CHAPTER 1

INTRODUCTION

Cancer is the leading cause of death in human population worldwide (Reddey *et al*., 2003). Cervical cancer is the second most common cancer in Malaysia (WHO, 2007) and fourth most common cause of women death in Malaysia (The Second Reports of National Cancer Registry, 2004). Other researcher found that cervical cancer ranks third of all cancers in Malaysia (Norhayati, 2003).

The most important risk factor in the development of cervical cancer is infection with a high-risk strain (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) of human papillomavirus. Types 16 and 18 are generally acknowledged to cause about 70% of cervical cancer cases (Munoz *et al.,* 2003). HPV 16 and 18 are two most common HPV types in Southeast Asia (WHO, 2007); although HPV 18 alone is relatively more frequent compared to the type distribution estimates in the rest of the world (Smith *et al*., 2007; Sriamporn *et al*., 2006).

The carcinogenic process has now been extensively investigated in terms of both biological and molecular alterations and a sequence of initiation, promotion, conversion and progression was recognized (Ito *et al.,* 1995). High-risk HPV (HPV16 and 18) infection contributes to carcinogenesis of cervical cancer and tumour progression predominantly through the actions of two viral oncogenes, E6 and E7. These oncogenes are consistently expressed in cervical cell lines and in human cancers (McMurray *et al*., 2001; Munger & Howley*,* 2004). Both of these oncogenes interact with and inhibit the activities of critical components of cell cycle regulatory systems (Philips & Vousden, 1999; Milde *et al*., 2004; McMurray, 2001). As p53 and pRB normally control cellular proliferation, differentiation, and apoptosis, the abrogation of their normal biological activities places such a cell at a risk of malignant progression. In addition, high-risk HPV E6 and E7 expressing cells have a decreased ability to maintain genomic integrity (White *et al.*, 1994; Margaret *et al*., 2008).

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 reduced immune recognition of HPV infected cells. The pathways that are targeted by E6 in HPV-associated cancers have provided important insight to identify the critical mutations that are commonly found in other tumours (Howie *et al*., 2009).

The expression of E7 requires integrity of the p53 tumour suppressor pathway, but does not involve increased expression of p53-responsive genes (Eichten *et al*., 2004). E7 plays a central role in both viral life cycle and carcinogenic transformation. In the HPV life cycle, E7 disrupts the intimate association between cellular differentiation and proliferation in normal epithelium, allowing for viral replication in cells that would no longer be in the dividing population. This function is directly reflected in the transforming activities of E7, including tumour initiation and induction of genomic instability (Helt and Galloway, 2001; Helt *et al*., 2002; Margaret *et al*., 2009).

Available evidence indicates that the most effective way to fight cancer has been to prevent cancer from developing in the first place, either by removing pre malignant lesion, or by chemoprevention via food supplements eats (Milchele and Harvey, 2004). The therapies used in the treatment of cervical cancer are radiotherapy and chemotherapy. Radiotherapy works by damaging a cancer cell's DNA, making it unable to multiply. Radiation therapy can damage nearby healthy cells, cancer cells are highly sensitive to radiation and typically die when treated. Healthy cells that are damaged during radiation are resilient and are able to fully recover. Side effects of radiation vary from patient to patient. It all depends on how often treatment is given. The three most commonly experienced side effects are fatigue skin problems and loss of appetite (Bucci *et al*., 2005).

Chemotherapy is a cancer treatment that utilizes anticancer drugs. Chemotherapy works by detecting cancerous cells and destroying them. Unfortunately, many chemotherapy drugs cannot discern between healthy and cancerous cells, thus causing side effects like hair loss and stomach upse (Fischer *et al*., 2003; Skeel *et al*., 2003).

Natural products play a relevant role in cancer therapy today with substantial number of anticancer agents used either in the natural form or derived from natural products from varius sources such as plants, animals and microorganism (Cheryl *et al*., 2000). There is a long history of medicinal use of plants in Southeast Asian countries, some of which have been proven to be useful as pharmaceuticals (Burkill, 1966). That is why there is a need to look for agents from commontly consumed natural resources such as vegetable.

Research on the natural products for cervical cancer therapy represents an area of great interest in which plants are the most important source. Besides fulfilling the

physiological needs, routine or habitual consumption of vegetables may deliver significant benefits to human health. Dietary modification by increasing the consumption of wide variety of fruits and vegetables daily is a practical strategy for consumers to optimize their health and reduce the risk of cancer. Use of dietary supplements, nutraceuticals and functional foods is increasing as industry is responding to consumers demand (Rui, 2004).

Numerous epidemiological studies have demonstrated a lower risk of cancer among individuals whose diet includes a relatively large amount of vegetable, fruits and plant products, all containing different vitamins and macronutrients with ability to prevent carcinogenesis by interfering with detrimental actions of mutagens, carcinogens and tumour promoters (Hanausek *et al*., 2001).

In the cancer field, a number of important new commercialized drugs have been obtained from natural sources, by structural modification or natural compounds, or by the synthesis of new compounds, designed following a natural compound as a model. The search for improved cytotoxic agents continues to be important in the discovery of modern anticancer drug (Nobili *et al*., 2009).

The aims of the present study were:

a. to screen the crude methanol and water extracts from 40 selected vegetables for cytotoxic potentials against cervical cancer-derived cell lines, CaSki (HPV 16-containing cell lines), HeLa (HPV 18-containing cell lines) and normal cell lines MRC5 (human lung fibroblast) using the neutral red cytotoxic assay.
b. to screen the crude methanol and water extracts from 40 selected vegetables for the ability to suppress or inhibit the expression of HPV 16 E6 oncoprotein in CaSki cell line using the 3-step indirect avidin-biotin immunoperoxidase immunocytochemistry technique.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

With 10.9 million new cases and 6.7 million deaths per year, cancer is a devastating disease, presenting an immense disease burden to affected individuals and their families as well as health care system (Parkin *et al*., 2005). Cancer is mass of abnormal cells that have lost, partially or fully, the normal regulatory growth control mechanism. This disorder disrupts the normal process of cell division, controlled by the genetic material (DNA) of the cells (Becker *et al*., 1995; Reddy *et al*., 2003).

Cancer stem from abnormal cell growth within the body, which results from damage to DNA. This damage could be due to a number of factors ranging, from environmental factors such as asbestos inhalation or smoking, to inherited damaged/mutated DNA. The DNA cannot be repaired in abnormal cells ultimately leading to uncontrollable proliferation. These abnormal cells rapidly outgrow normal cells, spreading to other parts of the body (Jiade, 2004).

Neoplasm can be classified as benign or malignant based on their likelihood of spreading to distant parts of the body. Benign tumours are typically encapsulated nodules of neoplastic tissue and therefore do not spread, whereas malignant tumours often invade neighboring tissue and damaging these tissue and organs. Cancer cells can break away and enter the blood stream to form new tumours in other parts of body. This type of spread of cancer is called metastasis (Becker *et al*., 1995). Table 2.1 shows the contrast between benign and malignant neoplasms.

Table 2.1: The characteristics of benign and malignant neoplasms

Characteristics	Benign	Malignant
Growth rates	Usually slow Low mitotic rate Normal mitoses Normal nucleoli	Usually rapid High mitotic rate Abormal mitoses Large nucleoli
Differentiation	Resembling normal Maintains normal functions	Often poor Loss or altered function
Spread	No invasion No metastases	Local invasion Metastases common
Boundaries	Often encapsulated Circumscribed	Non-encapsulated Irregular, ill-defined
Relationship to surrounding tissues	Merely compresses normal tissue	Invades and destroys normal tissue

(Adapted from Tanaka, 1997)

The patterns of cancer occurrence can be grouped in two categories. The first comprise of hormone-dependent cancers such as those of female breast, endometrium, ovary, prostate, testis colon and male lung cancer. The second comprised of cancers linked to biological agents such as cancers of the liver with hepatitis B and C which are associated to with viruses; nasopharyngeal cancer and Burkitt lymphoma which are associated with Epstein-Barr-virus; Kaposis sarcoma and non-Hodgkin lymphoma which are associated with human immunodeficiency virus (HIV) and cervical cancer which is associated with human papillomavirus. These tumours are common in Asia, Latin and South America as well as Africa (IARC, 1997). The occurance of most cancers such as breast, prostate, testis, colon, lung, and thyroid cancer, as well as brain tumours, non Hodgkin lymphomas are increasing worldwide. The occurance of other cancers such as stomach or cervical cancer is decreasing (Annie, 2008).

2.2 Carcinogens

Carcinogens are substances which may increase the risk of getting cancer by altering cellular metabolism or damaging DNA directly in cells, interfering biological processes, and inducing the uncontrolled, malignant division, ultimately leading to the formation of tumours. Usually DNA damage, if too severe to be repaired, leads to programmed cell death, but if the programmed cell death pathway is damaged, the damage persist causing transformation of the mutant cells (Parkin *et al*., 2005).

Carcinogens include both natural and man made or man-enhanced substances (Eric, 1993). They range from chemicals, viruses, hormone, ionizing radiation, solid materials to mineral fiber (Peter and Curtis, 1994: Reddy *et al*., 2003). Chemical carcinogens can be converted into highly reactive compounds that can damage DNA and other cell components, or they can be detoxified and thus prevented from causing cellular damage. The metabolic fate of chemical carcinogens is linked to the activities of particular enzymes, protein molecules in the body that help chemical reactions to occur but are not themselves changed in the reactions. The activities of these enzymes can differ among individuals because of the occurrence of genetic polymorphisms (different forms of the genes that code for the enzymes) and the differing activities can either

increase or decrease a person's susceptibility to environmental carcinogens (Holland *et al*., 1997).

Carcinogens may be divided into several classes (Table 2.2). Genotoxic carcinogens react with nucleic acids and directly affect cellular constituents. Ionizing radiation is a genotoxic carcinogen. Procarcinogens on the other hand, require metabolic activation to induce carcinogens. Epigenetic carcinogens are those that are not genotoxic. Other carcinogens may change DNA expressesion without affecting its structure directly, or may increase susceptibility to DNA damage from other causes. These are known as nongenotoxic carcinogens or promoters. Arsenic and estrogen are nongenotoxic carcinogens. Still other carcinogens, such as nickel, may interfere with cell division and changing the number or structure of chromosomes in new cells after cell division (Zur Hausen, 2001; Parkin, 2006).

Susceptibility to the action of carcinogens is very complex and is affected by genetic heritage, behavior, physiology, nutrition, external exposures, and other factors of the host. For example, some chemicals are carcinogenic in their original form (direct carcinogens), while some must be metabolized in the body to their active form (indirect carcinogens). In such cases, individual susceptibility to a chemical carcinogen is affected by the rate at which the chemical metabolizes in the body into a cancer-causing form or into a harmless form. This rate varies from person to person (Holland *et al*., 1997)**.**

Table 2.2: Types of carcinogens

Type	Example
Genotoxic carcinogens	
Primary, direct-acting alkylating agents	Dimethysulfate, ethylene imine, 3-propiolactonel
Procarcinogens	
Polycyclic aromatic hydrocarbons, nutrosamines, hydrazine, inorganic	benzo[α]pyrene, dimethynitrosamine, 1,2-dimethylhydrazine, cadmium, plutonium
Epigenetic carcinogens/nongenotoxic	
Promoters, solid state, hormones, immunosuppressant, cocarcinogens	phorbol ester, saccharin, bile acids, asbestos, plastic, estrogens, purine analogues, catechol
Unclassified	peroxisome proliferators clofibrate, phthalate ester

(Adapted from: Reddy *et al*., 2003)

2.3 Carcinogenesis

Carcinogenesis refers to the development of neoplasia (Zur Hausen, 2001; Parkin, 2006). Once inside the body, most chemical carcinogens are metabolized. They are transformed by the physical and chemical processes in the body (Holland *et al*., 1997). Although there are many different forms of cancer, the basic multistage process by

which various tumours develop is similar for all cancers.

Carcinogenesis is a multistage process driven by genetic damage and apigenetic change (Zur Hausen, 2001; Parkin, 2006). Recently, multistage carcinogenesis processes have been advocated, but the two-stage model is considered very convenient for the detection of carcinogenic and anticarcinogenic substances. Since the time Berenbelum describe two-stage mouse skin carcinogenesis model in 1994, the secrets of chemically induced carcinogenesis in several animal models have been well studied and the carcinogenesis process has become clearer, especially in the past decade (Tanaka, 1997). Cancer develops through four definable stages: initiation, promotion, malignant conversion and progression (Figure 2.1). These stages may progress over many years.

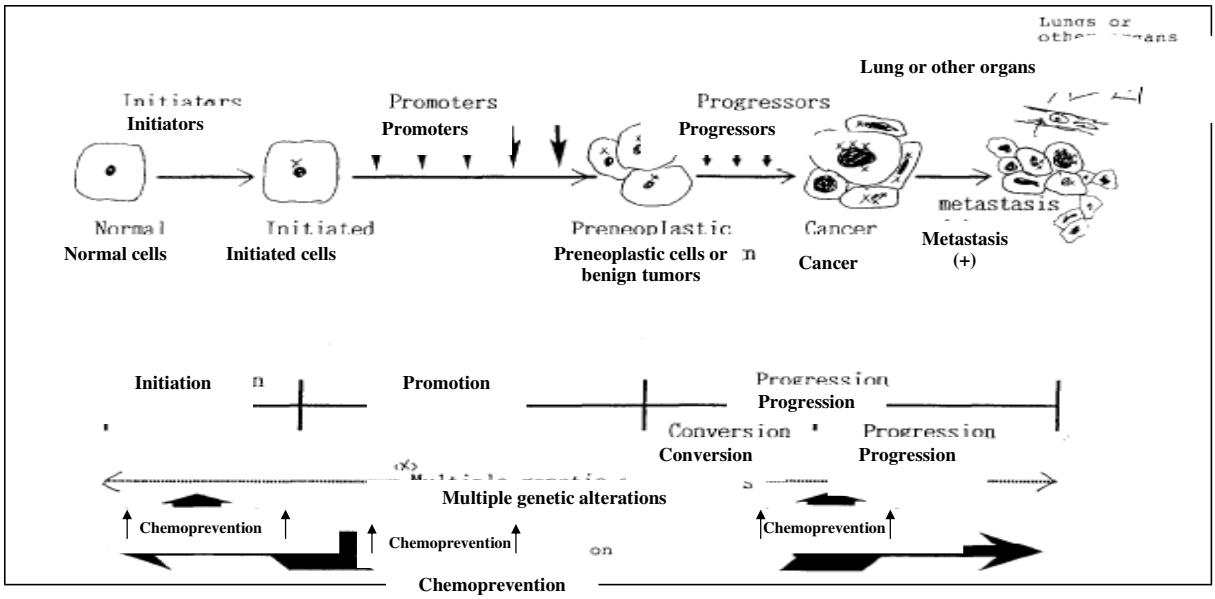

Lung or other organs
Initiators
Promoters
Progressors
Normal cells
Initiated cells
Preneoplastic cells or benign tumors
Cancer
Metastasis (+)
Initiation
Promotion
Progression
Conversion
Progression
Multiple genetic alterations
Chemoprevention
Chemoprevention
Chemoprevention
Chemoprevention

Figure 2.1:

Initiation

The first stage, initiation can result from a single application of a sub-carcinogenic dose of a carcinogen and it is a direct action on the target cell. Tumour initiation begins in cells through mutations from exposure to carcinogens (Peter and Curtis, 1994). An initiating agent is defined as chemical, physical or biological agents capable of directly altering in an irreversible manner, the native molecular structure of the genetic component (DNA) of the cells (Vimala, 1995).

