CHAPTER 5

DISCUSSION AND CONCLUSION

Natural products research continues to provide a tremendous variety of lead structures which are used as template for the development a new drugs by the pharmaceutical industry. Advances in bioassay technology and in chemical methodology have combined to make natural products a cost effective source.

The screening of natural products is one of the earliest steps in drug discovery, namely "lead" identification. A lead compound, as the term is currently used, is a compound with many of the characteristics of a desired new drug which will be used as a model for chemical modification (Borris, 1996).

Natural occurring compounds especially those derived from consumable plants including vegetables are ideal and effective chemopreventive agents because they are safe and non-toxic to human for long-term application (August, 2003). Chemical chemoprevention is a concept defined as the prevention of cancer by the administration of natural or synthetic pure chemical. Some natural compounds block, reverse or prevent the sequential event of carcinogenesis of invasive cancer (Greenwald *et al.*, 1990; Colic *et al.*, 2000).

The present study investigated the cytotoxic and anti-HPV 16 E6 oncoprotein activities of crude extracts of selected vegetables against two cervical-derived cancer cell lines. The cytotoxic activity of crude extracts from selected vegetables, were evaluated

using the Neutral Red cytotoxic assay. The anti-HPV 16 E6 oncoprotein activity was evaluated using, the 3-step Indirect Avidin-Biotin Immunocytochemical technique. Crude methanol and crude water extracts from 40 selected vegetables were prepared. The extracts were then analysed and evaluated separately for their potential cytotoxicity against HeLa, CaSki and MRC5 cell lines and anti-HPV 16 E6 oncoprotein activity against cervical cancer-derived, HPV 16 E6 containing, CaSki cell line.

5.1 **Preparation of Crude Extracts**

Forty selected vegetables were extracted successively using methanol and water in a cold extraction method. This method is widely used for the extraction of plant material using particular solvent and is often being applied when exhaustive sequential extraction of a ground plant material with a range of solvents of increasing polarity is desired (Houghton and Raman, 1998). In the present study, as mentioned earlier, cold extraction method (28°C or at room temperature) was used. The cold extraction method enables unstable or heat-labile compouns to be retained. The compounds are otherwise be destroyed if the hot extractions method is used.

The extraction procedure generated two crude extracts from each vegetable species. Results showed that the yield percentages from methanol extracts were found higher as compared to the water counterparts. Similar results were reported by Yen and Duh (1996), when the polarity of the solvent increases, yield of extract will also increase. The high polarity methanol and water is adapted to extract components such as sugar, amino acids and glucoside from samples (Houghton and Raman, 1998). Different polarity of extraction solvent results in different compounds being extracted out as "like dissolve like". A polar solvent is more effective and referable than water when attempting to remove polyphenols (Krinsky, 1992).

In the present study, evaporation under reduced pressure by rotary evaporator used to concentrate the methanol extracts. This method has a number of limitations. Volatiles from the extracts may be loss, particularly where heat is applied. To prevent such loss, 40°C-60°C was applied in the present study. It is difficult to concentrate aqueous solvent unless a very low pressure can obtain. The freeze-drying technique used for aqueous samples and generally gives a drier and less sticky residue. In most cases the dry residue is a fluffy solid. To prevent the dry residue from being sucked out, the frozen extracts were covered by filter paper and series of small holes made in the film before the samples were placed in the freeze-drier (Houghton and Raman, 1998).

In the present study, the crude methanol extracts which contain mainly polar compounds were subsequently dissolved in dimethylsulfoxide (DMSO) due to its poor solubility in aqueous solution. While, crude water extracts were dissolved in distilled water. 1 % DMSO is the most common solvent used to dissolve a low polarity crude extract and is usually non-toxic to the cells (Houghtan and Raman, 1998). According Riddle *et al.*, (1986), the final concentration of DMSO used was 1%v/v or below to ensure the growth of culture is not affected.

5.2 Aseptic Techniques

Culture techniques must be carried out under a strict septic condition, because animal cells grow much less rapidly than many of the common contaminants, such as bacteria, mold, and yeasts (Freshney, 2000). In this study, aseptic conditions were ensured with the use of laminar-flow hood. Flask, dishes and boxes that were brought into the laminar-flow or other sterile work station were thoroughly swabbed with 70% alcohol before use. In the present study any spillage was immediately moped up and the area also swabbed with 70% ethanol.

All apparatus (beakers, bottles, swinnex filter units, conical flasks, centrifuge tubes and eppendorf tips) were sterilized by autoclaving at 1000 psi at 120°C for 20 minutes. These apparatus were placed in a hot air oven to dry any water residue left. This procedure usually destroyed microorganism in all apparatus during autoclaving.

Glass pipettes were used in the present study because they are significantly cheaper but they have to deplugged and replugged each time after using. Glass pipettes in pipette canisters were sterilized using dry heat in a hot air oven at 180°C for 2 hours, avoiding the possibility of both chemical contamination from steam condensate and corrosion of pipette cans. To retard growth of microorganism, most tissue culture reagents (serum and trypsin) were stored at -70 ° C and culture media were kept at 4°C.

5.3 The Neutral Red Cytotoxic Assay

Cytotoxic screening models provide important preliminary data to help select vegetable extracts with potential antitumour properties for future work as a part of a permanent screening program for natural products with anticancer properties as the name implies *in vitro* cytotoxic screening utilized cell lines. Cytotoxic assay is economic, rapid, standardized, sensitive and then well known quantitative colorimetric methods (Borenfreund and Puerner, 1985). The sensitivity of the cytotoxicity assay differs depending on the different mechanisms, which lead to cell death. Some of these differences might be minimized by longer incubation with the agents, but in every case it is important to have a close look on the expected mechanism to get the right decision for or against an assay.

There are various assay methods for evaluating the effects of chemical on cultured cells. Cytotoxic assay include LDH (lactate dehydrogenase) assay, MTT (tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and neutral red cytotoxic assay.

Measuring the lactate dehydrogenase (LDH) release from lysed tumour cells has the same limitation as vital dry exclusion because they both measure necrotic cells with damaged membranes (Decker and Cohmann, 1988). The LDH assay give satisfactory responses by using cell membrane damaging agents like triton X-100, but can be misleading if the toxic agent only influences intracellular activities e.g. sodium azide which inhibits the respiratory chain (Weyermann *et al.*, 2005).

