reader (Titertek Multiscan MCC/340, Findland). All tests were carried out in triplicates to determine the cytotoxic activity of each vegetable extracts. The percentage of inhibition by each vegetable extract was calculated according to the following formula:

$$\% \text{ of inhibition} = \frac{\text{OD control} - \text{OD samples}}{\text{OD control}} \times 100$$

The average data from triplicates for each vegetable extract concentration was expressed in term of killing percentage relative to negative control. Dose-response curve were plotted to determine the IC_{50} values for all the extracts. The IC_{50} value was then extrapolated from each curve. IC_{50} value refers to the effective dose (concentration of extracts in µg/ml) that inhibits 50% of cell growth. Extracts having an IC_{50} value equal to or less than 20 µg/ml are considered active for cytotoxicity assay against cells (Geran *et al*., 1972; Chiang *et al*., 2003).

3.5 Analysis for Anti-HPV 18 E6 Oncoprotein Activity of Vegetable extracts

3.5.2 Cell Lines

The cell line used in this study was the cervical cancer-derived, HPV-16 containing CaSki cells.

3.5.3 Serial Dilution of Vegetable Stock Solution

Five different concentrations were used to study the anti-HPV 16 activity of vegetables extracts. Aliquots of 180 µl from each vegetable stock solution (20 mg/ml) was diluted in 2.82 ml of sterile distilled water to produce a stock concentrations of 100, 50, 25, 10, 1 µg/ml. The calculations to produce stock concentrations of 100, 50, 25, 10 and 1 µg/ml are shown in Figure 3.1.