Initiation process is divided into three substages. First, macromolecular binding and DNA adducts. Administration of chemical carcinogens to living animals or cells, whether by topical, subcutaneous, intragastric, intraperitoneal or any other route, results in absorption, transport, entry into cells and metabolism, generally with excretion of detoxified products (Laconi *et al*., 2008). The genotoxic groups of chemical carcinogens can be divided into direct and nondirect acting species, depending on whether not they require metabolism for reactive species to be generated. The enzymes involved include the phase I and phase II drug metabolizing species (Ito *et al*., 1995). The products of phase I may be more electrophilic then the parent and will bind to macromolecules in the cell. Hormones, irradiation, UV light and physical agents or procedures such as *in situ* freezing know to be capable of initiation.

Second, DNA repair and cell proliferation. From oxidative process alone, production of adducts by endogenous sources is exceedingly high and evoluttion has presumably demanded and ability of cells to remove or repair damage to their DNA (Laconi *et al*., 2008). A number of repair systems have been generated. If cell devision occurs before repair can take place then any changes in one DNA strand will be replicated and no longer recognizable as and error. In fact it has even been postulated that proliferation is a requirement for initiation to occur (Ito *et al*., 1995).

Third, specific mutation / tissue character. Specific mutation has been linked with specific carcinogens and in the N-Methyl-N-nitrosourea (MNU) case. H-ras is apparently activated in 100% of induced mammary carcinomas. Transplancental induction of a specific H-ras mutation is described as critical for natal carcinogenesis and the same oncogen maybe involved in generation of esophageal papillomas by methylbenzylnitrosamine. Similarly, Ki-ras has been implicated in gastrointestinal dysplasias. For initiation of skin carcinogenesis covalent binding of adenine residues for 3,2-dimethyl-4-aminobiphenyl (DMBA) and guanine for benz[a] pyrene have been described to be of particular importance (Ito *et al*., 1995).

Promotion

The second stage known as the promotion stage is the longer stage. This stage can be divided into cell proliferation and altered phenotypic expression (Ito *et al*., 1995). The cell proliferation involves repeated application of irritating and promoting agent. A promoting agent is an agent that alters the expression of genetic information of the cells (Pitot *et al*., 1982). Examples of such agents include hormones, drugs and plant products, which themselves do not directly react with the genetic material but rather affects its expression by a variety of mechanism including their interaction with cell surface receptors, with cytoplasmic and nuclear protein receptors or by alteration of other cellular components and function (Ito *et al*., 1995 and Laconi *et al*., 2008).

On the other hand, in alterated phenotypic expression, the process of promotion is mainly a quantitative phenomenon (many cells arising from a single cell). While no qualitative changes are necessarily implied, it is a fact that cell populations in early nodules, papillomas, or polyps are rather homogenous in size and shape or in the expression of specific biochemical markers (Ito *et al*., 1995).

The mutated cell is stimulated to grow and divide faster and becomes a population of mutated cells. The lesions are known as premalignant lesions (Holland *et al*., 1997). Eventually a benign tumor becomes evident. In human cancers, hormones, cigarette smoke, or bile acids are substances involved in promotion (Schottenfeld and

Fraumeni, 1996). This stage is usually reversible as evidenced by the fact that lung damage can often be reversed after smoking stops (Holland *et al*., 1997).

Conversion

The process that transforms a preneoplastic cell into one that possesses malignant phenotype is known as the conversion process. This process starts with the invasion of cells outside their normal tissue boundaries into surrounding tissues and often show changes in nuclear morphology (Kulesz-Martin, 1997). If the tumour does not break through the boundaries between tissues, it is known as "in situ" cancer (Holland *et al*., 1997). Increasing rate of exposure to DNA damaging agents and cell division can be mediated by the activation of proto-oncogens and inactivation of tumour suppressor genes.

Progression

During progression, there is further growth and expansion of the tumour cells over normal cells. The genetic material of the tumour is more fragile and prone to additional mutations (genetic alteration). A variety of consequences for the tumour cells ensue, including chromosome or gene amplification, translocations and rearrangement, deletion, and possibly also proto-oncogene activation and tumour suppressor inactivation (Ito *et al*., 1995). Karyotypic instability appears unique to the progression stage.

In situ tumours can develop further mutations, break through tissue boundaries, and invade surrounding tissues; at this stage, they become malignant tumours that can send cells throughout the body to establish new tumours (metastasis). During the development of a malignant tumour, DNA damage occurs as an accumulation of mutations in as many as six or more genes (Holland *et al*., 1997).

2.4 Cervical Cancer

Epidemiology

Cervical cancer is the second most frequent cancer type in women with yearly 450, 000 newly diagnosed women and 230,000 deaths worldwide. Approximately 75-80% of cancer cases are found in developing countries (Chris *et al*., 2009). The countries of Western Asia (2.9%), China (3.8%) and Japan (2.8%) have similar rates to those of European countries (4.4%) (Simcock and Shafi, 2007). On the other hand, sub-Saharan Africa, most of central and southern Asia, and south and Central America have higher incidence and mortality rates for cervical cancer (IARC, 2005).

Histological Types of Cervical Cancer

Invasive and preinvasive cervical lesions are classified by appearance of cells morphology. There are two main types of cervical cancers, namely *squamous cell carcinoma* and *adenocarcinoma*. About 80% to 90% of cervical cancers are squamous cell carcinoma (Jiade, 2004). Development of squamous cervical carcinoma is preceded by cervical intraepithelial neoplasia (CIN) of which CIN 3 in the most advanced (Bosch *et al*., 2002; Wright *et al*., 2002; Chris *et al*., 2009). Squamous cell carcinoma comprise of squamous cells that live the surface of the exocervix. Squamous cell carcinomas most often begin where the exocervix joins the endocervix (Kim, 2008).

The remaining 10% to 20% of cervical cancers are adenocarcinomas. Adenocarcinomas are becoming more common in women born in the last 20 to 30 years (Kim, 2008). Cervical adenocarcinoma develops from the mucus-producing gland cells of the endocervix. Less commonly, cervical cancers have features of both squamous cells carcinomas and adenocarcinomas. These are called adenosquamous carcinomas or mixed carcinomas (Jiade, 2004).

International Classification of Cervical Carcinoma

The activity of anticancer drugs is evaluated by measuring changes in tumour size in response to treatment (Therasse, 2002; Park *et al*., 2003). Tumour size has traditionally been estimated from bidimensional measurements (the product of the longest diameter and its longest perpendicular diameter for each tumour) (James *et al*, 1999; Park *et al*., 2003). In the early 1980s, the World Health Organization (WHO) developed recommendations in an attempt to standardize criteria for response assessment, and the WHO response criteria were adopted as the standard method for evaluating tumour response (Miller, 1981; Park *et al*., 2003). Table 2.3, shows the International Federation of Gynecology and Obstetrics (FIGO) staging of cervical cancer and have four satges in this classification. There are stages I, stage II, stage III and stage IV.

Table 2.3: International Federation of Gynecology and Obstetrics (FIGO) staging of cervical cancer (Simcock and Shafi, 2007).

Stage I: carcinoma strictly confined to the cervix - Stage IA Invasive cancer identified only microscopically; all gross lesions, even with superficial invasion - Stage IA1 Measured invasion of the stroma ≤ 3 mm depth and ≤ 7 mm diameter - Stage IA2 Measured invasion of stroma > 3 mm but ≤ 5 mm depth and ≤ 7 mm diameter - Stage IB cancers; invasion is imited to measured stromal invasion with a maximum depth of 5 mm and no wider than 7 mm - Stage IB Clinical lesions confined to the cervix or preclinical lesions greater than stage IA - Stage IB1 Clinical lesions ≤ 4 cm - Stage IB2 Clinical lesions > 4c m
Stage II: carcinoma that extends beyond the cervix but has not extended onto the pelvic wall; the carcinoma involves the vagina, but not as far as the lower-third section - Stage IIA No obvious parametrial involvement; involvement of as much as the upper two-thirds of the vagina - Stage IIB Obvious parametrial involvement, but not onto the pelvic sidewall
Stage III: carcinoma that has extended onto the pelvic sidewall and/or involves the lower third of the vagina; all cases with hydronephrosis or a non-functioning kidney should be included, unless they are known to be due to other causes - Stage IIIA No extension onto the pelvic sidewall, but involvement of the lower third of the vagina - Stage IIIB Extension onto the pelvic sidewall, or hydronephrosis or non-functioning kidney
Stage IV: carcinoma that has extended beyond the true pelvis or has clinically involved the mucosa of the bladder and/or rectum - Stage IVA Spread of the tumour onto adjacent pelvic organs - Stage IVB Spread to distant organs

Symptoms

Early-stage disease may be asymptomatic and detected on a smear or large loop excision of the transformation zone procedure. The classical symptoms are irregular

vaginal bleeding, especially post-coital but symptoms may be absent until the cancer is in the advanced stage. Invasive cancer is rare in women with post-coital bleeding i.e vaginal bleeding that occurs after sexual intercourse, but assessment is merited as it much more common in this group than in the general population. Discharge and pain are often associated with more advanced disease (Simcock and Shafi, 2007).

2.4.1 Risk Factor for Cervical Cancer

Epidemiologic studies have identified a number of factors that play a significant role in the development of cervical intraepithelial neoplasma (CIN), a precursor to cervical cancer. There are a sexually trasmited diseases (STDs), sexual behaviour, having blood relative, aging and tobacco (Munoz, 2006).

Infection by HPV is basically a sexually transmitted disease (STDs). As such, both man and women are involved in the epidemiological chain of infection and at the same times are able to be asymptomatic carriers, transmitters and also victims of the infection by HPV and other viruses such as *Chlamydia trachomatis*, *Trichomonos* and *Cytomegalovirus* which are also associated with cervical cancer (Castellsague *et al*., 2003; Muñoz, 2006).

Women also increase their risk for CIN by engaging in other behaviors known to suppress the immune system. Suppression of the immune system due to HIV infection also is an important risk factor because it makes the cells lining the lower genital tract (vulva, vagina and cervix) more easily infected by the cancer-inducing types of HPV. There is substantial evidence that HIV-positive women are at increased risk of developing cervical cancer as well (Munoz, 2006). In two studies, both from high HIV prevalence areas, a statistically significant association between HIV and CIN was reported. Because the number of adolescents, as well as adults, with HIV is rising in most countries where cervical cancer is largely untreated, it can be expected that cervical cancer rates will continue to increase, especially in areas where STDs and HIV/AIDS rates are high. Other less common conditions that cause immunosuppression include those requiring chronic corticosteroid treatments, such as asthma (Castellsague *et al*., 2002 & 2003; Munoz, 2006).

An analysis stratified by HPV status in Colombia and Spain studies showed that risk factors of cervical cancer are associated with the individual's sexual behavior. The most important are early age at the start of the first sexual relationships, high number of sexual partners throughout life, sexual contacts with high risk individuals (in men, frequent contact with women that practice prostitution and in women, frequent contacts with men with multiple sexual partners) (Castellsague *et al*., 2002; Hogewoning *et al*., 2003; Bleeker *et al*., 2003). It is important to emphasize that at young ages and at the most sexually active ages in spite of very frequent infection by HPV, the great majority of infected women (more 90%) resolve the infection spontaneously and the infection persists in only a small fraction of women (Bosch *et al*., 2003; Elfgren *et al*., 2000).

In Malaysia, a cross-sectional school survey of 12-19 year old adolescents is 5.4% (of which 8.3% were males and 2.9% were females) reported having had sexual intercourse (Lee *et al*., 2006). Median age at first sexual intercourse was 15 years; however, this estimate may be underreported given that talking about sex is culturally taboo subject in Malaysia.

Another risk factor is having a blood relative (mother or sister) with cervical cancer. Magnusson, *et al*., (1999) compared the incidence of dysplasia and carcinoma *in-situ* (CIS) in relatives of women with disease and in age-matched controls (Munoz, 2006). They found a significant familial clustering among biological, but not adoptive, relatives.

In many developing countries, women who have abnormal Pap smears frequently do not receive treatment at an early stage when cervical cancer could be prevented because of there are long delays in reading and reporting the results. Furthermore it is difficult to locate the patient once the report becomes available. Besides that the cost of treatment is not affordable for many women, even when simple outpatient procedures are used and there is a lack of equipment as well as service providers trained to use and maintain it. As a consequence, even in countries where Pap smears are available, many women may not get the treatment they need in a timely manner. According to the World Health Organization (WHO) Health Survey 2001/2002, Pap smear coverage was only 23%. The highest Pap smear uptake was among women aged 30-39 years (36.6%) as compared to women in other age group: 18-29 years (14.6%), 40-49 years (28.8%), 50-59 years (20.9%) and 60-69 years (5%) (WHO, 2002).

The number of women who smoke has increased dramatically in recent years especially among teenage girls. A survey carried out in 2005, showed that in Malaysia current smoker prevalence rate was 18.6% (Rosliza and Khadijah). Nicotine and the byproducts of smoking are thought to increase a woman's relative risk for cervical cancer because they concentrate in the cervical mucus and decrease the immune capability of Langerhan's cells to protect cervical tissue from invading oncogenic factors, such as HPV infection (Munoz, 2006).

2.5 Human Papillomavirus

Papillomaviruses consists of small double-stranded circular DNA genomes of approximately 8-kilobase pairs (Rapp and Chen, 1998; Castellsague, 2008). The human papillomavirus (HPV) is a non-enveloped virion with an icosahedral capsid. The HPV genome encodes eight early genes products, two late genes products (Table 2.4) and upstream regulatory region (URR, non-coding) (Soliman, 2004). These viruses infect squamous epithelial of a variety of species. Approximately 200 human papillomavirus (HPV) types have been described. HPVs cause a range of epithelial hyperplastic lesions and can be classified into two groups: mucosal and cutaneous. These groups can be further divided into low-risk and high-risk depending on the associated lesion's propensity for malignant progression (Margaret *et al*., 2008).

Table 2.4: Functions of the products of HPV early region open reading frames (Anderson, 2003).

Early region open reading frame	Protein function
E1	• Two proteins required for extrachomosomal DNA replication and completion of viral life cycle • Work with E2 products
E2	• Two proteins required for extrachromosomal DNA replication • Work with E1 products • Full length protein acts as a transcriptional activator and binds to DNA in the URR to increase transcription of the early region • Smaller protein inhibits transcription of the early region
E4	• Important for the maturation and replication of the virus • Expressed in later stages of infection, when complete virions are being assembled
E5	• Interacts with cell membrane receptors, such as EFG and PDGF • Might stimulate cell proliferation in infected cells
E6	• Crucial for viral replication, host cell immortalization and transformation • Binds to p53 and stimulates p53 degradation through ubiquitin-dependent proteolytic pathway
E7	• Crucial for viral replication, host cell immortalization and transformation • Binds to Rb proteins and dissociates E2F-Rb complex stimulating transcription of cellular genes

Figure 2.2 shows the electron micrograph of purified virus from a human skin wart in a cutaneous or mucosal epithelial cell negatively stained with potassium phosphotungstate (Howley, 1996).