The MTT assay is simple to use. Using this assay, the metabolic activity of the mitochondria can be determined. Unfortunately, this assay shows more or less the same disadvantages as the LDH assay (Weyermann *et al.*, 2005). This method is a rapid

colometric assay based on tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide, which is cleared and converted to a blue formazon by the mitochondrial dehydrogenases in active motochondria, but this method effect decrease with exposure time. No toxicity is seen in cells exposed 24 hours and above (Komissorava *et al.*, 2005). Therefore, the MTT assay seemed to be more sensitive using 24 hours exposure.

The *in vitro* neutral red cytotoxic assay is a rapid and simple assay based on the incorporation of NR dye into lysosome of viable cells and can conveniently carried out in 96-well microtitre plates (Borenfreund and Puerner, 1986). Neutral red is an inexpensive possibility to measure cell death. In some cases, this assay is less sensitive (excitoxic model) and not recommended in ion channel studies. This assay can be used to measure relative cytotoxicities of a spectrum of agents including surfactants, pharmaceutical, industrial chemicals and aquatic pollutants (Borenfreund *et al.*, 1988).

It is known that different cell lines might exhibit different sensitivities towards a cytotoxic compound. Therefore the use more than one cell lines was considered necessary in the detection of cytotoxic compounds. Bearing this in mind, two cervical cancer-derived cell lines of different types HPV; HeLa (HPV 18), CaSki (HPV 16) and human lung fibroblast (MRC5) as a normal cell line (non-cancerous cell lines) were used to evaluate the cytotoxic potentials of the vegetable extracts in the neutral red cytotoxic assay method.

5.4 Screening of Cytotoxic Activity in Selected Vegetable Using *In vitro* Neutral Red Cytotoxic Assay

Several new approaches in cancer prevention strategies based on knowledge of biological processes have been developed and tested (Fujiki *et al.*, 1997; Morrow and Cowman, 2000, Jiade, 2004). Many naturally occurring agents have been used for the prevention and treatment of cancer (Sinha, 2003), which can function as cancer chemopreventive agents because they are safe and non-toxic to human for the long term application (August, 2003).

Some agents such as curcumin and resveratrol act as both blocking and suppressing agents (Surh, 1999). Different phenolic substances present in various types of vegetables, fruits and plants possess antioxidant and anti-inflammatory properties. Blocking agents such as isothiocyanates, ellagic acid and flavonoids are able to prevent initiation by either inhibiting the formation of carcinogens from precursor molecules or hindering carcinogens from interacting with cellular target molecules (Surh, 1999). On the other hand, suppressing agents such as β -carotene, gingerol, epigallocatechin-3-gallate (EGCG), are thought to prevent carcinogenic expression of cells in promotion or progression stage (Surh, 1999).

In the present study, 40 vegetable extracts were evaluated for their cytotoxic capacity against cervical cancer derived cell lines (HeLa and CaSki) and and the human lung fibroblast-derived cell lines, (MRC5). The results obtained from NR assay were expressed as IC_{50} values which refer to the effective concentrations of extracts (µg/ml), to inhibit the proliferation of cells by 50%. In the present study, the IC_{50} values was established using five different concentrations from each extracts (1, 10, 25, 50 100 µg/ml) tested separately HeLa, CaSki and MRC5. The IC_{50} values were extrapolated from the

dose-response curves, plotted from percentage inhibition values. Vegetable extracts having an IC₅₀ value less than 20 μ g/ml is considered active (Geran *et al.*, 1972).

In the present study, neutral red cytotoxic assay was performed after 72 hours of treatment of selected vegetable crude extracts on HeLa, CaSki and MRC5 cell lines. According Riddle *et al.*, (1986), this test period 72-hour was recommended as compared to below 48 hours incubation with test material. This is because the latter may give an unacceptable number of false negative indications of cytotoxicity. The neutral red assay also depends on the increasing number of cell and so a short incubation period (24 hours) is not enough to discriminate between chemicals which have genuinely different cytotoxicities. Not all treatment in NR assay performed in 24 hours, some bioactive compounds may need a longer time to express their cytotoxic effects as they require metabolic activation. Similarly observation was reported by Kamissarova *et al.*, (2005), who evaluated the effects of exposure time on arsenite cytotoxicity using the NR assay. They noted statistically significant increases in cell viability after a 24 hours exposure to arsenite concentrations. These effects diminished with longer incubation times. Toxicity increases with time of exposure, especially between 24 and 48 hours.

Cultured cell lines used for this assay are normally allowed to be 60-70% subconfluent. This is to ensure that the cells are fully exposed to the testing agents (vegetable extracts). At this time, the fresh serial diluted extracts were prepared daily and added to the cells culture at varying concentration (1, 10, 25, 50, 100 µg/ml). Incubation was carried out without medium change for 3 days.

In 1985, Borenfreund and Puerner reported that NR-medium need to be preincubated in the dark place at 37°C to remove fine precipitate and dye crystals which might appear in the mixture. The deposition of NR crystal onto the cells cultured will interfere the assay and gave false-positive results. Therefore in this study, the 10% NR-medium was preincubated overnight at room temperature in the dark. The medium was then centrifuged twice for 10 minutes each at 1000 rpm twice before use on cells to facilitate removal of precipitate or crystal. After a 3-hour incubation period, cells were rapidly rinsed with washing solution to eliminate extracellular NR, as well as to prevent detachment of cells during the subsequent extraction procedure.

5.4.1 Family Liliacea

In the present study, three species from family Liliacea, namely Asparagus officinalis, Allium fistulosum, Allium tuberosum were evaluated for their cytotoxic activities against HeLa and CaSki and MRC5 cells.

Results revealed that extracts of *A. officinalis* were found to exhibit effective cytotoxic activity against CaSki in both crude methanol and water extracts with IC₅₀ values 7.8 μ g/ml and 16 μ g/ml respectively. The finding of the present study supports the finding of other studies. The extracts have been demonstrated to possess certain biological activities including cytotoxic and antiviral properties (Wong *et al.*, 2002; Hibasami *et al.*, 2003 and Chin, 2006). Shao *et al.*, (1996) found the methanol extract of the *A. officinalis* significantly inhibited human leukemia HL-60 cells. Among the asparagus, hydroxycinnamic acid, especially ferulic acid, may be beneficial in the prevention of disorder linked to oxidative stress including cancer (Zhao and Moghadasian, 2008). Both

methanol and water extracts of *A. officinalis* were not active in suppressing the proliferation rate of HeLa cell and only crude water extract were not cytotoxic against MRC5 cells.