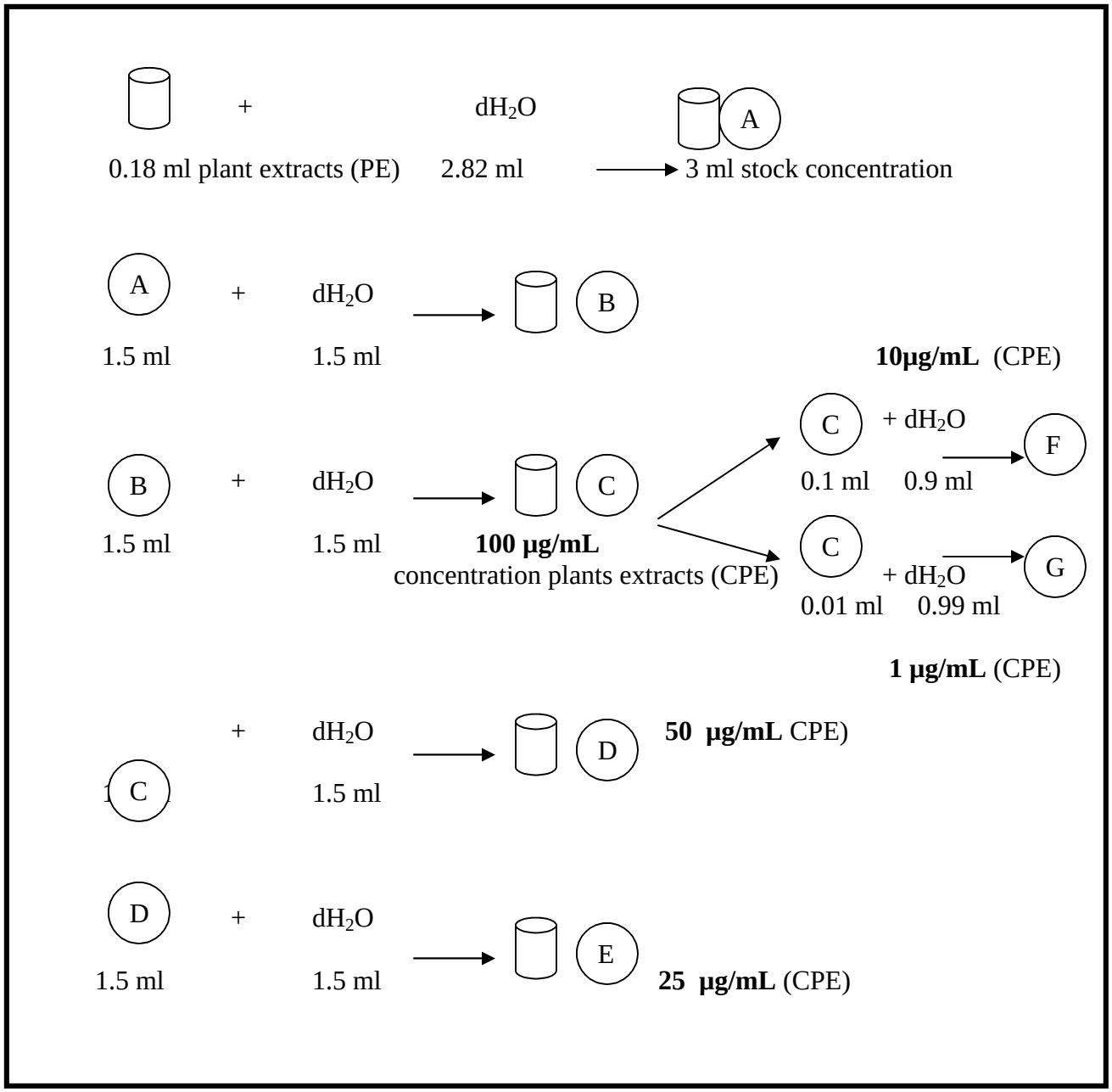


Figure 3.1: The calculations to produce stock concentrations of 100, 50, 25, 10 and 1 µg/ml

3.5.4 Incubation of CaSki Cells with Vegetable Extract

CaSki cells were cultured in 25 cm^3 tissue cultured flask, and maintained in 10% supplemented RPMI 1640 medium. After 24 hours, the cells were treated with different concentrations of the crude vegetable extracts (1, 10, 25, 50, 100 µg/ml) and incubated at 37°C, 5% CO_2 incubator for another 72 hours. Negative control consisted of CaSki cells without addition of any vegetable extracts. Positive control consisted of CaSki cells were treated with Doxorubicin.

3.5.5 Fixation of CaSki Cells onto slide

At the end of the incubation period (72 hours), the culture media were removed from the flask and cells were trysinized and detached from the flask. The cell pellet was obtained by centrifugation 1000 rpm for 5 minutes and suspended in 1 ml of PBS. One drop of the cells suspension in PBS (pH 7.6) was placed in each well of the Teflon coated slide. Slides were left to dry at room temperature for 1 day. The slide were then fixed with cold acetone (-20°C) for 8-15 minutes and kept at -20°C prior to immunocytochemical detection.

3.5.6 Detection of Human Papilloamvirus (HPV) E6 Protein Using 3- Step Indirect Avidin-Biotin Immunoperoxidase Method

The immunocytochemistry technique was carried out using the Labelled Streptavidin Biotin (LSAB**)** peroxidase kit and the DAB substrate system (Dako, Japan) according to the specifications described by the manufacturer with some modifications. All washing steps

required constant shaking and incubations with reagents were carried out in a humidified chamber. Before slide rehydration, the slides were left at room temperature for a few minutes. Slides used in this assay are those which contain cells treated with vegetable extracts and those which contain CaSki cells not treated with vegetable extracts.

The cells were rehydrated in decreasing concentrations of ethanol (Scharlau)-100%, 95%, 90%, and 80% at 2 minutes each, followed by a 5 minutes-wash with PBS (pH 7.6) on the rocker platform (Bellco Biotechnology). The surrounding areas of each well were blotted dry using filter paper (Whatman). Incubation with a few drops per well of 3% hydrogen peroxide (BDH) was carried out for 10 minutes to remove endogenous peroxidase activity. Cells were then rinsed in PBS for 5 minutes as described before. After the areas surrounding each well were blotted dry, 30 µl of anti-HPV E6 16 monoclonal antibody (Chemicon), diluted 1:40 in sterile PBS were added to selected wells, while the remaining wells were added with 30 µl of distilled water. These cells not incubated with monoclonal antibody serum as negative controls. Cells were then incubated for 1 hour at 37°C. Following this, the slides were washed twice in PBS for 15 minutes each before the addition of secondary antibody (biotinylated link anti-mouse and anti-rabbit immunoglobulin, Dako) into all wells. Incubation was allowed to proceed for 10 minutes at 37°C. The slides were then washed again in PBS for 5 minutes before incubation with streptavidin-horse radisd peroxidase (Dako) conjugate for 10 minutes at 37°C. The cells were then washed in PBS for 5 minutes. The cells were then incubated with 50 µl/ml of 3'-diaminobenzidine tetrahydrochoride (DAB) (Dako) at 37°C for 10-20 minutes during which the colour reaction was monitored until the desired colour intensity developed.

The slides were then rinsed with distilled water, counterstained with Mayer hematoxylin solution (Fluka) for 2 minutes, rinsed again with distilled water and immersed into ammonia solution (Systerm) for 10 seconds. After the final rinse with distilled water, the slides were mounted with pre-warmed glycergel (56°C) (Dako). The slides were then left to dry in the dark before being analysed under the light microscope (Olympus).