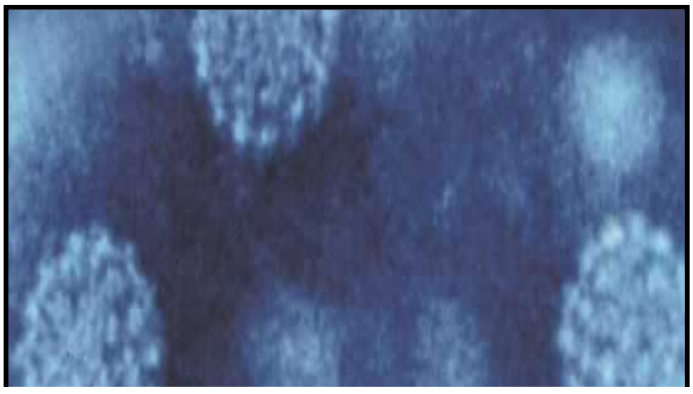


Figure 2.2: Electron micrograph of purified free (HPV) from a human skin wart negatively stained with potassium phosphotungstate (Howley, 1996).

2.5.1 Diversity Amongst Human Papillomavirus

Over 100 different HPV types have been described, with new types classified based on nucleotide and amino acid sequence alignment (Bernard, 2005; Castellsague, 2008). Intratypic variation may also occur with a specific HPV type, and these natural variants may demonstrate different biological

properties. The distribution of these variants shows geographic variability and non-prototypic variants have been shown in some studies to increase the risk of cervical lesions and cancer. Although papillomavirus classification is based on nucleotide sequence homology, the differences between evolutionary groups are reflected to some extent in the differences that exist in the biology of the different viruses (Meyers *et al*., 1994). Approximately 35 HPV types have been isolated from the anogenital epithelium. The remaining HPV types have been associated with benign lesion (i.e. hand and plantar warts) and malignant skin lesion that occur in a rare condition known as epidermodysplasia verruciformis. Table 2.5 shows the association of HPV types with lesion categories (Anderson, 2002).

Table 2.5: Association of HPV types with lesion categories

Lesion	HPV types
Common warts	1, 2, 3, 4
Plantar warts	1, 4
Flat warts	3, 10
Butcher's warts	3, 7
Epidermodysplasia verruciformus	3, 5, 8, 9, 10, 12-15, 17, 19, 20-25, 28, 29
Respiratory	6, 11, 30
Genital	6, 11, 16, 18, 30, 31, 33-35, 39, 40, 42, 43-45, 51-59, 66, 68, 70

(Adapted from Anderson, 2002)

Anogenital HPV types are often classified as low-risk or high-risk depending on their association with lesion types (Margaret *et al*., 2008). Low-risk viral types such as HPV 6 and HPV 11 are associated with low grade lesion, primarily with benign lesion such as condylomas which rarely progress to cancer. The high-risk HPV types, including HPV type 16 (HPV-16) and 18 (HPV-18) are commmonly associated with lesions that can progress to high-grade cervical intraepithelial neoplasia (CIN) and ultimately to cervical carcinoma and detected in almost 100% of squamous carcinomas and adenocarcinomas of the uterine cervix (Tahir, 1994; Rapp and Chen, 1998; Walboomers *et al*., 1999 and Pirog *et al*., 2000; Castellsague, 2008). The stratification of HPV types into low-risk and high-risk group is summarized in Table 2.6.

Table 2.6: Classification of HPV into low-risk and high-risk groups

Risk group	Type
Low	6, 11, 42, 43, 44
High	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, and 70

(Adapted from Margaret *et al*., 2008).

The progression of high risk HPV-induce lesions is depicted in Figure 2.3 and is mainly based on experimental findings within various stages of progression (De Villiers, 1992). This figure has shown the stepwise progression of high risk HPV induce lesion that can progress to low grade CIN, high grade CIN, carcinoma *in situ* and ultimately to invasive cancer. The inner circles schematically represent initially infected cells. The subsequent circle symbolized progressively modified clones with increased and growth potential.

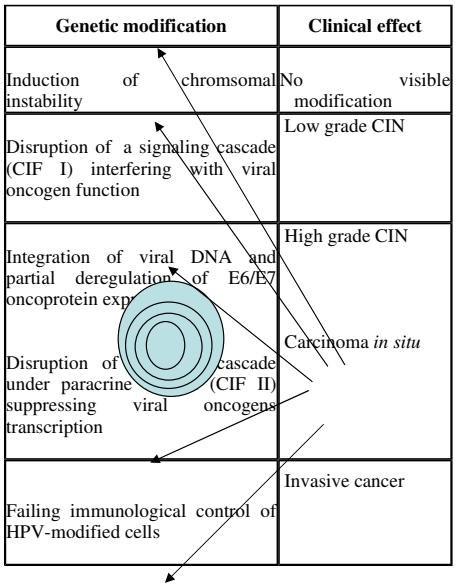
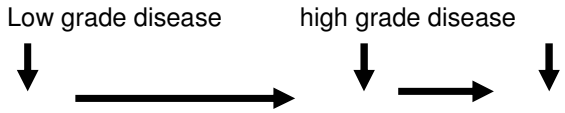


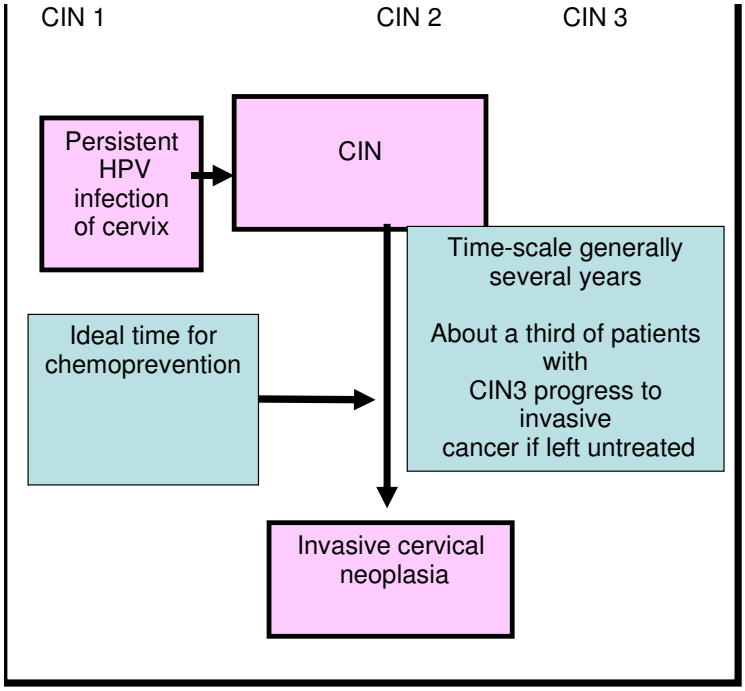
Figure 2.3 Stepwise progression of high risk HPV-induce lesion. The inner circle schematically represent initially infected cells, subsequent circles symbolize progressively modified clones with increased and growth potential.
(de Villiers, 1992).

2.5.2 Human Papillomavirus and Cervical Cancer

Human papillomaviruses (HPVs) are seen as the primary cause of cervical cancer because HPV genomes are detected in about 90% of all cervical cancer and encode proteins with molecular properties required for cellular transformation in cell culture and *in situ*. The development of cervical cancer intraepithelial neoplasm (CIN) and cervical cancer was reported be asociated with HPV 16 and frequently detected in cervial intraepithelial neoplasia (CIN) and invasive cervical carcinoma (ICC) (Igor *et al*., 2003). Invasive cancer is preceded by a progressive spectrum of abnormalities of the cervical epithelium which are considered cancer precursors and classified as CIN 1 (mild dysplasia), CIN 2 (moderate dysplasia) and CIN 3 (severe dysplasia and carcinoma *in situ*). Flat cervical condylomas are considered part of the spectrum of CIN 1.

Progression of cervical intaepithelial neoplasia (CIN) to invasive disease is shown in Figure 2.4. CIN is classified in grades namely low grade squamous intraepithelial lesion (CIN 1) and high grade squamous intraepithelial lesions (CIN 2 and 3). CIN can start in any of the three stages, and can either progress to the next grade, or regress (Kumar *et al*., 2007). Most cases of CIN remain stable, or are eliminated by the host's immune system without intervention. However a small percentage of cases progress to become cervical cancer, usually cervical squamous cell carcinoma (SCC), if left untreated. However most of CIN spontaneously regress. About 50% of CIN 2 will regress within 2 years without treatment (Agorastos *et al*., 2005).





gure 2.4: Progression of cervical intraepithelial neoplasia (CIN) to invasive disease

.dapted from Stanly and Margaret, 2003)

Figure 2.5 shown annual numbers of cases of various cancers worldwide. Gray bars show the total annual number of cases of various cancers worldwide. The fraction of cancers estimated to be induced by HPV types is shown in red. For example, nearly all cases of cervical cancer and a substantial majority of anal cancers are believed to be the caused by HPV (Parkin 2006).

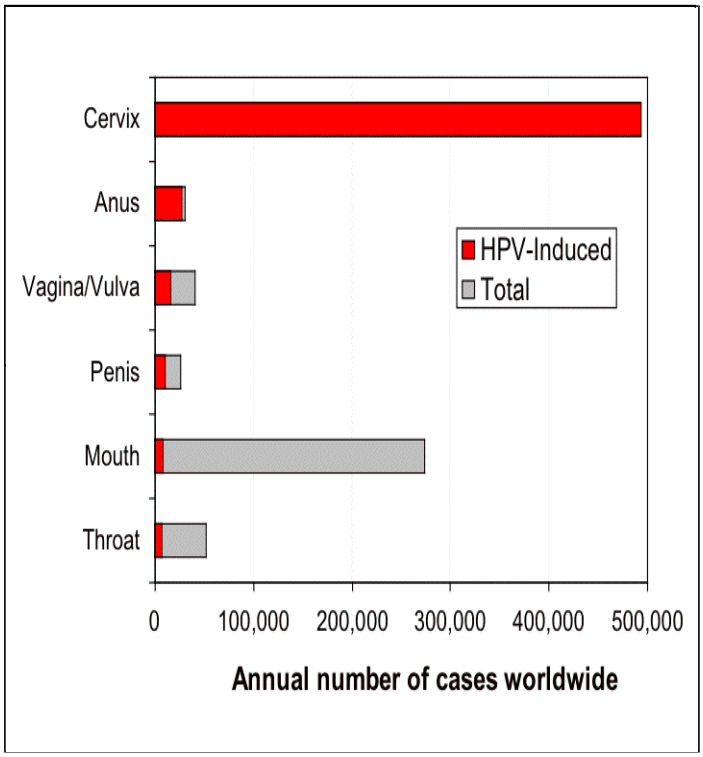


Figure 2.5 Annual numbers of cases of various cancers worldwide (Adapted from Parkin, 2006).

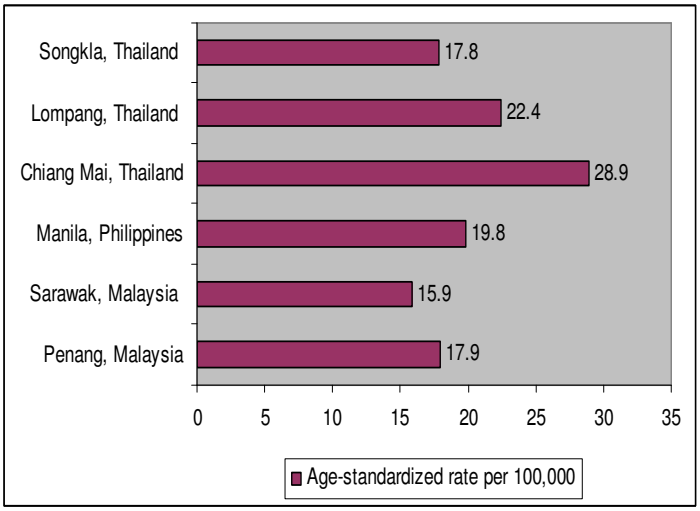


Figure 2.6: Age-standardized (world) incidence rates of cervical cancer by cancer registries (1998-2002) in Malaysia, the Philippines, and Thailand (Curado *et al*., 2007).

Figure 2.6 shows age-standardized (world) incidence rates of cervical cancer by cancer registries (1998-2002) in Malaysia, the Philippines, and Thailand (Curado *et al*., 2007). Malaysia has a population of 8.49 millions women ages 15 years and older who are at risk of developing cervical cancer. Current estimates indicate that every year 1492 women are diagnosed with cervical cancer and 766 die from the disease. Cervical cancer is the second most frequent cancer among women between 15 and 44 years of age (WHO, 2007). In Malaysia, the overall incidence rate in 2002 is 19.7 per 100,000 women, however differs by ethnic group. Ethnic Chinese women have the highest ASR of 28.8 per 100,000, followed by ethnic Indians with 22.4 and ethnic Malays (includes Peninsular Malaysia but not East Malaysia) with 10.5 per 100,000 women (Second reports of National Cancer Registry, 2004; Ferly *et al*., 2004).

2.5.3 Biochemical Properties of HPV E6 and E7 Oncoproteins

HPV 16 is a virus that infects several hundred thousand patients per year. Walter *et al*., 1999; was reported the E6 protein contains 158-amino-acid residue and contains two hypothetical Cys-X2-Cys-X29-Cys-X2-Cys (where X represents any amino acid and the number represents the number of residues) zinc fingers.

The oncogenic activities of papillomavirus E6 proteins have been reflected in many biological assays. These include immortalization of primary cells, transformation of established cell lines, resistances to terminal differentiation, tumorigenesis and abrogation of cell checkpoint (Rapp and Chen, 1998). The E6 protein by itself or in cooperation with HPV 16 E7 gene product can immortalize primary keratinocytes, fibroblast and epithelial cells (Halberd *et al*., 1991).

A Patscan search reveals that this sequence motif is unique for papillomavirus E6 as well as for E7 proteins (Rapp and Chen, 1998). E6 binding protein (E6BP) (also known as ERC-55) is a calcium binding protein localized in the endoplasmic reticulum, with possible consequences for intracellular signaling. E6 has also been described to activate or, alternatively to repress transcription, stimulate telomerase, immortalize primary cell culture and interfere with the differentiation of human keratinocytes (Walter *et al*., 1999).

The E6 protein promotes the degradation of the p53 tumour suppressor protein, whereas the E7 protein inactivates the Rb protein and related pocket protein, p107 and p130 (Munger *et al*., 2001). Alteration in the p53 gene including deletion, insertation and point mutation are the most frequent genetic events in many different carcinomas (Takashi, 1992). However, the tumorigenic properties of the E6 and E7 proteins may not necessarily be limited only to the Rb and p53 to related pathway (Igor *et al*., 2003).