In the present study, it was found that for the *A. fistulosum* samples, only crude methanol extracts were found active with IC_{50} value of 17.8 µg/ml. Water extract of *A. fistulosum* was found to be not cytotoxic against HeL, CaSki and MRC5 cells. On the other hands, only water extracts of *A. tuberosum* were not cytotoxic against HeLa, CaSki and MRC5 cells. Kim *et al.*, (2008) suggested that tiosulfinates from *A. tuberosum* to be cytotoxic and induced apoptosis in human prostate cancer cells.

No data related to cytotoxic activities of *Asparagus officinalis, Allium fistulosum, Allium tuberosum* against HeLa, CaSki and MRC5 cells had been reported. One study reported that Allium vegetables showed an inverse relationship with overall cancer risk (Greenwald *et al.*, 2001). It is necessary to evaluate the cytotoxic effect of these Liliacea extracts against other types of human cancer in order to evaluate possible cell-type selectivity.

5.4.2 Family Solanacea

Five selected vegetables from the Solanacea family were tested for their cytotoxic potential against HeLa, CaSki and MRC5 cells. They were *C. annum (lada merah), C.*

frutescens (cili padi), L. esculentum (tomato), S. melongena (terung) and S. tuberosum (kentang).

Observation in the present study showed that 4 out of 10 extracts namely methanol and water extracts of *C. annum*, methanol of *C. frutescens* and *L. esculentum* were found to be cytotoxic of HeLa cell lines. Only 3 crude extracts were found effective against CaSki cell lines. They were water extracts of *C. annum* and methanol extracts of *S. melongena* and *S. tuberosum*. Other researchers reported that *S. tuberosum* significantly inhibited the development of breast cancer cells grown in monolayer culture (Hakimuddin *et al.*, 2004; Lee & Zhu, 2006; Neto, 2007 and Thompson, 2009). None of the extracts are active against MRC5 cell lines. Overall results indicate that *C. annum* showed the best cytotoxic affect against cervical cancer cell lines compared to other Solanacea extracts evaluated here. No previous data to date has been reported on the cytotoxic activities of these vegetables against these cell lines.

5.4.3 Family Cucurbitacea

C. sativus, *C. moschata*, *L. sceraria* and *M. charantia* from the family cucurbitacea were evaluated for their cytotoxic potentials against HeLa, CaSki and MRC5 cells. The present findings suggest that water extracts of *L. sceraria* with IC₅₀ value 6 μ g/ml were potent in cytotoxic activities against HeLa cell lines while methanol extract of *C. moschata* were found to be active in retarding the proliferation of CaSki cell lines with IC₅₀ values 19.8 μ g/ml and 14.1 μ g/ml respectively. On the other hand, the both methanol extracts of *C. sativus*, *M. charantia* and water extracts of *M. charantia* were found to be not cytotoxic against all cell lines tested in present study. Similarly Norhanom *et al.*, 1999 reported that

crude methanol extracts of *M. charatia* were found not cytotoxic against EBV activation in Raji cells. In comparison, other researchers have shown that aqueous extracts of *M. charantia* caused inhibition of prostatic adenocarcinoma growth and exerted cytostatic as well as cytotoxic activities against human leukemic lymphocytes (Groverand and Yadav, 2004).

5.4.4 Family Composiateae

C. caudatus is a popular *ulam* and is also known as *ulam rajah*. In traditional medicine, *C. caudatus* is used for cleansing blood and to strengthen the bones. *L. sativa* is known as salad or salada and usually eaten raw as *ulam*. In the present study, *C. caudatus* and *L. sativa* were evaluated for their cytotoxic activity against CaSki, HeLa and MRC5 cell lines.

Crude extracts of *C. caudatus* and *L. sativa* demonstrated varying degrees of cytotoxicity against HeLa cells. The crude methanol extracts of *C. caudatus* was found to be cytotoxic against HeLa cells, while the crude water extracts of *C. caudatus* was found to be cytotoxic against CaSki cells. In contrast, both methanol and water extracts of *L. sativa*, tested were found not to be cytotoxic against HeLa and MRC5 cells, with IC₅₀ values more than 20 μ g/ml. No report of cytotoxic activities on CaSki, HeLa and MRC5 cell by *C. caudatus* and *L. sativa* extracts have been previously documented.

5.4.5 Family Leguminosae

Archidendron jiringga, Neptunia prostrata, Pachyrrhizus erosus, Psophocarpus tetragonolobus, Parkia speciosa, Sesbania grandiflora, Vigna sinensi, Phaseolus vulgaris and Pisum sativum were evaluated for their cytotoxic potentials on HeLa, CaSki and MRC5 cells.

Among the 9 Leguminosae being evaluated in this present study, only crude methanol extracts of *V. sinensis* was found to show potent cytotoxic effects against HeLa cell lines. Data obtained showed that the crude methanol and water extracts of, *Neptunia prostrata, Pachyrrhizus erosus* and *Parkia speciosa* in this family were not cytotoxic against HeLa cells. On the other hand, when tested against CaSki cell lines, both crude methanol and water extracts of *N. prostrata* and *V. sinensis* showed effective cytotoxicity.

No data related to cytotoxic activities against the HeLa, CaSki and MRC5 cells of the Archidendron jiringga, Neptunia prostrata, Pachyrrhizus erosus, Psophocarpus tetragonolobus, Parkia speciosa, Sesbania grandiflora, Vigna sinensi, P vulgaris and Pisum sativum had been done previously. In a different study Norhanom *et al.*, (1999) reported that crude chloroform extracts of *P. speciosa*, *A. jiringga* and *V. sinensis* to inhibited EBV activation in Raji cells. Crude methanol extracts of *A. jiringga* were shown to be cytotoxic against EBV. On the other hand, crude petroleum ether extracts of *P. speciosa* showed cytotoxic against EBV in Raji cells.

5.4.6 Family Amaranthacea

Amaranthus gangeticus and Amaranthus viridis from the family Amaranthaceae were evaluated for their cytotoxic potential on HeLa, CaSki and MRC5 cells. Only the

crude methanol extracts of *Amaranthus* were found to be cytotoxic against CaSki and HeLa cells giving IC_{50} values less than 20 µg/ml. The water extracts on the other hand, were not cytotoxic against the cell lines evaluated.

Other researcher has evaluated the cytotoxic potential of *Amaranthus* species in other cell lines. Sani *et al.*, (2004), reported cytotoxic assay using MTT assay. In that study, aqueous extracts of *A. gangeticus* was shown to be cytotoxic against liver cancer cell line (HepG2) and breast cancer cell line (MCF-7). The IC₅₀ values were reported to be 93.8 μ g/ml and 98.8 μ g/ml for HepG2 and MCF-7, respectively. The cytotoxic effect was also observed in colon cancer cell line (Caco-2), but lower percentage compare to HepG2 and MCF-7. For normal cell line (Chang liver), there was not cytotoxic effects. Similar results were reported by Temple and Basu, (1988) that *Amaranthus gangeticus* (*bayam pasir*) and *A. viridis* (*bayam merah*) have been found to contain various phytochemical with possess anticarcinogenic activity. Carotenoid from *Amaranthus* species have been shown to protect against cancer, increase immune system, decrease nuclear damage and stop mutagenesis (Ong, 2003).

5.4.7 Family Convolvulaceae

Ipomea batatas also known as *keledek* and *Ipomea reptans* is know *kangkung* from Family Convulvulacea are widely used in Malaysian cuisine. In the present study, only crude methanol extracts of *I. reptans* showed to be cytotoxic activity against HeLa and CaSki cells with IC_{50} values of 5.9 µg/ml and 17.8 µg/ml, respectively. No reports on cytotoxic activities against HeLa, CaSki and MRC5 cells of *I.reptans* have been previously documented.

Water extract of *I. reptans* were found not to be cytotoxic on cancer cells were tested in this study. In another study, Norhanom *et al*, 1999, found that crude chloroform extracts of *I reptans* showed moderate activity against EBV activation in Raji cells. On the other hand, Saha *et al.*, (2008) evaluated the antitumour activity of methanol extracts of *Ipomoea reptans* on Ehrlich's Ascites Carcinoma (EAC) model in mice. Treatment with *I. reptans* significantly reduced tumour volume and viable cell count compared to those of EAC control mice. Experimental results revealed that *I. reptans* possessed significant cytotoxic activity indicating it to be a potent antitumour agent. In the *in vitro* cytotoxic study, I. *reptans* showed direct toxicity on the EAC cell lines, in a concentration-dependent manner in the dose range of 100-500 μ g/ml, and the IC₅₀ value was found to be 281.15 μ g/ml.

In comparison, no cytotoxicity observed in this study of crude methanol *I. batatas* against HeLa cells and both methanol and water extracts against Caski cells. The crude water extracts and both methanol and water extract of *I. batatas* were found not to be cytotoxic against HeLa and MRC5 cells. This is not in agreement with Ong (2003) who reported that carotenoid from *I. batatas* may help protect against cancer, increase immune system, decrease nuclear damage and stop mutagenesis (Ong, 2003). Further study is needed to evaluate the cytotoxic potential of *I. batatas* against other cancer cell lines.

5.4.8 Family Umbeliferae

Apium graveolens, Centella asiatica, Daucus carota and Oenanthe javanica from the family Umbeliferae were evaluated for their cytotoxic potentials against HeLa, CaSki and MRC5 cells. *Centella asiatica (pegaga)* is one of the most popular ulam consumed by the Malays. It is believed to stimulate appetite and aids in digestion (Joseph *et al.*, 2005). In the present study C. asiatica extracts were not to be cytotoxic against CaSki, MRC5 and HeLa cells. In another study, all crude extracts of C. asiatica showed not cytotoxic against EBV activations in Raji cells (Norhanom et al., 1999). Other investigators reported that C. asiatica extracts possess very high antioxidant activity (Joseph et al., 2005; Gupta and Plura, 2006) and antipoliferative properties (Yoshida et al., 2005). The extract of C. asiatica has certain bioactive terpene acids such as asiatic acid, medecassic acid and their respective glycoside, asiaticoside, madecassoside (Inamdar et al., 1996). In primary screening an ethanolic extracts of the plant showed significant wound healing activity. Asiaticoside showed a promising wound healing activity whereas madecassoside was found to be inactive (Shukla et al., 1999). Asiatic acid decreased the viability and induced apoptosis in human melanoma SK-MEL-2 cells (Park et al., 2005).

Oenanthe javanica is known as *selom*. The Department of Food Science and technology in South Korea reported that this vegetable has antihypertensive property (Joseph *et al.*, 2005). Both methanol and water extracts of *O. javanica* were evaluated for their cytotoxic potential against HeLa, CaSki and MRC5 cells. Results revealed that methanol extracts were found not to be cytotoxic activities against HeLa, MRC5 and CaSki cells.

Observations made in present study showed that only extracts of *D. carota* were not cytotoxic activities against CaSki, HeLa and MRC5 cells. Numerous researchers have found that carotenoid from *D. carota* that may help protect against cancer, increase immune system, decrease nuclear damage and stop mutagenesis (Ong, 2003). Therefore it is necessary to evaluate the cytotoxic effect of *D. carota* extracts against other types of human cancer.

5.4.9 Family Malvacea, Oxilidacea, Euphorbeacea, Portulacea, Chenopodeacea, Rutacea and Cruciferace

H. esculentus from the family Malvacea, *A. carambola* from the family Oxilidacea, *M. esculenta* from the family Euphorbiaceae, *P oleracea* from the family Portulacea, *B. vulgaris* from the family Chenopdeacea, *C. aurantifolia* from the family Rutacea and *B. oleracea* var. *alboglabra* and *P. crispum* from the family Cruciferacea were evaluated for their cytotoxic activity potentials against CaSki, HeLa and MRC5 cells.

In the present study, it was found that both crude methanol and water extracts of *H*. *esculentus*, demonstrated to be cytotoxic against CaSki cells while water extract were active against HeLa cells. Our results are consistent with previous study which found that extracts of *H*. *esculentus* demonstrated to be cytotoxic against EBV in Raji cells (Norhanom *et al.*, 1999).

Only crude methanol extracts of *A. carambola* were found to be cytotoxic activity against CaSki cells. No reports on activities on HeLa, CaSki and MRC5 cells by *A. carambola* have been previously documented.

Observations made in this study showed that the all crude extracts of *M. esculenta* possessed not to be cytotoxic against HeLa, CaSki and MRC5 cells. The finding of this study support the findings of other studies, which reported ethanolic extracts of *M. utilisima* (shoot) to exhibit antioxidant activity and to be able inhibit the viability of (Breast cancer cell line) MCF-7 cell line. It showed that *M. utilisima* (tapioca shoots) have potential as anticancer agent against certain breast tumour (Asmah *et al.*, 2003).