The E6 and E7 products from oncogenic viral types like HPV 16 and HPV 18 can binds to inactivate and cause the degradation of cellular tumours suppressor gene products p53 and Rb. This process plays an important role in the development of cervical cancer by a variety of mechanisms including altering host gene expression, releasing cell from cell cycle checkpoints, affecting the DNA repair process and/or activating the expression of telomerase (Rapp and Chen, 1998 and Anderson, 2003).

The viral E6 and E7 oncoproteins can alter the keratinocyte terminal differentiation phenotype and abrogate negative cell cycle controls to induce cells to enter S-

phase. In doing so, the E6 and E7 proteins are establishing conditions in which viral DNA synthesis can occur in the differentiating epithelial cells. Cells constitutively expressing E6 and E7 proteins can pass by normal cell cycle checkpoints resulting in the accumulation of genetic damage which could ultimately result in malignant progression (Rapp and Chen, 1998).

P53 mutation is the most commonly reported change in human oncogenesis. Wild-type p53 has distinct DNA binding and transcription factor properties. The p53 protein levels and DNA binding activity are inducible by DNA damaging agents such as actinomycin D and irradiation. Induction of p53 is followed by the transcriptional activation of genes involved in DNA repair. Wild-type p53 also block cell cycle progression in response to DNA damage and has been implicated in apoptosis. Mutation or inactivation by viral oncogenes inhibit the transactivation function of p53 and subsequently affects its control on cell growth, cell cycle progression and apoptosis. The major mechnanism of control of p53 expression appears to be through protein stability (posttranslational modification) and several observations support the hypothesis that HPV E6 modulates p53 function by targeting the protein for ubiquition and rapid degradation (Maria *et al*., 1999).

2.6 Treatment and Prevention of Cervical Cancer

The last few decades have seen a significant progress in the management of various cancers. In addition to traditional treatments such as radiation, surgery, and chemotherapy, several new approaches based on knowledge of biological processes have been developed and tested and are being constantly refined.

Radiation therapy also called radiotherapy, x-ray therapy, or irradiation is the use of a certain type of energy (called ionizing radiation) to kill cancer cells and shrink tumours. Radiation therapy injures or destroys cells in the area being treated (target tissue) by damaging their genetic material, making it impossible for these cells to continue to grow and divide (Bucci *et al*., 2005). Although radiation damages both cancer cells and normal cells, most normal cells can recover from the effects of radiation and function properly. The goal of radiation therapy is to achieve maximum toxicity in the tumour while limiting injury to the surrounding normal tissues. On the other hand, radiation sensitivity of normal tissues is affected by different tumour and patient factors, principally vascular co-morbidities and genetic susceptibility (Jiade, 2004).

Historically, the three main divisions of radiotherapy are external beam radiotherapy or teletherapy, brachytherapy or internal radiotherapy, and systemic radioisotope therapy or unsealed source radiotherapy (Bucci *et al*., 2005). The differences relate to the position of the radiation source; external is outside the body, brachytherapy uses sealed radioactive sources placed precisely in the area under treatment, and systemic radioisotopes are given by infusion or oral ingestion (Jiade, 2004). The radiation used in external radiation therapy can come from a variety of sources, including an x-ray, electron beam or gamma rays (Galvin *et al*., 2004).

Chemotherapy is a systemic therapy with anticancer drug in the treatment of cancer. Chemotherapy destroys cancer cells anywhere in the body. It even kills cells that have broken off from the main tumour and traveled through the blood or lymph systems to other parts of the body (Fischer *et al*., 2003). Chemotherapy can cure some types of cancer. In some cases, it is used to slow the growth of cancer cells or to keep the cancer from spreading to other parts of the body (Skeel *et al*., 2003). When a cancer has been removed by surgery, chemotherapy may be used to keep the cancer from coming back (adjuvant therapy). Chemotherapy also can ease the symptoms of cancer, helping some patients have a better quality of life (Fujiki *et al*., 1997; Morrow and Cowman, 2000).

Robotic surgery is the use of robots in performing surgery. Three major advances aided by surgical robots have been remote surgery, minimally invasive surgery and unmanned surgery. Some major advantages of robotic surgery are precision, miniaturization, smaller incisions, decreased blood loss, less pain, and quicker healing time (Pott *et al*., 2005). Further advantages are articulation beyond normal manipulation and three-dimensional magnification. Robotic surgery is a minimally invasive alternative to laparoscopy for the surgical treatment of endometrial cancer and cervical cancer (Pott *et al*., 2005).

The Papanicolaou test (also called Pap smear, Pap test, cervical smear, or smear test) is a screening test used in gynecology to detect premalignant and malignant (cancerous) processes in the ectocervix. Significant changes can be treated, thus preventing cervical cancer. The test was invented by and named after the prominent Greek doctor Georgios Papanicolaou. Prior to the introduction of the Pap test, carcinoma of the cervix was a leading cause of death in women (Coste *et al*., 2003). Since the introduction of the Pap test, deaths caused by carcinoma of the cervix have been reduced by up to 99% in some populations wherein women are screened regular (DeMay,

2007).

2.7 Natural Products and Anticancer Drug

Natural products from plants are used for centuries to cure various ailments. Today, the use of bioactive plant-derived compounds is on the rise worldwide. This is due to the main apprehension in side effects from the use of synthetic drugs developed by pharmaceutical industries. There side effects can be more dangerous than the diseases, these drugs are claimed to cure. The demonstration of the presence of natural products such as polyphenols, alkaloids, flavonoids and other secondary metabolites in medicinal plants will provide a scientific validation for their popular use and may serve as guide to assist in the selection of the plants with anticarcinogenic activity (Swayamjot *et al*., 2003).

Plants have played an important role as a source of effective anticancer agents, and it is significant that over 60% of currently used anticancer agents are derived in one way or another from natural sources, including plants, marine or organisms and microorganism (Cragg *et al*., 2005; Newman *et al*., 2003). The search for anticancer agents from plant sources started in earnest in the 1950s with the discovery and development of the vinca alkaloids, vinblastine and vincristine, and the isolation from the Madagascar periwinkle, *Catharanthus roseus* (Apocynaceae) which was used by various cultures for the treatment of diabetes (Cassady and Douros, 1980; Gueritte and Fahmy, 2005).

Each of these points should be considered when developing a new protocol or in using an unfamiliar protocol for the first time (Bruce, 1993). The process of drug discovery and development of anticancer agents involve substantial time, effort and resources. The approaches to identify the new therapies are constantly being evaluated and modified.

Plant alkaloids are antitumour agents derived from plants. These drugs act specifically by blocking the ability of a cancer cell to divide. Although they act throughout the cell cycle, some are more effective during the S-and M phase. Examples of plant alkaloids used in chemotherapy are actinomycin, doxorubicin and mitomycin.

The literature indicated that many natural products are available as chemoprotective agents against commonly occurring cancers worldwide. A major group of these products are the powerful antioxidants, others are phenolic in nature and remainder includes reactive groups that confer protective properties. These natural products are found in vegetables, fruits, plant extracts and herbs. Although the mechanism of the protective effect in unclear, the fact that the consumption of fruit and vegetables lowers the incidence of carcinogenesis is widely reported (Reddy, 2003).

The antioxidants, vitamin C, E and the provitamin β-carotene from vegetables and herbs exert chemopreventive properties. Antioxidants are substances or nutrients in foods which can prevent or slow the oxidative damage to body. When the body cells use oxygen, they naturally produce free radicals (by-products) which can cause damage. Antioxidants act as "free radical scavengers" and hence prevent and repair damage done by these free radicals (Gueritte and Fahmy, 2005).

Chemoprevention trials investigate the abilities of specific dietary constituents or synthetic compounds to block or suppress the initiation or progression of carcinogenesis. Chemoprevention trials test potential cancer inhibitory agents in initial phase I (pharmacological and toxicological profile) and phase II (biomarker endpoint) trials to determine which agents have the most potential with regard to high efficacy and low toxicity. If findings in phase I and II clinical studies of a chemoprevention agents support the initial hypothesis, a phase III study is conducted to determine specific clinical outcomes (Greenwald *et al*., 1990).

Numerous epidemiological studies have demonstrated a protective effect of vegetable and fruit consumption on cervical cancer risk. Increase in vegetable intake up to 50% of all dietary products will decrease up to 50% risk of cancer (Ong, 2003) and eating at least five fruits and vegetables a day may also help prevent cervical cancer (Berkely, 2003).

Amaranthus gangeticus have been found to be one of diserable source of effective cancer preventive agents because high frequency of inhibitory activity towards liver cancer (HepG2) and breast cancer (MCF7) cell lines (Sani *et al*., 2004). *A. gangeticus* extracts exhibited strong activity toward tumour promoter 12-O-hexadecanoylphobol-13-acetate (HPA)-induced Epstein Barr virus activation in Raji cells (Mukarami *et al*., 2000). The inhibitory effects were also observed in colon cancer cell line (Caco-2) but at a lower percentage compared to HepG2 and MCF7. In normal cells line (Chang liver), there are no inhibitory effects were observed (Sani *et al*., 2004).

Asparagus (*Asparagus oficinalis*) is a popular vegetable consumed in most parts of the world. Asparagus shoots which are frequently used in salads. The crude

saponins from the shoots of asparagus (asparagus crude saponins; ACS) were found to have antitumour activity. The ACS inhibited the growth of human leukemia HL-60 cells in culture and macromolecular synthesis in a dose and time dependent manner (Shao, 1996). *A. officinalis* extracts have been found to exhibit moderate activity toward tumour promoter 12-O-hexadecanoylphobol-13-acetate (HPA)-induced Epstein Barr virus activation in Raji cells (Mukarami *et al*., 2003).

Citrus aurantifolia have been found showed inhibition of human colon cancer (SW-480) (Patil, 2009) and human lymphoblastoid B cell line (RPMI-8866). The citrus extracts also inhibited the Epstein-Barr virus early antigen (EBV-EA) activation induced by 12-O- tetradecanoylphorbol 13-acetate (TPA) as a useful screening method for antitumour promoters. Other researcher found that *C. aurantifolia* extracts exhibited weakly activity toward tumour promoter 12-O-hexadecanoylphobol-13-acetate (HPA)-induced Epstein Barr virus activation in Raji cells (Mukarami *et al*., 2003).

Sesbania grandiflora extracts displayed cytotoxic activity against the HeLa, human cervical carcinoma cell lines (Mackeen, 2007). Other researchers showed that *S. gandiflora* extracts exhibited no activity toward tumour promoter 12-O-hexadecanoylphobol-13-acetate (HPA)-induced Epstein Barr virus activation in Raji cells (Mukarami *et al*., 2003).

2.8 Antitumour Compounds of Vegetables

Glycosinolates

Cruciferous vegetables are a rich source of glucosinolates and their hydrolysis products, including indoles and isothiocyanates, and high intake of cruciferous vegetables has been associated with lower risk of lung and colorectal cancer in some epidemiological studies (Fedrick *et al*., 2003). Glucosinolate hydrolysisproducts alter the metabolism or activity of sex hormones in ways that could inhibit the development of hormone-sensitive cancers. Isothiocyanates and indoles derived from the hydrolysis of glucosinolates, such as sulforaphane and indole-3-carbinol (I3C), have been implicated in a variety of anticarcinogenic mechanisms. Epidemiological studies indicate that human exposure to isothiocyanates and indoles through cruciferous vegetable consumption may decrease cancer risk, but the protective effects may be influenced by individual genetic variation (polymorphisms) in the metabolism and elimination of isothiocyanates from the body (Greenwald, 2001)

Carotenoids

Epidemiological studies have shown that people with high β-carotene intake and high plasma levels of β-carotene have a significantly reduced risk of lung cancer. However, studies of supplementation with large doses of β-carotene in smokers have shown an increase in cancer risk (possibly because excessive β-carotene results in breakdown products that reduce plasma vitamin A and worsen the lung cell proliferation induced by smoke (Alija *et al*., 2004).

Carotenoids were present in *Daucus carota* (carrot) and *Lycopersicum esculentum* (tomato). Blood levels of micronutrients beta-carotene (precursor vitamin A) have been inversely correlated with the development of cancer (Greenwald *et al*., 1990; Fredrick *et al*., 2003). Carotenoids incuding ß-carotene may affect cell transformation and differentiation, enhance cell to cell communication, and enhance immune responeses. ß-carotene is simply a marker for the actual substances in vegetables and fruits that may inhibit cancer development (Greenwald *et al*., 2001). Consumption of carotenoid juice reduce oxidative DNA damage in human lymphocytes by various mechanisms, possibly reduceding cancer risk (Greenwald *et al*., 2001).

Vitamin E

Vitamin E consists of 8 different molecules. There are 4 tocotrienols and 4 tocopherols. The vitamin E molecules all have right-handestereochemistry (McVean, 1999). Vitamin E has been associated to decrease risk for lung and cervical cancers (World Cancer research Fund, 1997). Vitamin E succinate (VES), a drivate of vitamin E, has been shown to trigger apoptosis of human prostate carcinoma cells *in vitro* (Israel, 2000). Report shows that vitamin E inhibited cell proliferation (Azzi, 2000).

Vitamin C

Early epidemiologic evidence indicated that high intakes of vitamin C, rich fruit and vegetables and a high vitamin C concentration in serum are inversely associated with the risk of some cancers (Lee e*t al*., 2003). A recent epidemiologic study by Khaw *et al*., 2001, showed that a high vitamin C concentration in plasma had an inverse relation with cancer-related mortality. In 1997, expert panels at the World Cancer Research Fund and the American Institute for Cancer Research estimated that vitamin C can reduce the risk of the stomach, mouth, pharynx, esophagus, lung, pancreas, and cervical cancers (World Cancer Research Fund, 1997).

Selenium

Selenium is effective in reducing cancer incidence in animal models, and epidemiologic data, as well as supplementation trials, have indicated that selenium is likely to be effective in humans (Holben, 1999). Experiments in variety of animal models have demonstated that selenium can inhibit carcinogenis. For example, selenium supplied as high-selenium broccoli significantly decreased the incidence of chemically-indicative of colon cancer, in rat (Finley, 2000). Selenium is a component of numerous selenoproteins (e.g. glutathione peroxidase, thioredoxin reductase) that function as enzymes in redox reactions that may affect cancer risk (Holben, 1999).

Retinoids

Apart from chemoprevention, retinoids could have a place in cancer chemotherapy or as radiation sensitizers. Radiation sensitizers act in a number of ways to make cancer cells more susceptible to death by radiation than surrounding normal cells, and several such compounds are now available for the treatment of solid tumours (Park *et al*., 2003). In human cervix, retinoid have been shown to interact with the HPV proteins E6 and E7, thus restoring the tumour suppressive roles of pRB and p53 proteins. The oncogenicity of HPV is attributable to the E6 and E7 proteins and expression of these proteins is higher in tumours than in healthy tissue. Increased expression of E6 and E7 has also been shown in high grade cervical lesions such as CIN 3. Clearly, the retinoids are promising class of drugs that will potentially aid in combating cancer (Dianne, 2007).