Evidence from this study showed that the both crude methanol and water extracts of *P. olerecea* was found to be cytotoxic against CaSki cells with IC_{50} value 16 µg/ml and 20 µg/ml, respectively. The other extracts were not effective against HeLa and MRC5 cells. Chan and her collage (2000) were reported that the 10% ethanolic extracts of *P. oleracea* showed significant anti-inflammatory and analgesic.

B. vulgaris (beetroot) is particularly beneficial and may be eaten raw, cooked or in juice. Raw vegetables juice, which many include carrots, celery and parsley, together with beetroot are an excellent way of providing concentrated antioxidants and plant enzymes (Reddy, 2003). In this present study, showed that both methanol and water extracts of *B. vulgaris* were cytotoxic against Caski cells. No cytotoxic activity observed against HeLa cells.

Frohlieh *et al.*, (1996) were reported that *C. aurantifolia* are associated with a decreased risk of cancer, particularly of epithelial tumours but these finding do not support findings. Of the present study observations showed that extracts of *C. aurantifolia* were not to be cytotoxic against CaSki, HeLa and MRC5 cells. In contrast, Yoko *et al.*, (2008) reported that *C. aurantifolia* oil showed cytotoxic against activity human dermal fibroblast as evaluated using MTT assay. Patil *et al.*, (2009) supported this finding, where *the C. aurantifolia* oil showed 78% inhibition of human colon cancer cells (SW-480) at 100 µg/ml.

B. oleracea var. *alboglabra* from family Cruciferacea were evaluated for their cytotoxic potential. Results showed that only crude methanol extracts were cytotoxic against CaSki cells. Lin and Ng (2008), were reported that seeds of *B. oleracea* var. *alboglabra* significantly to be cytotoxic against hepatoma (Hep G2) and breast cancer (MCF 7) cells with IC₅₀ at 2.7 μ M and 3 μ M respectively.

P. crispum were evaluated for the cytotoxic potential against HeLa, CaSki and MRC5 cells. All the extracts tested were found to be not cytotoxic against all the cell lines evaluated here. No data on the cytotoxic of *P. crispum* activities against HeLa, CaSki and MRC5 cell lines.

5.5 Screening of Anti-HPV 16 E6 Oncoprotein Activity in selected vegetable Using Immunocytochemistry Technique

A number of important epidemiologic risk factors have been identified as contributing to the development of cervical invasive neoplasia (CIN) and invasive cervix carcinoma. Of the key importance is infection with HPV, which is the primary risk factor (Rock *et al.*, 2000). Treatment of this virally caused neoplasia is still inadequate in spite of the long-term establishment of surgical techniques. The ability of high-risk HPV E6 proteins to inactive the cellular tumour suppressor p53 has been suggested as a mechanism by which the viral protein promotes cell growth and proliferation (Rock *et al.*, 2000).

E6 from the high risk HPVs has been shown to inactivate p53, block apoptosis, activate telomerase, disrupt cell adhesion, polarity and epithelial differentiation, alter transcription and G-protein signaling, and reduce immune recognition of HPV infected cells. The pathways that are targeted by E6 in HPV associated cancers have provided important insights to identify the critical mutations that are commonly found in other tumours (Howie *et al.*, 2008).

Women infected with high risk human papillomavirus, are more likely to recover from the infection and thus not go on to develop cervical cancer if they have high blood levels of certain nutrients, such as beta carotene and lycopene that found in fruits and vegetable (Berkely, 2003).

The cervical cancer-derived cell lines; CaSki cell contains HPV 16 DNA. E6 oncoprotein is expressed in cervical-cancer precursor lesions, cervical intraepitial neoplasia (CIN) I, CIN II, CIN III and carcinoma *in situ* (CIS) where its presence was found to be increase with increasing severity of the lesion (Meijer *et al.*, 2009). It continues to be highly expressed in HPV-associated cervical cancer and cervical cancer-derived cell lines (Anderson, 2003). This strongly supports that E6 protein indeed plays a vital role in carcinogenesis of cervical cancer. Therefore, suppressing it should either slow down or

inhibit the progression of cervical cancer development. HPV 16 oncoprotein, E6 is responsible for the onset and maintenance of the transformed state and therefore, represent appropriate targets for therapeutic vaccines (Fehrmann and Laimins, 2003).

Twenty crude extracts from 10 species of vegetables were screened for anti-HPV 16 E6 oncoprotein activities by using 3-step indirect avidin biotin immunoperoxidase immunocytochemistry technique. The immunocytochemistry technique was carried out using the Labelled Streptavidin Biotin (LSAB) peroxidase kit and the DAB substrate system. DAB substrate-chromogens are unstable at temperature greater than 8°C. LSAB peroxidase kit was stored in freezer (4°C). LSAB peroxidase kit and DAB substrate are sensitive to contamination from variety of oxidizing agents (Meijer *et al.*, 2009). Therefore, these solutions were not exposed to any potential source of contamination. In this study, pipette not directly use from the bottle. DAB solution were always pour out regarding require amount into a clean container and pipette from it. The excess of DAB solution were would not return to the primary storage container.

There is an inverse relationship between incubation time and antibody titer- when the higher the antibody titer, the shorter the incubation time required for optimal results (Boenish, 2001). In this method, cells were incubated at 37°C with 1:40 anti-HPV 16 E6 antibody for 1 hour to allow antibody to react sufficiently strong with bound antigen. Consistent timing of the primary antibody incubation times can cause variation in the overall quality and intensity of staining. Some researchers prefer to incubate at high temperature because equilibrium in antigen-antibody reactions is reached more quickly compared room temperature (Boenish, 2001). In this study, slides were analysed after 1 or 2 days of immunocytochemistry staining procedure to avoid fading of colour stains if slides are exposed to strong light over few days. To minimized fading, slides were stored at in the dark places at room temperature

In this method, some time appeared false-positive and false-negative staining occur using the immunocytochemistry methods. The false-positive staining which attribute to the masking of HPV 16 E6 activity may be cause by over fixation of the CaSki. Control slide done with incubation anti-HPV monoclonal antibody showed very dark reddish-brown stain. This indicated that the HPV 16 E6 oncoprotein was highly express in untreated CaSki cells. The negative control in this study included untreated CaSki cells (not treated with extracts). The negative control omitting anti-HPV 16 E6 monoclonal antibody did not demonstrate any reddish-brown stain. This correlates well with the principle of immunocytochemistry staining where the absent of specific primary antibody caused failure in detecting E6 oncoprotein. In this study monoclonal antibody will combine with E6 oncoprotein to give reddish-brown stain in the end of process.

PBS solution was used in the washing procedure after which the slide was gently tapped to remove the excess solution. Some time colour on the slide are very weak staining, it was because of wells on the slide retain too much washing solution (PBS pH 7.6). To avoid this, the slide should be gently tapped off of excess the washing solution before wiping around the wells with filter paper or cotton bud. In this present study, the humidity chamber was used to avoid drying of samples during staining procedure. Dried cell sections may display increased nonspecific staining.

The false-positive staining may be resulted from incomplete elimination of endogenous enzyme activity. Peroxidase is naturally found in the cells. The anticipated endogenous enzyme activity can be inhibited and suppressed by incubating cells with 3% hydrogen peroxide prior to application of the primary antibody.

5.6 Anti-HPV16 E6 Oncoprotein Activity of the Methanol Vegetable Extracts

Asparagus officinalis, Beta vulgaris, Manihot esculenta, Pachyrrizus erosus, Sesbania grandiflora, Solanum tuberosus, Ipomea batatas, Petroselium crispum, Phaseolus vulgaris and Poutulaca oleracea of crude methanol extracts were evaluated at varying concentrations (1, 10, 25, 50 and 100 μ g/ml) for their anti-HPV 16 E6 oncoprotein activity in CaSki.

All the methanol extracts tested produced suppression against HPV 16 E6 oncoprotein but at different levels. Some produced good suppression, some produced moderate suppression and some produced weak suppression. The resulting staining intensity of CaSki cell treated with extracts showed suppressive effects against HPV 16 E6 by crude methanol extracts of *Asparagus officinalis*, *Beta vulgaris*, *Manihot esculenta*, *Pachyrrizus erosus*, *Sesbania grandiflora* and *Solanum tuberosum* and that suppression of HPV 16 E6 oncoprotein increased with increasing concentrations of methanol extracts. On the other hand, crude methanol extracts of *Ipomea batatas* exerted strong suppression effects against HPV 16 E6 oncoprotein at all concentrations tested. *Petroselium crispum* only weakly suppressed of the expression of E6 oncoprotein at all the concentrations tested.

Phaseolus vulgaris and *Poutulaca oleracea* showed different patterns suppression. Both exerted suppression at lower concentrations. The suppression however decreased at higher extracts concentrations. Results indicate that crude methanol extracts of *I. batatas*, *S. grandiflora*, *P. oleracea*, *M. esculenta* and *B. vulgaris* exhibited strong suppression of anti-HPV 16 E6 oncoprotein.

5.7 Anti-HPV 16 E6 Oncoprotein Activity of the Water Vegetable Extracts

Suppression of the HPV E6 oncoprotein was observed in CaSki cells treated with water extracts of *Asparagus officinalis*, *Beta vulgaris*, *Ipomea batatas*, *Pachyrrizus erosus*, *Petroselum crispum*, *Phaseolus vulgaris*, *Portulaca oleracea*, *Sesbania grandiflora*, *Solanum tuberosum* and of *M. esculenta*. Of the ten crude water extracts of *A. officinalis*, *B. vulgaris*, *M. esculenta* and *P. erosus* possess the most potent anti-HPV 16 E6 oncoprotein activities.

The water extract of *P. oleracea* produced strong inhibition of HPV 16 E6 oncoprotein at concentrations as low as 1 μ g/ml and 10 μ g/ml. The inhibition then became very strong at higher concentrations. In contrast, the water extract of *M. esculentus* exhibited strong suppression of HPV 16 E6 oncoprotein at all concentrations tested. The integrity of CaSki cells treated with *P. crispum* and *P. vulgaris* extracts were negatively affected where the cells lysed at concentrations as low as 1 μ g/ml and 10 μ g/ml.

5.8 Anti-HPV 16 E6 Oncoprotein Activity among Methanol and Water Vegetable Extracts.

Different vegetable extract produced different degree and pattern of suppression of HPV 16 E6 oncoprotein. In general, the crude methanol extracts were more suppressive than water extracts in inhibiting the expression of HPV 16 E6 oncoprotein. The different polarity of the solvent caused different groups of bioactive compounds being sequential extracted from the vegetables. Therefore it would be beneficial if further investigation can be carried out to isolate and identify the specific bioactive compounds which possess HPV 16 E6 activity. Among all crude vegetables extracts tested here, only 3 out of 10 selected vegetables possessed potent anti-HPV 16 E6 activities in both methanol and water extracts. They were *B. vulgaris*, *M. esculenta* and *P. oleracea*.

5.9 Conclusion

Several reports have indicated that a variety of naturally occurring compound from vegetables may play partial roles in the prevention or therapy of human cancer. Since cancer is a disorder of deregulated cell proliferation and cell survival, inhibiting cell proliferation in tumour are effective strategies for preventing tumour growth.

Vegetables are an effective and inexpensive food source to sustaining health. Some vegetables have been reported to possess anti-cancer activities. Increasing the consumption of fresh vegetable and fruits can reduce the cancer risk.

In the present study, the *in vitro* neutral red cytotoxic assay and 3-Step Indirect Avidin-Biotin Immunoperoxidase technique was successfully applied to evaluate the cytotoxic and anti-HPV 16 E6 oncoprotein activities in selected vegetables. Even though the results from *in vitro* assay are not sufficient to predict the final effectiveness of a potential natural product in cancer chemoprevention, this practical screening tool can help in identifying active and non-active extracts from a large quantity of samples. Furthermore, many assays are needed to confirm the potential of these vegetables as chemopreventive agent. Cytotoxicity assay reveal that some of the vegetable evaluated showed signs of selectivity for the cell lines used in this study, HeLa and CaSki. For this reason it is essential also to evaluate and determine the cytotoxic effect of these extracts on other types human cancer cell lines.

Only 41 out of 120 vegetables extracts, both in water and methanol extracts were actively cytotoxic against HeLa, CaSki and MRC5 cells with IC_{50} value less than 20 µg/ml and some of the selected vegetable extracts namely, *I. batatas, M. esculenta, P. oleracea,* and *S. grandiflora* have very strong anti-HPV 16 E6 oncoprotein activities when tested in immunocytochemistry technique.