2.9 Bioactivities of Selected Vegetables

Antigenotoxic Activity

Centella asiatica has been reported to exert antigenotoxic properties (Siddique *et al*., 2007). In, 2008 Siddique found that *C. asiatica* plant extract can modulate the genotoxicity of cyproterone acetate (CPA) of human lymphocytes *in vitro.* CPA is not only a tumour promoting agents but also a genotoxic chemical by generating free radicals and the excess of reactive oxygen species leads to the DNA damage. Therefore it can be concluded that *C. asiatica* extracts has the potential to reduce the genotoxic damage induced by CPA in cultured human lymphocytes (Siddique *et al*., 2008).

Apoptosis activity

Allium tuberosum contains thiosulfinates, which is used as folk medicine as well as to flavor foods. Thiosulfinates are known in the Allium species as unstable intermediates in the enzymatically initiated degradation of S-alk(en)yl-L-cysteine sulfoxide (Kim, 2008). Thiosulfinates significantly induced cell death in dose-and time-dependent manners in HT-29, colon cancer cells and PC-3 cells, human prostate cancer cells which is associated with apoptosis. Thiosulfinates activated the initiator caspase-8, and-9 and the effector caspase-3. Thiosulfinates were found to increase the expression of AIF, a caspase-independent mitochondrial apoptosis factor and induced DNA fragmentation and chromatin condensation in HT-29 cells (Leea *at al*., 2009).

Antioxidant activity

Recently, there has been increasing interest in the protective biochemical function of phytochemical, especialy flavonoids from *Pisum sativum* and their related compounds for the prevention of oxidative damage to an organism caused by reactive oxygen species (ROS). Imbalance between ROS and antioxidant defence systems may lend to chemical modification of biologically relevant macromolecules like DNA. This pathobiochemical machanisme involved in initiation or development of various diseases including cancer (Troszyrisa *et al*., 2002).

Kudo were reported that *Solanum tuberosum* have antioxidant activities (Kudo *et al*., 2009). Similar result were observed in *Asparagus officinalis* where the composition of bioactive compounds and the antioxidant activity of a fibre-rich product obtained from asparagus by-product, which may be used as an ingredient in the preparation of additional food products (Alventosa, 2009).

Antifungal activity

An antifungal drug is a medication used to treat fungal infectin. A drug that has been used for 40 years for the treatment of skin fungus has been found to be a possible cancer treatment. The antifungal drug, griseofulvin, has been shown to inhibit the growth of cancer cells in laboratory (Wilson, 2005).

Brassica oleracea var *alboglabra* were contain napin-like polypeptide (Ngai, 2004) were showed inhibited mycelial growth in number of fungal species including *Fusarium oxysporum, Helminthosporium maydis, Mycosphaerella arachidicola and Valsamali* and exhibited pronounced thermostability and pH stability (Lin and Ng, 2008).

Antiproliferative

Antiproliferative was used or tending to inhibit cell growth and effect on tumour cells and cruciferous vegetables are an important source of compounds that may be useful for chemoprevention (Brandi *et al*., 2005). *Brassica oleracea* var *alboglabra* inhibited proliferation of hepatoma (HepG2) and breast cancer (MCF7) cells with an IC_{50} of 2.7 mM and 3.4 mM and the activity of HIV-1 reverse transcriptase with an IC_{50} of 4.9 mM (Brandi *et al*., 2005; Lin and Ng, 2008). Antiproliferative activities were also studied *in vitro* using HepG(2) human liver cancer cells in *Amaranthus* sp (Spinach), *Capsicum annum* (red pepper) and *Amaranthus* sp showed the highest inhibitory effect, than the Capsicum annum (Brandi *et al*., 2005).

Antibacterial activity

Investigation of the traditional uses of *Momordica charantia* (Cucurbitaceae) in Togo (West Africa) showed that it is one of the most important local medicinal plants both for ritual and ethnomedical practices. There was a high degree of consensus (>50%) for use in the treatment of gastrointestinal and viral disease (Beloin *et al*., 2005).

Leaf extracts (water, ethanol and methanol) of *Momordica charantia* have demonstaretd broad-spectrum antimicrobial activity (Khanna *et al*., 1998). *In vitro* antimicrobial activity of leaves extracts was seen against *E. coli*. An extracts of entire plant was also shown to have antiprotozoal activity against *Entamoeba histolitica* (Grover *et al*., 2004). Saeed and Tariq, 2005 were reported that the skin and seeds of *Pisum sativum* (green pea) exhibited good antibacterial activity with average zone of inhibition (Saeed and Tariq, 2005).

Antiviral activity

Medicinal plants have been traditionally used for different kinds of ailments including infectious diseases. There is an increasing need for substances with antiviral activity since the treatment of viral infections with the available antiviral drugs often leads to the problem of viral resistance (Vijayan *et al*., 2003).

Momordica charantia and several of it isolated phytochemicals, eg. alpha and beta-momorcharin, have been documented to have *in vitro* antiviral activity against Epstein-Barr, Herpes and Human immunodeficiency virus (HIV) promising anti Human immunodeficiency virus activity has been attributed to a isolated protein known as MAP 30 (Grover *et al*., 2004). Beloin *et al*., (2005) reported that lyophilized extracts of *M. charantia* plants were exerted strong antiviral activities against Herpes simplex virus-1 (HSV-1) and Sindbis virus (SINV).

Analgesic activity

Many ethnic groups have used different species of *Portulaca oleracea* (Beremi), a member of the Family Portulacaceae, as vegetable and also herbal medicine against several diseases for many centuries. The 10% ethanolic extract of the aerial parts (dried leaves and stem) showed significant analgesic after intraperitoneal and topical but not oral administration when compared with the synthetic drug, diclofenac sodium as the active control (Chan *et al*., 2000).

Other researcher found that *Portulaca oleracea* were evaluated for their analgesic activity using the hot-plate and tail-flick respone on albino mice and wistar rats, respectively. Results showed that 10% ethanolic extracts showed significant analgesic effects and was comparable with that of sodium diclofenac solution (Chan *et al*., 2000).

2.10 List of Vegetable

Table 2.7 shows the distribution, medicinal uses and phytochemistry of selected vegetables studies. In the present study, all the vegetables were evaluated for their cytotoxic and anti-HPV activities. All the vegetables are commonly found in Malaysia and have been documented for their medicinal uses and phytochemical conten

Table 2.7: The distribution, medicinal uses and phytochemistry of selected vegetable

No	Scientific name	Family name	Common name	Distribution	Medicinal uses	Phytochemical contents
1	Liliaceae	*Asparagus officinalis*	Asparagas	Not naturalized in any country and widely distributed in the Mediterranean countries	In India the root has uses as astimulant, restorative, demulcent, diuretic and antidysentric	Green shoots contains an abundance of amino-succinamic acid
2	Liliaceae	*Allium fistulosum*	Spring onions	Its original country is unknown. It has been also commonly cultivated in India, Chi[illegible]l Japan	The bulb is antibacterial, antiseptic, diuretic, galactogue, stamachic, vermifuge and vulnerary	The leaves contains protein, fat, carbohydrate, vitamin B1 and vitamin C
3	Liliaceae	*Allium tuberosum*	Chinese chive	It has bee[illegible]ivated in East Asia	The plant is antibacterial, digestive and stimulant	Both leaves and flowering clums contains of vitamin A, B and C, Protein, fat and carbohydrate
4	Solanaceae	*Capsicum annum*	Chili	It is know from prehistoric in Peru and widely cultivated in Central and South America	Fruits use for circulatory stimulant and pain reliever. Roots in a compound decoction given for gonorrhoea.	Fruits of chilies rich of solanine and capsaicin and vitamins A and C
5	Solanaceae	*Capsicum frutescens*	The bird pepper	Occurs as weed from northern South America to Southern USA	To treat digestive complaints, relieving colds and headaches	Fruits of chilies contains oleoresins and capsaicin
6	Solanaceae	*Lycopersicum esculentum*	Tomato	Native to Central and South America and have spread as far north as Mexico	Fruit is beneficial skin wash for people with oily skin. Sliced fruits used treatment for buns, scalbs and sunburns	The seed contains oil and solanine. Solanine and saponin are present in the green parts of the plant
7	Solanaceae	*Solanum melongena*	Brinjal	Have been domesticated in India.	Fruits are prescribed for use haemorrhoids. The root is applied for ulcerationFruits contains carbohydrates and proteins	Fruits contains carbohydrates and proteins

2.7 continued

8	Solanaceae	*Solanum tuberosum*	The potato	It is native of the Andes. Ireland was the first country to cultivate potatoes	The fruits were reported for buns, corns, cough, cystitis, fistula, prostates, scurvy, spasm, tumour and warts	Potato contains the alkaloid solanine. The leaves and other green parts of the plant also contain solanine
9	Cucurbitacea	*Cucumis sativus*	Cucumber	The species was cultivated in France by the ninth century	The fruit is depurative, diuretic, emollient, purgative, and resolvent	Oil from seed contains linoleic acid, oleic acid, palmitic acid and stearic acid
10	Cucurbitacea	*Cucurbita moschata*	Muskmelon	The plant has been growing in America and it might be naturalized in Asia, Africa and America	The seed is vermifuge. It is eaten fresh or roasted for the relief of abdominal cramps	Fruits contains antioxidant, vitamin A, C, mineral and carbohydrate
11	Cucurbitacea	*Lagenaria sceraria*	The bottle gourd	Bottle gourd was carried from Africa to South America	The leaves have a purgative action. The bottle fruit is eaten for colic with fever. Externally, juice of the fruit with limejuice is an application for pimples. The pulp is applied to the head in delirium in India	The seeds contain saponin
12	Cucurbitacea	*Momordica charantia*	Bitter gourd	*M. chanrantia* is a genus about 42 species, mainly Africa, and several species are cultivated for their edible fruits	As a medicine, the vegetative parts a chiefly used. The fruits common to apply for skin. They are applied for buns, scalds, and to the abdomens of children for stomachache.	The bitter substance in the tissues is a glucoside. Along with it, in the leaves have small quantities of a oil and resin

Table 2[illegible]tinued

13	Cucurbitacea	*Luffa acutangula*	Loofah	Angled loofah is probably native to India where the wild from with bitter fruits occur	A tea of leaves is used as a diuretic, while juice of the fruits is used against internal hemorrhage	Fruits contain colocynthin. The seed contains oils nea[illegible] count.
14	Compositae	*Cosmos caudatus*	Ulam rajah	Native to scrub and meadow areas in Mexico, the southern United States, Central America and northern South America	The leaves recommended in the traditional medicine system for improving blood circulation	The leaves contains protein, calcium, fibre, vitamin A and essential oil
15	Compositae	*Lactuca sativa*	lettuce	It is through temperate and warm Europe, in Mediterranean and through the warmer temperate parts of western Asia	The seed is anodyne and galactogogue lettuce has acquired a folk reputation As an aphrodisiac, anodyne, carminative and diuretic	The seed of lettuce contains bland oil. It is rich in vitamin and minerals eg. sodium, phosphorus, calcium and magnesium
16	Leguminosae	*Archidendron jiringga*	Black pot	Native in Bangladesh, Borneo, Brunei, Burma, Indonesia, Kalimantan,	The jering fruit is used to treat hypertension. How ever, an overdose can result	The seeds contain volatile oil consisting of an allyl sulphur compound an alkaloid and toxic

				Malaysia, Myanmar, Singapore, Sumatra and Thailand	in toxicity that can cause kidney hyperemia and difficulty in urinating	jenkolic acid
17	Leguminosae	*Neptunia prostrata*	Winter mimosa	It is found almost as widely as the genus and in the Malay Peninsula	The Malays use the root in the late stages of Syphilis	Leaves contains carbohydrate, starch and vitamins

Table 2.7 continued

18	Leguminosae	*Pachyrrhizus erosus*	Yam bean	Native to Central America. It was brought to the Philippines and later become widely cultivated in India, China and east Africa.	The seeds are used in Java for applying to skin affection	From analysis, tubers c[illegible] 10% had carbohydrate[illegible] protein, fat, albumi and starchy matter
19	Leguminosae	*Psophocarpus tetragonolobus*	Botor bean	It came from the African side of the India Ocean, probably from Madagascar. The plant is now widely cultivated from India to New Guinea	Leaves used in a compound lotion for smallpox. The root is used in the Shan States for poulticing to cure vertigo	Seeds contain albuminiods, carbohydrates, oil, fat, starch and protein
20	Leguminosae	*Parkia speciosa*	Foul-smelling edible seeds	Native of Malaysia and often cultivated by the Malays	The petai seeds are used to treat diabetes and intestinal worms. The bark is considered to be tonic	Petai seeds contain β-carotene, thiamine, riboflavin, tannin, nitrates, nitrites, alkaloids, fatty acids and amino acids
21	Leguminosae	*Sesbania grandiflora*	Turi	It is a native of tropical north eastern Asia and planted widely from Africa to Pacific.	Bark, leaves and gums are considered medicinal. Resorted to be aperients, diuretic, emetic, emmenagouge, febrifuge, laxative and tonic	Leaf contains protein, fat, fiber, calsium, magnesium, thiamin, riboflavin, niacin and ascorbic acid
22	Leguminosae	*Vigna sinensis*	Long bean	It is probably a native of India and China and originally either Asiatic or African, and was spread to Europe early enough for the Greeks and Latins to grow it.	The juice of leaves is dropped hot into the ear forearm-ache	Bean contains fats and albuminoids

Table 2.7 continued

23	Leguminosae	*Pisum sativum*	Snow pea	Its origin can be traced to the stone age. The plant probably originated in Southwest Asia	Seed is contraceptive, fungistatic and spermicidal	Tender pods and [illegible] are highly nutritive and [illegible]in a percentage of digestible protein, carbohydrates, vitamin and minerals
24	Leguminosae	*Phaseolus vulgaris*	French bean	Widely cultivated of all beans in temperate regions and widely cultivated in semitropical regions	Beans are said to be used for acne, bladder, buns, cardiac, carminative, depurative, diabetes, diarrhea, diuretic	Beans contains 20% proteins and at least 50% carbohydrates
25	Amaranthaceae	*Amaranthus gangeticus*	Spinach	Its original home is India. The Chinese cultivate it throughout a large part of China and its cultivation is nothing new to them when undertaken in Malaysia	A decoction made from the roots of plant and of *Cucurbita pepo* is used to control the resulting haemorrhage	The whole plant contains 62 per cent of starch in it, with 6 per cent of fat. It contains substantial amounts of vitamins A, B, C and double the amount of iron found in spinaches
26	Amaranthaceae	*Amaranthus viridis*	White bayam	Virgin in Hawaii and Island	The decoction of the plant is used to dysentery and inflammation. The plant is emollient and vermifuge	The seed contains protein and fat. Leaves contains calories, protein, carbohydrate, fiber and calcium
27	Malvaceae	*Hibiscus esculentus*	Ladies finger	The plant originated from west or central Africa and spread to Europe and the far east in Christian times. It was brought to the new world in the 1600s	The fruits is used for gonorrhoea, dysuria, catarrh and urinary troubles	The fruit contains sodium, potassium, calcium, magnesium, phosphorus, iron, copper, zinc, carbohydrate, protein, carotene, thiamin, riboflavin and niacin

Table 2.7 continued

28	Oxalidaceae	*Averrhoa carambola*	Starfruits	The carambola is believed originated in Cylon and moluccas but it has cultivated in Southeast Asia and Malaysia for many centuries	The ripe fruits is administered to relieved bleding hemorrhoids and the dried fruits of the juice may be taken to counteract fevers	Fruits contains protein, fat, carbohydrate, fiber, iron, carotene, thiamin, riboflavin and ascorbic acid
29	Euphorbiaceae	*Manihot esculenta*	Tapioca	It was first domesticated in central or south	Fresh rhizome made into poultice is applied	The tubers contain starch with an average at about 26%with

				America. The cassava was taken to west Africa by the Portuguese	to sores, cancerous affections, condylomata, excrescences of the eye and tumour	the starch, there are 1.5 to 2% of proteins, fat, carbohydrate, calcium, manganese, and vitamins A, B and C
30	Portulacaceae	*Portulaca oleracea*	Beremi	A succulent herb, found as a weed throughout the warmer parts of the world, in the Peninsular Malaysia in all the more inhabited parts	The leaves are used as a remedy for rheumatism. The leave juice is given to children for relief in bronchitis and diarrhea	Both steam and leaves contains Bacosides A and B, Stigmasterol beta-sitosterol, sapogenins, flavonoids, triterpinoid, saponins, alkaloids, brahmine and herpertine, D-manitol, betulic acid, octacosane, nicotine and amino acids
31	Convulvulaceae	*Ipomoea batatas*	Sweet potato	The plant is native to tropical America. It spreads to the Pacific Island	The tops are used for poulticing	The root tubers are contains 65% of water, 20% of starch, sugar, 25% of fermentable matter

Table 2.7 continued

32	Convulvulaceae	*Ipomoea reptans*	Kangkung	Probably originated in India and distributed throughout the tropics of the world. It is very abundant in Asia and Malaysia	The young plants is laxative and recommended for piles, and fever and applies buds to ringworm	leaves and shoots contains amounts of iron
33	Umbeliferae	*Apium graveolens*	Celery	Its arrival in western Malaysia was from Europe. Celery is more cultivated in Java than in other parts of Malaysia	It is aromatic bitter tonic herb that reduces blood pressure, relieves indigestion, stimulates the uterus and is anti-inflamatory	Seeds contains glycoside-apiin and volatile oil
34	Umbeliferae	*Centella asiatica*	Pegaga	Native to Asia	Pegaga have been used for treating bronchitis, asthma and excessive secretion of gastric juice, dysentery, leucorrhoea, kidney trouble, urethritis and dropsy. This herb is said to have a direct action in lowering blood pressure	Pegega is reported to possess Asiatic acid, asiaticoside, madessiec acid, madecassoside, acorbic acid, β-sitosterol, stigmasterol, 3-glocosylkaempferol, hydroccotyline, riboflavin, carbohydrate, protein, calcium phosphorus and iron

Table 2.7 continued

35	Umbeliferae	*Daucus carota*	Carrot	It is ancient cultivation in the Mediterranean. It is native to Europe, Asia and North Africa	Roots are refrigerant and used in infusion for threadworm. The juice of the roots is applied to ulcers of the neck and uterus, cancer of bowels and stomach cancer	Carrots contains calcium and carotene (source of vitamin A)the leaves contain a volatile oil in small quantities
36	Umbeliferae	*Oenanthe javanica*	Shelum	An erect herb, which in the marshes of Java and is cultivate in Indo-China, Sumatra, and the Malaya Peninsula	The whole plant is depurative, febrifuge and styptic. A decoction is used in the treatment of epidemic influenza, fever and discomfort, jaundice, haematuria and metrorrhagia	The leaves a rich source of vitamins and minerals. The seed contains 3.5% essential oil
37	Chenopodiaceae	*Beta vulgaris*	Beetroot	Native to the Mediterranean but spread east world into west Asia but was only introduce into Germany and Britain around the sixteenth century	the roots as treatment for fever, constipation and aphrodisiac	Beetroot contains a large amount of sugar up to 8% mineral and boron
38	Chenopodiaceae	*Citrus aurantifolia*	Citrus	Native of eastern Malaysia. It was introduced to the Asian mainland early in historical time was and carried by Arab traders to the Middle East	Fruits are treat coughing headache, cold and flu	Contain limonene, terpinol bisabolene and essential oil

Table 2.7 continued

39	Rutaceae	*Brassica oleracea* var.	Chinese brocoli	It probably originated in the Mediterranean	Traditionally, the leaves use for breast cancer treatment	Leaves contai protein, carbohydra...

		alboglabra				calcium and v n C
40	Cruciferae	*Petroselium crispum*	Parsley	It probably originated in Spain and Portugal. It was introduced into Britain in 1548 from Sardinia	The Greeks used it only as a medicine. It was used as an edible plant dates in the Roman times	The leaves contains vitamin C

(Adapted from Joyce, 1991; Ong, 2003; Vimala *et al*., 2003; Reddy *et al*., 2003)

2.11 Tissue Culture

Cell culture is the process by which prokaryotic, or eukaryotic cells are grown under controlled conditions. In practice the term "cell culture" has come to refer to the culturing of cells derived from multicellular eukaryotes, especially animal cells. The historical development and methods of cell culture are closely interrelated to those of tissue culture and organ culture. Animal cell culture became a common laboratory technique in the 1950s, but the concept of maintaining live cell lines separated from their original tissue source was discovered in the 19th century (MacLeod, *et al.* 1999; Masters, 2002). The objective of tissue culture is to maintain viable single cells (cell culture) or a functional unit of cells (Organ culture) outside of their normal multicellular organism (Martin, 1994).

In tissue culture environment, the cells are protected from external pathogens. The tissue culture has a regular supply of nutrients and oxygen and the tissue metabolic-product, potentially toxic in high concentrations and removed at regular intervals. If the tissues are part of a mammal, its environment also included a constant temperature. In tissue culture, an artificial environment is created to replace the function of the missing tissue and organ systems (Martin, 1994).

2.11.1 HeLa Cell Line

A HeLa cell is an immortal cell line used in medical research. The cell line was derived from cervical cancer cells taken from Henrietta Lacks, who died from her cancer on October 4, 1951. HeLa cells contain 10-50 copies of DNA sequences HPV 18 and are the first human tumour cells to be established as a cell line (Massad *et al*., 1996). HeLa cells contain unintact genome of HPV 18, suggesting that the virus is not presenting high replication (tandem repeats) (Pater and Pater, 1985).

Karyotype (chromosome) analysis of HeLa cells from different repositories around the world show that different strains of HeLa cells are now very different from each other, probably due to the malignant nature of the cells and differences in culture conditions in different laboratories over the decade since this cell culture was established. Although HeLa cells provided a substantial foundation for today's knowledge of cells physiology, most analysis of cell structure and function in culture is now performed with non-transformed (non malignant) cells (Jones *et al*., 1971). The polyadenylated HPV RNA species present in HeLa cells were further analyzed by fraction in formaldehyde gels and found to be similar in size to those reported.

The HeLa cell line was derived for use in cancer research. These cells proliferate abnormally rapidly, even compared to other cancer cells. HeLa cells have an active version of the enzyme telomerase during cell division, which prevents the incremental shortening of telomeres that is implicated in aging and eventual cell death. In this way, HeLa cells circumvent the Hayflick Limit, which is the limited number of cell divisions that most normal cells can later undergo before dying out in cell culture (Terry, 2006).

2.11.2 CaSki Cell Line

CaSki cells are cancer cells derived from epidermoid carcinoma of human cervix, similar to HeLa cells, but contain 600 copies of HPV-16 DNA sequences (Lappalainen *et al*., 1994). In the CaSki cell lines the HPV-16 specific RNAs are abundant with three size classes measuring 0.7, 1.2 and 2.6 kb.

2.11.3 MRC5 Cell Line

MRC 5 cells are derived from normal lung tissue of a 14 week old male fetus (Terry, 2006). The cells are capable of 42 to 46 population doublings before the onset of senescence. The MRC-5 cell line was established in a growth medium consisting of Earle's Basal Medium in Earle's balanced salt solution supplemented with 10% calf serum. Following initial cultivation, subcultures were prepared twice weekly at a 1:2 ratio. When the cells reached approximately the 7th population doubling, the majority of the

cultures were harvested to be used to prepare (Terry, 2006).

2.12 Cytotoxicity

Cytotoxic screening evaluates drug-induced alterations in metabolic pathways or structural integrity which may or may not be related directly to cell death (Wilson, 2000). Some cytotoxic assay offer immediate interpretation, such as the uptake of dye by dead cells. This has been termed tests of viability, and is intended to predict survival rather than direct measurement of cytotoxicity (Wilson, 2000).

Cytotoxic screening models provided important preliminary data to select plant extracts with potential antineoplastic properties. Anticancer compounds are substances that inhibit proliferation of cancer cells. They could bind to DNA in the cancer cells, inhibit certain enzymes which are necessary for continual growth of cancer cells (enzyme inhibitors), alter the morphology of cancer cells (immunomodulators) or cause total death (cytotoxic drug) (Derelanko, 1995).

Cytotoxicity can be measured by the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, trypan blue (TB) assay, sulforhodamine B (SRB) assay, neutral red cytotoxic assay and clonogenic assay.

2.12.1 Neutral Red Cytotoxic Assay

Viable cells can be measured with colorimetric assays utilizing a substrate whose colour is modified by living cells, but not by dead cells (Kamissarova *et al*., 2005).

Neutral red assay is a rapid quantitative colorimetric test (Borenfreund and Puerner, 1985). The neutral red (NR) cytotoxic assay procedure is a cell survival/viability chemosensitivity assay, based on the capability of viable cells to integrate and bind (uptake of cationic supervital) neutral red (NR) (3-amino-7-dimethylamino-2-methylphenazine hydrochloride, a supervital dye into viable cells (Babich, 1987).

NR is a weak cationic dye that readily penetrates cell membrane by non-ionic diffusion, accumulates intracellularly in lysosomes, where it binds electrostatically to anionic sites in the lysosomal matrix (Bulyehev *et al*., 1978). Alteration of the cell surface or sensitive lysosomal membrane lead to lysosomal fragility and other changes that gradually becomes irreversible (Komissarova *et al*., 2005). Such changes brought about by the action of xenobiotics results in a decrease uptake and binding NR, making it possible to differentiate between viable intact cells and dead/damage cells, which is the basic of this assays. The NR assay was developed and successfully used to evaluate the cytotoxic effect of metal salts in mouse BALB/c 3T3 fibroblast (Borenfreund and Puerner, 1985).

The amount of NR dye accumulated can be extracted from lysosomes and quantitated spectrophotometrically and compared to NR dye recovered from untreated control cell culture (Borenfreund and Puerner, 1985). The results are expressed as IC_{50} values which can be obtained from dose-respone curve. IC_{50} values refer 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 then 20 µg/ml are considered active for cytotoxicity assay against target cells (Geran *et al*., 1972; Chiang *et al*., 2003).

2.13 Immunocytochemistry

Immunocytochemistry (ICC) is a common laboratory technique which uses antibodies that target specific peptides or proteins antigens in the cell via specific epitopes. These bound antibodies can then be detected using one of several methods. ICC allows researchers to evaluate whether or not cells in a particular sample express the antigen in question. In cases where an immunopositive signal is found, ICC also allows researchers to determine which sub-cellular compartments are expressing the antigen. Immunocytochemistry differs from immunohistochemistry in that the former is performed on samples of intact cells that have had most, if not all, their surrounding extracellular matrix removed. This includes cells grown within a culture, deposited from suspension, or taken from a smear. In contrast, immunohistochemical samples are sections of tissue, where each cell is surrounded by tissue architecture and other cells normally found in the intact tissue.

There are many methods to obtain immunological detection on tissues, including those tied directly to primary antibodies or antisera. A direct method involves the use of a detectable tag (e.g., fluorescent molecule, gold particles, etc.) directly to the antibody that is then allowed to bind to the antigen (e.g., protein) in a cell.

Alternatively, there are many indirect methods. In one such method, the antigen is bound by a primary antibody which is then amplified by use of a secondary antibody which binds to the primary antibody. Next, a tertiary reagent containing an enzymatic moiety is applied and binds to the secondary antibody. When the quaternary reagent, or substrate, is applied, the enzymatic end of the tertiary reagent converts the substrate into a pigment reaction product, which produces a colour (many colours are possible; brown, black, red, etc) in the same location that the original primary antibody recognized that antigen of interest. Presence of coloured product application of chromagen indicates a positive results or presence of the antigen under study. Immunocytochemical stains are used mainly when an H&E (Hematoxylin and Eosin) diagnosis cannot be made (Babich and Borenfreund, 1987).

Some examples of substrates used (also known as chromogens) are AEC (3-Amino-9-EthylCarbazole), or DAB (3,3'-Diaminobenzidine). Use of one of these reagents after exposure to the necessary enzyme (e.g., horseradish peroxidase conjugated to an antibody reagent) produces a positive immunoreactions product. Immunocytochemical visualization of specific antigens of interest can be used when a less specific stain like H&E (Hematoxylin and Eosin) cannot be used for a diagnosis to be made or to provide additional predictive information regarding treatment.

CHAPTER 3

MATERIALS AND METHODS

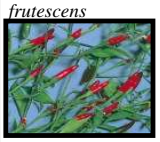
3.1 Vegetable Samples

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

'able 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,	Muskmelon,	

			labu perang, ketola, labu manis	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

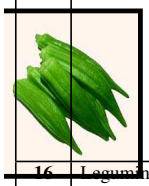
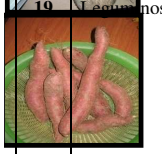
15	Compositae 	*Lactuca sativa* 	salad, selada	Lettuce	Whole plant
16	Leguminosae 	*Archidendron jiringga*	jering	Black pot	Fruit
17	Leguminosae 	*Neptunia prostrata*	akar kemen hantu, keman ayer, kangkong puteri, tangki, keman gajah	Ghost or wild mimosa, winter mimosa	Leaves

Table 3.1 Continued

		*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

27	Malvaceae	*Hibiscus esculentus*	kacang bendi, sayur bendi, kacang lendir	Ochro gomb ladies finge
28	Oxalidaceae	*Averrhoa carambola*	belimbing buluh, belimbing asam, belimbing besi	Starfr
29	Euphorbiaceae	*Manihot esculenta* 	ubi kayu, ubi benggala, ubi belanda	The cassa mani and tapio plant
30	Portulacaceae	*Portulaca oleracea*	gelang pasir, rumput beremi, segan jantan	Beren
31	Convulvulaceae	*Ipomoea batatas*	keladek, keledek, ubi keladek	Swee potat
32	Convulvulaceae	*Ipomoea reptans*	kangkong, kangkung	Kang

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

Table 3.1 Continued

23	Leguminosae	*Pisum sativum*	kacang puteh	Snow pea, sugar pea	Fruit
24	Leguminosae	*Phaseolus vulgaris*	kacang buncis	French bean	Fruit
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
33	Umbeliferae	*Apium graveolens*	saderi, selderi	Celer
34	Umbeliferae	*Centella Asiatica*	pegaga	Pegag
35	Umbeliferae	*Daucus carota*	lobak merah	Carro
36	Umbeliferae	*Oenanthe javanica*	shelum, selom, piopo	Shelu
37	Chenopodiaceae	*Beta vulgaris*	bit	Bitro
38	Chenopodiaceae	*Citrus*	limau	Citrus
39	Cruciferace	*Brassica oleracea* var. *alboglabra*	kai lan, gailan	Chine cabba Chine broco
40	Cruciferae	*Petroselium crispum*	parsley	Parsl

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.