The results of this study suggest that *B. vulgaris* and *P. oleracea* not only possess strong cytotoxic potentials against cervical cancer-derived cell lines, HeLa and CaSki but also anti-HPV 16 E6 oncoprotein activity on CaSki cells. These two vegetables could further be exploited for their antitumour properties. However, further study is needed to be carried out to identify and isolate the specific active components bearing this activity and to discover the underlying mechanism of action.

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APPENDIX

Appendix A

Preparation of Media and solutions for Cytotoxic and Immunocytochemistry Assay Basic RPMI 1640 Medium

One bottle of RPMI powder, 10.39 g (Sigma) were dissolve in 1000 ml of distilled water. Two grams of Natrium bicarbonate (NaHCO3, Merk, Germany) was added to the medium. The pH of the medium was calibrated to PH 7.4 using a pH meter (Hanna Instrument 8417). The medium was then filter sterilized through 0.02 μ m filter membrane (Orange Scientific) and stored at 4°C for up to 2 months.

Basic EMEM Medium

One bottle of EMEM powder, 10.39 g (Sigma) without Natrium bicarbonate (NaHCO3, Merk, Germany) was made up to one litre with distilled two grams of Sodium

hydrogen carbonate was added to the medium. The medium was filter sterilized using 0.02 µm pore size filter membrane (Orange Scientific) and kept at 4°C for up to 2 months.

10% Supplemented RPMI 1640 Medium and EMEM Medium

One hundred milliliters of 10% supplemented RPMI 1640 medium and EMEM medium ere prepared using basic medium, supplemented with 10% (v/v) heated Foetal Calf Serum (FCS, PAA lab, Austria). 1 ml (100 μ g/ml) of streptomycin and (100 IU/ml) penicillin (PAA Lab., Austria) were added followed by filteration of the media using, 0.22 μ m filter membrane and stored at 4°C for up to 2 weeks.

20% Supplemented RPMI 1640 Medium and EMEM Medium

20% supplemented RPMI 1640 medium or EMEM medium were prepared using 50 ml 0f 10% supplemented medium and added with 5 ml inactive FCS (PAA Lab., Austria). The medium as sterilized using a 0.22 μ m filter membrane and stored at 4°C for up to 2 weeks. This 20% supplemented medium was used for the reviving of the cells.

Cryopreservation of Cells

Freezing medium was prepared by adding 40% of basic medium, followed by 10% DMSO (Sigma) as cryoprotectant and 50% Foetal calf serum (FCS) (PAA Lab, Austria). The mixture was filter-sterilized using 0.22 μm filter membrane and stored at 4°C.

Preparation of Phosphate Buffered saline (PBS)

Phosphate Buffered Saline (PBS) was prepared using 1.52 g of Sodium phosphate anhydrous (NaHPO₄, Merck), 0.58 g of Potassium dihydrogen orthophosphate (KH₂PO₄. Merck) and 8.5 g of Natrium chloride (NaCl, BDH Analar) in distilled water. The pH of the buffer was adjusted to 7.2 using pH meter (Hanna Instruments 84171, USA). The buffer was then filtered using Whatman filter paper and stored at room temperature until required.

Preparation of Trypsin-EDTA

Trypsin-EDTA solution was prepared by dissolving 0.25 g trypsin (Amresco, USA) and 0.03 g of EDTA (sigma, USA) in 100 ml of distilled water, and filter sterilized using 0.22 μ m pore size filters and stored at -20°C.

Dimethy sulfoxide (DMSO) 10%

One of Dimethyl sulfoxide (DMSO) was dissolve in 9 ml distilled water. The solution was kept in Falcon tube, wrapped with aluminium foil and kept in dark place at room temperature.

Preparation of Trypan Blue 0.4%

0.4% stock solution of Trypan blue was prepared by dissolved 0.2 g Trypan blue powder in 50 ml of dH₂0 and stored at room temperature. Trypan blue will stain the dead cells while the living cells will appear colourless cells (unstained).

Preparation of Natural Red Stock solution

4 mg powder of natural red powder was dissolved in 10 ml distilled water in beaker, and stored at 4°C until required.

Neutral Red Medium for Cytotoxic Assay

12 ml of 10% supplemented RPMI medium or EMEM medium were mixed with 150 μ l of NR stock (1:80). The solution was centrifuge at 1000 rmp for 10 minutes to remove any fine needle like precipitate of dye crystal. Neutral red medium were incubated at room temperature in a dark place overnight.

Neutral Red washing solution

One gram of Calcium chloride (CaCl₂, Sigma) was dissolved in 500 μ l formaldehyde (Sigma) and 99.5 ml of distilled water and kept at 4°C.

Neutral Red resorb solution

One ml of glacial acetic acid (BDH, Analar) was dissolved in 50 ml of absolute ethanol (Hamburg) and 49 ml of distilled water. The solution was kept at 4°C.

Preparation of Phosphate Buffered saline PBS (pH 7.6)

Phosphate Buffered saline (pH 7.6) was prepared using 1.5 g Sodium phosphate anhydrous (Na₂HPO₄, Merck), 0.2 g of potassium dihydrogen orthophosphate (KH₂PO₄,

Merck) and 7.75 g of Sodium chloride (NaCl, BDH Analar) in 1 liter of distilled water. The pH was adjusted to 7.6 using a pH meter (Hanna Instrument 84171, USA). The buffer was then filtered using Whatman filter paper and stored at room temperature until required.

3% hydrogen peroxidase (H₂O₂)

0.3 ml hydrogen peroxidase (H2O2) was added with 9.7 ml distilled water and kept in room temperature.

Appendix C

	Concentration of extracts (µg/ml)	Morphology of cells	Intensity of reddish- brown stain
	100	Intact	+
	50	Majority intact but some showing lysis	2+
	25	Majority intact but some showing lysis	3+
Sold and a second secon	10	Intact	4+
	1	Intact	4+

Appearanxce of CaSki cells after treatment with methanol of Asparagus officinalis

CaSki cells treated with methanol extracts of Asparagus officinalis (400x)

Concentration of extracts (µg/ml)	Morphology of cells	Intensity of reddish- brown stain
100	Lysis	3+
50	Intact	3+
25	Intact	3+
 10	Intact	3+
1	Intact	3+

Appearance of CaSki cells after treatment with water of Asparagus officinalis

CaSki cells treated with water extracts of Asparagus officinalis (400x)

Concentration of extracts (µg/ml)	Morphology of cells	Intensity of reddish- brown stain
100	Intact	+
50	Intact	+
25	Intact	+
10	Intact	3+
1	Intact	3+