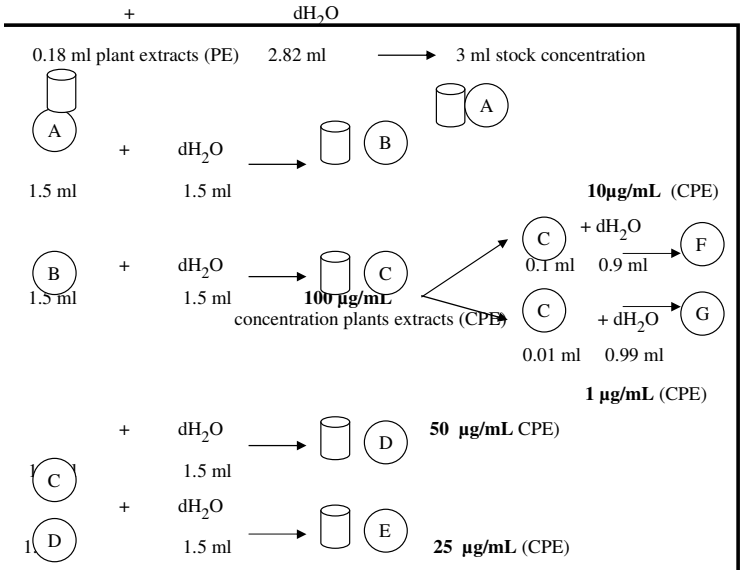


Figure 3.1: The calculations to produce stock concentrations of 100, 50, 25, 10 and 1 µg/m

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).

CHAPTER 4

RESULTS

4.1 Yield of Crude Extracts

Forty species of vegetables were extracted using two solvents systems namely, methanol and water. The yield of extracts obtained from methanol and water extraction

are shown in Table 4.1.

The total yield percentages range from 5.72% to 48.78%. The *Lycopersicum esculentum* showed the highest total yield percentage while *Centella asiatica* showed the lowest. Generally, the yield percentages of the crude methanol extracts (ranged 1.74% to 37.44%) were higher than that of the water extracts (ranged 2.01% to 27.90%).

Table 4.1 Yield of crude extracts obtained from selected vegetable species

No	Plant	Extraction Solvent	Weight (g)	Yield (%)	Total yield (%)
1	*Asparagus officinalis*	Methanol Water	3.23 3.82	6.16 9.10	15.26
2	*Allium fistulosum*	Methanol Water	2.71 3.01	13.58 15.08	28.66
3	*Allium tuberosum*	Methanol Water	0.96 1.09	4.83 5.48	10.32
4	*Capsicum annum*	Methanol Water	4.92 3.16	24.63 15.81	40.44
5	*Capsicum frutescens*	Methanol Water	0.50 0.95	2.50 4.79	7.30
6	*Lycopersicum esculentum*	Methanol Water	7.48 2.26	37.44 11.34	48.78
7	*Solanum melongena*	Methanol Water	4.15 3.01	20.79 15.07	35.87
8	*Solanum tuberosum*	Methanol Water	2.61 1.09	13.08 5.46	18.55
9	*Cucumis sativus*	Methanol Water	3.16 2.78	15.83 13.93	29.76

Table 4.1 Continued

10	*Cucurbita moschata*	Methanol Water	4.57 4.94	22.89 24.73	47.63
11	*Lagenaria sceraria*	Methanol Water	2.36 1.05	11.84 5.25	17.10
12	*Momordica charantia*	Methanol Water	3.37 3.10	16.89 15.50	32.40
13	*Luffa acutangula*	Methanol Water	1.31 2.11	6.56 10.59	17.15
14	*Cosmos caudatus*	Methanol Water	1.39 1.34	1.39 1.34	13.70
15	*Lactuca sativa*	Methanol Water	2.31 1.94	2.31 1.94	21.32
16	*Archidendron jiringga*	Methanol Water	1.66 1.51	1.66 1.51	15.89
17	*Neptunia prostrata*	Methanol Water	1.61 1.51	1.61 1.51	15.66
18	*Pachyrrhizus erosus*	Methanol Water	5.61 5.58	5.61 5.58	55.98
19	*Psophocarpus tetragonolobus*	Methanol Water	0.60 1.00	0.60 1.00	8.03
20	*Parkia speciosa*	Methanol Water	3.82 2.20	3.82 2.20	30.14
21	*Sesbania grandiflora*	Methanol Water	2.98 2.35	2.98 2.35	26.71
22	*Vigna sinensis*	Methanol Water	1.05 2.09	1.05 2.09	15.76
23	*Pisum sativum*	Methanol Water	1.68 2.76	8.40 13.82	22.22
24	*Phaseolus vulgaris*	Methanol Water	0.98 1.67	4.90 8.39	13.30
25	*Amaranthus gangeticus*	Methanol Water	1.65 2.77	8.25 13.86	22.11
26	*Amaranthus viridis*	Methanol Water	0.87 3.77	4.36 18.87	23.23
27	*Hibiscus esculentus*	Methanol Water	3.95 0.87	19.77 4.35	24.12
28	*Averrhoa carambola*	Methanol Water	1.67 0.91	8.37 4.56	12.93
29	*Manihot esculenta*	Methanol Water	0.38 2.79	1.94 13.95	15.90
30	*Portulaca oleracea*	Methanol Water	2.37 1.44	11.86 7.21	19.07
31	*Ipomoea batatas*	Methanol Water	2.91 2.77	14.58 13.89	28.47

Table 4.1 Continued

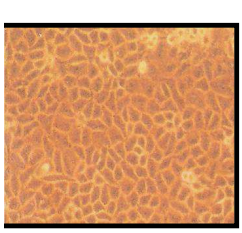
32	*Ipomoea reptans*	Methanol Water	2.77 2.61	13.89 13.08	26.97
33	*Apium graveolens*	Methanol Water	1.05 1.07	5.29 5.39	10.68

34	*Centella asiatica*	Methanol Water	0.70 0.40	3.50 2.011	5.72
35	*Daucus carota*	Methanol Water	4.76 1.00	23.82 5.01	28.84
36	*Oenanthe javanica*	Methanol Water	3.16 2.62	15.80 13.10	28.91
37	*Beta vulgaris*	Methanol Water	2.61 1.95	13.07 9.76	22.83
38	*Citrus aurantifolia*	Methanol Water	1.98 1.39	9.93 6.97	16.91
39	*Brassica oleracea* var. *alboglabra*	Methanol Water	1.98 2.49	9.93 12.48	22.41
40	*Petroselium crispum*	Methanol Water	0.34 3.38	1.74 16.92	18.67

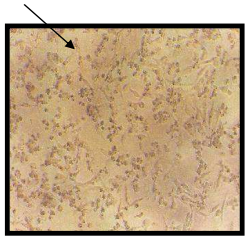
4.2 Screening For The *In vitro* Cytotoxic Activity of Crude Methanol and Water Extracts of Selected Vegetables

In the present study, 80 crude methanol and water vegetable extracts were evaluated for their *in vitro* cytotoxic activity against two cervical cancer-derived cell lines (HeLa and CaSki) and human fibroblast cell line (MRC5) using the neutral red cytotoxic assay. HeLa and CaSki cells were grown in 10% supplemented RPMI 1640 medium and MRC5 cells grown in 10% supplemented EMEM medium were incubated with methanol and water extracts of selected vegetables at varying concentrations (1, 10, 25, 50, 100 µg/ml) for 72 hours in a 5% CO_2 incubator at 37ºC. The negative controls consisted of cells not treated with vegetable extracts. Figure 4.1 shows human cancer cell lines, HeLa, CaSki and MRC5 cells treated with 100 µg/ml of vegetables crude extracts. The negative controls exhibited normal proliferation rate and showed no sign of death after the incubation time of 72 hours, while HeLa, CaSki and MRC5 cells treated with 100 µg/ml of vegetables crude extracts exhibited decrease in proliferation rate and showed signs of death after the incubation period of 72 hours.

The cytotoxicity results obtained were presented as inhibition percentages relative to the negative control and the IC_{50} values were extrapolated from the dose response curves plotted from the percentage of inhibition values. The IC_{50} value is the concentration of extract that inhibits the growth 50% of cells. An extract which gives IC_{50} value of 20 µg/ml and below is considered cytotoxically active (Geran *et al*., 1972; Chiang *et al*., 2003).



(a)



(b)

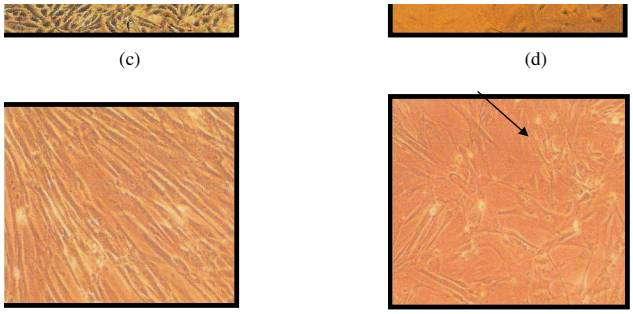


Figure 4.1: Photomicrograph (100X) of: (a): Untreated CaSki cells incubated (negative contol) (b): CaSki cell treated with 100 µg/ml of crude extract of *Beta vulgaris* (c): untreated HeLa cells incubated (negative control) (d) CaSki cell treated with 100 µg/ml of crude extract of *Portulaca oleracea* (e): untreated MRC5 cells incubated in EMEM (Negative control) (f): MRC5 cells treated with 100 µg/ml of crude MeoH extract of *Capsicum annum*

4.2.1 Cytotoxic Activity of Doxorubicin Against HeLa and CaSki Cells and MRC5 Cell Lines

The human cervical cancer-derived cell lines, (HeLa and CaSki) and human fibroblast cell line (MRC5), were treated with doxorubicin, a positive control for the neutral red cytotoxic assay.

Cells treated with doxorubicin exhibited decreased proliferation rate and showed signs of death after the incubation of 72 hours. Results showed that the doxorubicin shown significant cytotoxic effects against HeLa, CaSki and MRC5 cells (Table 4.2, Table 4.3, Figure 4.2). Doxorubicin to be cytotoxic of all cell lines tested in a dose-dependent manner with inhibition percentages of 5.89-44.99% at $1x10^{-5}$ µg/ml, 13.08-45.46% at $1x10^{-4}$ µg/ml, 23.86-47.68% at $1x10^{-3}$ µg/ml, 46.81-69.91% at $1x10^{-2}$ µg/ml, 46.95-76.36% at $1x10^{-1}$ µg/ml, 50.91-84.54% at 1x10 µg/ml, 53.10-87.82% at $1x10^{1}$ µg/ml and 63.39-89.48% at $1x10^{2}$ µg/ml.

The inhibition percentages obtained were presented as dose-respone curves. The *in vitro* growth inhibition of doxorubicin against HeLa, CaSki and MRC5 cell lines are shown in Figure 4.2. The IC_{50} values were estimated from the graph extrapolation and summarized in Table 4.3. The IC_{50} value refers to the effective dose (concentration of extracts in µg/ml) that inhibits 50% of cells growth. Extracts having an IC_{50} value equal to or less than 20 are considered active for cytotoxic assay against cells (Geran *et al*., 1972).

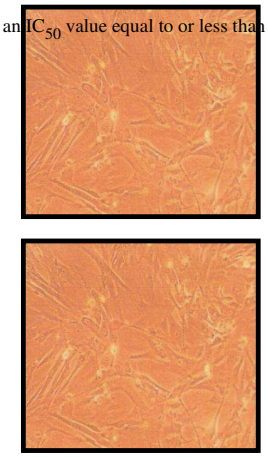


Table 4.2: Inhibition of HeLa, CaSki and MRC5 cells treated with doxorubicin

Cell line	Percentage of inhibition ± standard deviation (%) at different concentration (µg/ml)							
	$1x10^{-5}$	**$1x10^{-4}$**	**$1x10^{-3}$**	**$1x10^{-2}$**	**$1x10^{-1}$**	**1**	**10**	**100**
HeLa	20.1±5.5	21.6±9.4	26.5±1.7	46.8±2.8	47.0±0.9	53.6±0.5	55.3±0.7	63.3±1.3
CaSki	5.9±3.7	13.1±1.3	23.9±0.5	69.9±1.5	76.4±0.5	84.5±0.2	87.8±0.4	89.5±0.4
MRC5	45.0±0.12	45.5±0.6	47.7±0.4	48.3±0.3	49.0±0.5	50.9±0.5	53.1±0.4	68.4±1.4

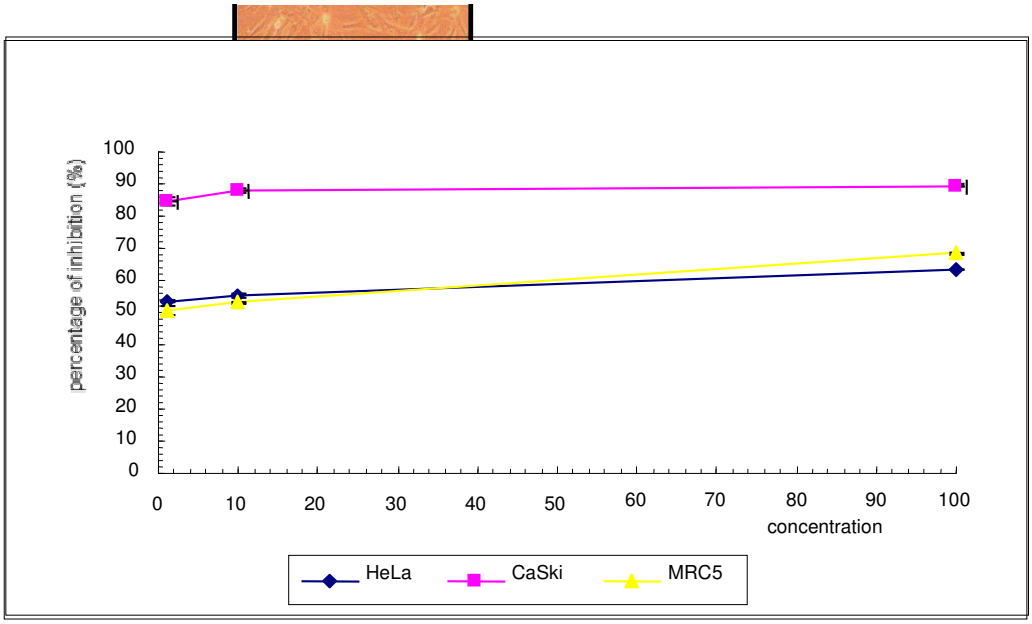


Figure 4.2: Dose-response curve showing *in vitro* growth inhibition of HeLa, CaSki and MRC5 cells by doxorubicin (positive control)

Table 4.3: The IC_{50} values of doxorubicin against various cell lines

Cell lines	IC_{50} value (µg/ml)
HeLa	3.00
CaSki	0.05
MRC5	4.00

4.2.2 *In vitro* Cytotoxic activity of Selected Vegetable Extracts Against HeLa Cells

The *in vitro* growth inhibition of HeLa cells by crude methanol extracts of the 40 vegetables are shown as dose-response curves in Figures 4.3(a) to 4.3(h). The IC_{50} values were extrapolated from the dose-response curves and summarized in Table 4.4. The inhibition activities ranged from 4.57-41.36% at 1µg/ml, 9.7-65.66% at 10 µg/ml, 14.95-81.93% at 25 µg/ml, 23.65-84.92 at 50 µg/ml and 28.17-87.78 at 100 µg/ml. Out of 40 vegetables evaluated only 7 vegetables (17.5%) methanol extracts, namely *Capsicum annum (lada merah), Capsicum frutescent (cili padi)s, Lycopersicum esculentum (tomato), Cosmos caudatus (ualm rajah), Vigna sinensis (kacang panjang), Amaranthus viridis (bayam merah)* and *Ipomoea reptans* (*kangkung*) were actively cytotoxic against HeLa cell with IC_{50} values less than 20 µg/ml. The IC_{50} values are 13 µg/ml, 17.5 µg/ml, 17.4 µg/ml, 18.02 µg/ml, 15.9 µg/ml, 15 µg/ml and 5.9 µg/ml, respectively.