Appearance of CaSki cells after treatment with methanol of *Beta vulgaris*

CaSki cells treated with methanol extracts of *Beta vulgaris* (400x)

Concentration of extracts (µg/ml)	Morphology of cells	Intensity of reddish- brown stain
100	Intact	+
50	Intact	+
25	Intact	+
10	Intact	2+
1	Intact	2+

Appearance of CaSki cells after treatment with water of *Beta vulgaris*

CaSki cells treated with water extracts of *Beta vulgaris* (400x)

	Concentration of extracts (µg/ml)	Morphology of cells	Intensity of reddish- brown stain
Section of the sectio	100	Intact	+
	50	Intact	+
	25	Intact	+
	10	Intact	+
	1	Intact	+

Appearance of CaSki cells after treatment with methanol of *Ipomea batatas*

CaSki cells treated with methanol extracts of *Ipomea batatas* (400x)

Concentration of extracts (µg/ml)	Morphology of cells	Intensity of reddish- brown stain
100	Intact	2+
50	Intact	2+
25	Intact	2+
10	Intact	4+
1	Intact	4+

Appearance of CaSki cells after treatment with water of Ipomea batatas

CaSki cells treated with water extracts of *Ipomea batatas* (400x)

Concentration of extracts (µg/ml)	Morphology of cells	Intensity of reddish- brown stain
100	Intact	+
50	Intact	+
25	Intact	+
10	Intact	2+
1	Intact	2+

Appearance of CaSki cells after treatment with methanol of Manihot esculenta

CaSki cells treated with methanol extracts of Manihot esculenta (400x)

Concentration of extracts (µg/ml)	Morphology of cells	Intensity of reddish- brown stain
100	Intact	2+
50	Intact	2+
25	Intact	2+
10	Intact	2+
1	Intact	2+

Appearance of CaSki cells after treatment with water of Manihot esculenta

CaSki cells treated with water extracts of Manihot esculenta (400x)

Concentration of extracts (µg/ml)	Morphology of cells	Intensity of reddish- brown stain
100	Lysis	2+
50	Lysis	2+
25	Majority intact but some showing lysis	3+
10	Intact	4+
1	Intact	5+

Appearance of CaSki cells after treatment with methanol of Pachyrizus erosus

: CaSki cells treated with methanol extracts of *Pachyrizus erosus* (400x)

Concentration of extracts (µg/ml)	Morphology of cells	Intensity of reddish- brown stain
100	Intact	+
50	Intact	+
25	Intact	2+
10	Intact	2+
1	Majority intact but some showing lysis	3+

Appearance of CaSki cells after treatment with water of Pachyrizus erosus

CaSki cells treated with water extracts of *Pachyrizus erosus* (400x)

Concentration of extracts (µg/ml)	Morphology of cells	Intensity of reddish- brown stain
100	Lysis	3+
50	Majority intact but some showing lysis	3+
25	Majority intact but some showing lysis	4+
10	Majority intact but some showing lysis	4+
1	Majority intact but some showing lysis	5+

Appearance of CaSki cells after treatment with methanol of *Petroselium crispum*

: CaSki cells treated with methanol extracts of *Petroselium crispum* (400x)

Concentration of extracts (µg/ml)	Morphology of cells	Intensity of reddish- brown stain
100	Lysis	3+
50	Majority intact but some showing lysis	3+
25	Lysis	4+
10	Lysis	4+
1	Majority intact but some showing lysis	5+

Appearance of CaSki cells after treatment with water of *Petroselium crispum*

CaSki cells treated with water extracts of *Petroselium crispum* (400x)

Concentration of extracts (µg/ml)	Morphology of cells	Intensity of reddish- brown stain
100	Intact	5+
50	Intact	4+
25	Intact	2+
10	Intact	+
1	Intact	+

Appearance of CaSki cells after treatment with methanol of *Phaseolus vulgaris*

CaSki cells treated with methanol extracts of *Phaseolus vulgaris* (400x)

Concentration of extracts (µg/ml)	Morphology of cells	Intensity of reddish- brown stain
100	Lysis	+
50	Lysis	2+
25	Lysis	4+
10	Lysis	5+
1	Intact	5+

Appearance of CaSki cells after treatment with water of *Phaseolus vulgaris*

CaSki cells treated with water extracts of *Phaseolus vulgaris* (400x)

Concentration of extracts (µg/ml)	Morphology of cells	Intensity of reddish- brown stain
 100	Intact	2+
50	Intact	2+
25	Intact	+
10	Intact	+
1	Intact	+

Appearance of CaSki cells after treatment with methanol of Portulaca oleracea

CaSki cells treated with methanol extracts of Portulaca oleracea (400x)

Concentration of extracts (µg/ml)	Morphology of cells	Intensity of reddish- brown stain
100	Intact	+
50	Intact	+
25	Intact	+
10	Intact	2+
1	Intact	2+

Appearance of CaSki cells after treatment with water of Portulaca oleracea

CaSki cells treated with water extracts of Portulaca oleracea (400x)
	Concentration of extracts (µg/ml)	Morphology of cells	Intensity of reddish- brown stain
	100	Intact	+
	50	Lysis	+
	25	Lysis	2+
	10	Intact	2+
200	1	Intact	2+

Appearance of CaSki cells after treatment with methanol of Sesbania grandiflora

CaSki cells treated with methanol extracts of Sesbania grandiflora (400x)

Concentration of extracts (µg/ml)	Morphology of cells	Intensity of reddish- brown stain
100	Intact	+
50	Intact	+
25	Intact	2+
10	Intact	3+
1	Intact	5+

Appearance of CaSki cells after treatment with water of Sesbania grandiflora

CaSki cells treated with water extracts of Sesbania grandiflora (400x)

	Concentration of extracts (µg/ml)	Morphology of cells	Intensity of reddish- brown stain
	100	Intact	+
	50	Intact	+
	25	Intact	+
2 9 B	10	Intact	4+
	1	Intact	4+

Appearance of CaSki cells after treatment with methanol of Solanum tuberosum

CaSki cells treated with methanol extracts of Solanum tuberosum (400x)

Concentration of extracts (µg/ml)	Morphology of cells	Intensity of reddish- brown stain
100	Intact	+
50	Intact	+
25	Intact	+
10	Intact	4+
1	Intact	4+

Appearance of CaSki cells after treatment with water of Solanum tuberosum

Figure 4.23: CaSki cells treated with water extracts of *Solanum tuberosum* (400x)