Crude methanol extracts of *Asparagus officinalis (asparagus), Solanum tuberosum (ubi kentang), Cucumis sativus (timun), Cucurbita moschata (labu merah), Lagenaria sceraria (labu ayer), Momordica charantia (peria), Luffa acutangula (petola segi), Lactuca sativa (salada), Neptunia prostrate (tangki), Pachyrrizus erosus (sengkuwang), Parkia speciosa (petai), Amaranthus gangeticus (bayam pasir), Apium graveolens (selderi), Centella Asiatica (Pegaga), Daucus carota (lobak), Beta vulgaris (bit), Citrus aurantifolia (limau), Pisum sativum (kacang putih), Brassica oleracea* var *alboglabra (kalian), Phaseolus vulgaris kacang buncis),* and *Petroselum crispum (parsley)* possessed inhibited HeLa cells with percentage inhibition less than 50% at all concentrations tested in this study. Therefore, IC_{50} values could not be determined from the existing dose-response curve

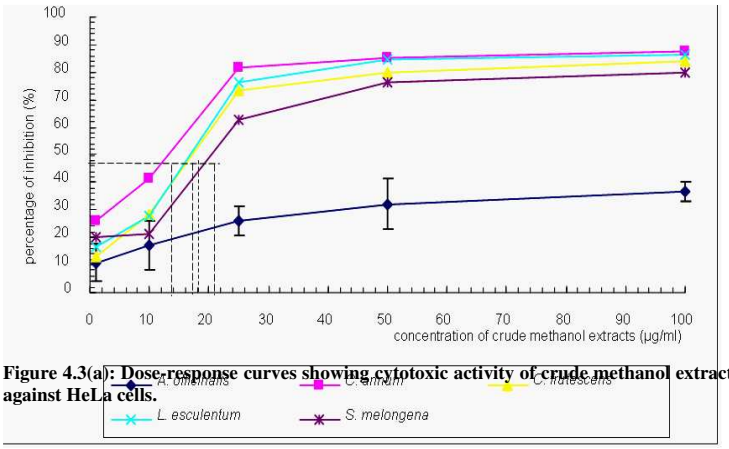


Figure 4.3(a): Dose-response curves showing cytotoxic activity of crude methanol extracts of *A. officinalis*, *C. annum*, *C. frutescens*, *L. esculentum* and *S. melongena* against HeLa cells.

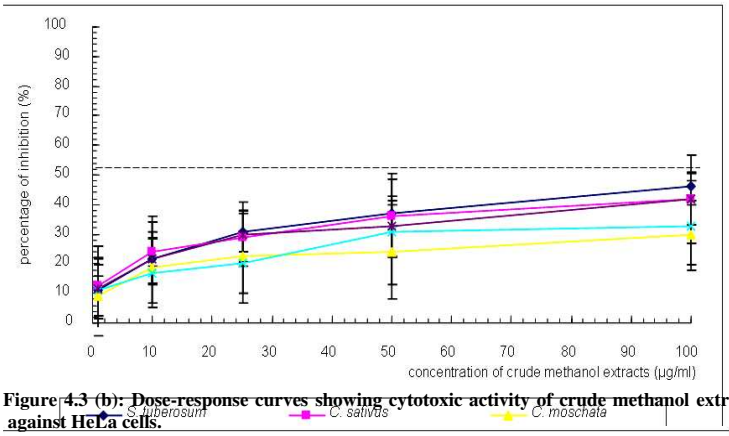


Figure 4.3 (b): Dose-response curves showing cytotoxic activity of crude methanol extracts of *S. tuberosum, C. sativus, C. moschata, L. sceraria* and *M. charantia* against HeLa cells.

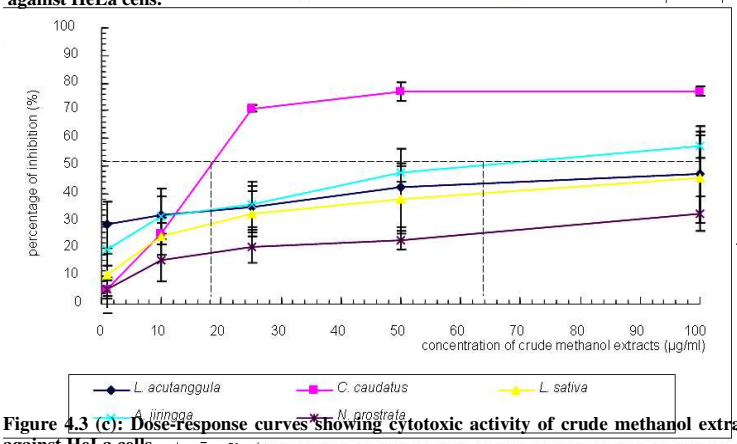


Figure 4.3 (c): Dose-response curves showing cytotoxic activity of crude methanol extracts of *L. acutanggula, C. caudatus, L. sativa, A. jiringga* and *N. prostrate* against HeLa cells.

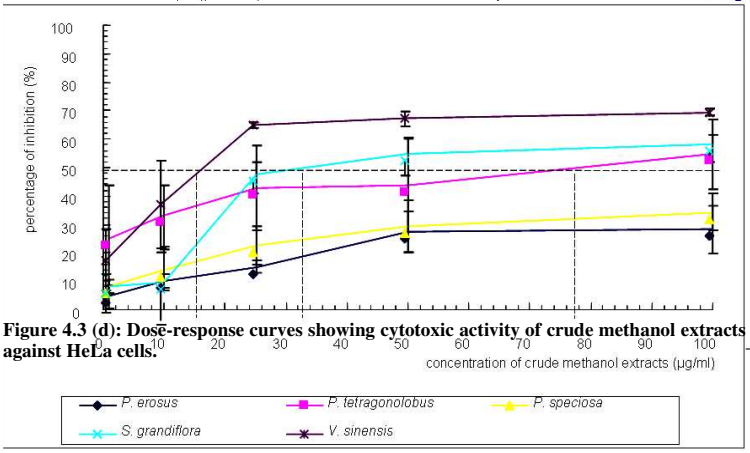


Figure 4.3 (d): Dose-response curves showing cytotoxic activity of crude methanol extracts of *P. erosus, P. tetragonolobus, P. speciosa, S. grandiflora* and *V. sinensis* against HeLa cells.

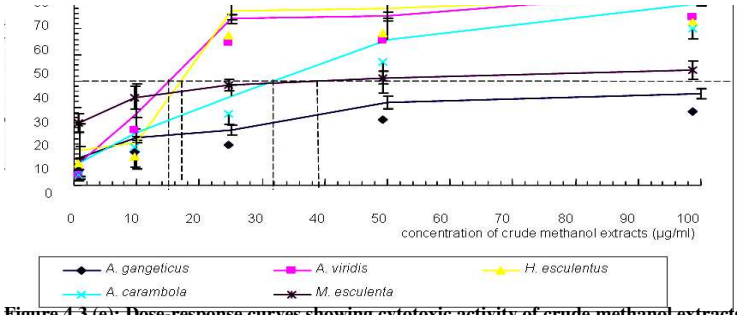


Figure 4.3 (e): Dose-response curves showing cytotoxic activity of crude methanol extracts of *A. gangeticus, A. viridis, H. esculenthus, A. carambola* and M. esculenta against HeLa cells.

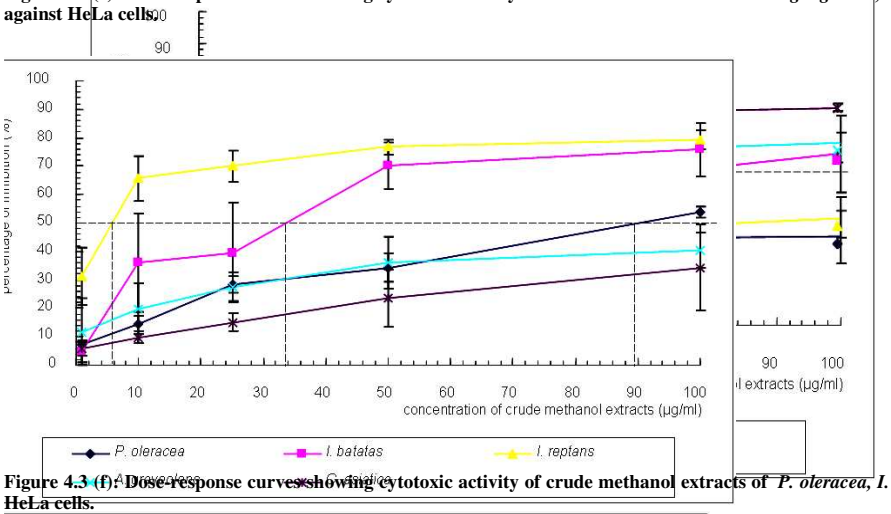


Figure 4.3 (f): Dose-response curves showing cytotoxic activity of crude methanol extracts of *P. oleracea, I. batatas, I. reptans, A. graveolens* and *C. asiatica* against HeLa cells.

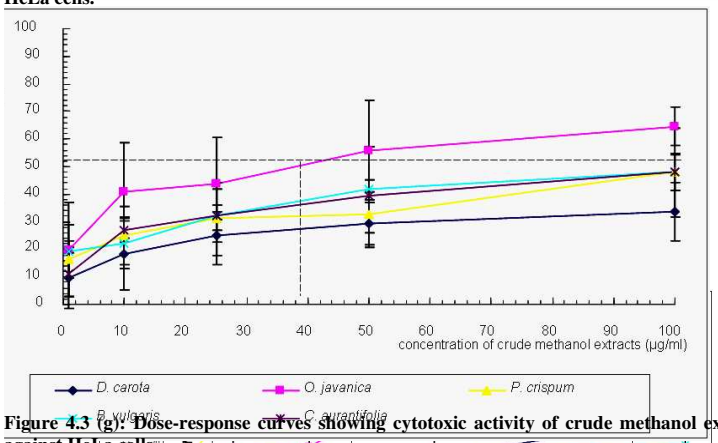


Figure 4.3 (g): Dose-response curves showing cytotoxic activity of crude methanol extracts of *D. carota, O. javanica, P. crispum, B. vulgaris* and *C. aurantifolia* against HeLa cells.

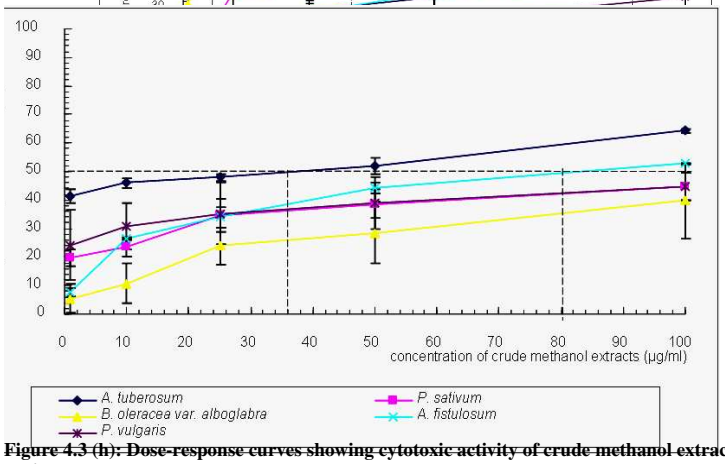


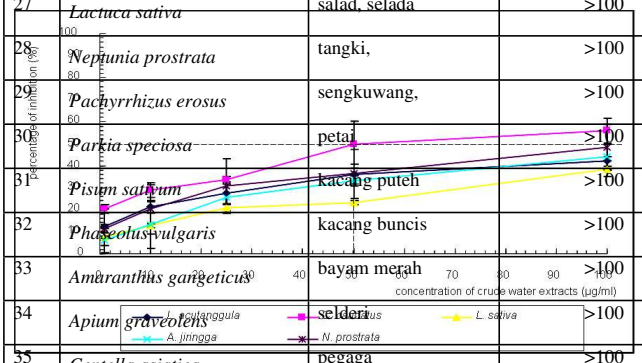
Figure 4.3 (h): Dose-response curves showing cytotoxic activity of crude methanol extracts of *A. tuberosum, P. sativum, B. oleracea var. alboglabra* and *P. vulgaris* against HeLa cells

Table 4.4: IC_{50} values of crude methanol extracts of selected vegetables against HeLa cells

No	Plant	Local name	IC_{50} (µg/ml)
1	*Ipomoea reptans*	kangkung	**5.90**
2	*Capsicum annum*	lada merah,	**13.00**
3	*Amaranthus viridis*	bayam pasir	**15.00**
4	*Vigna sinensis*	kacang panjang	**15.90**
5	*Hibiscus esculentus*	bendi	**17.00**
6	*Lycopersicum esculentum*	tomato	**17.40**
7	*Capsicum frutescens*	chabai, lada api, lada kerawit	**17.50**
8	*Cosmos caudatus*	ulam rajah	**18.02**
9	*Solanum melongena*	terong	20.05
10	*Averrhoa carambola*	belimbing besi	32.00
11	*Sesbania grandiflora*	turi	33.50
12	*Ipomoea batatas*	keledek	33.80
13	*Allium tuberosum*	Ku chai	36.00
14	*Oenanthe javanica*	shelum	38.50
15	*Manihot esculenta*	ubi kayu	39.00
16	*Archidendron jiringga*	jering	64.00
17	*Psophocarpus tetragonolobus*	kacang botor,	78.00
18	*Allium fistulosum*	Daun bawang	80.00
19	*Portulaca oleracea*	beremi	89.50
20	*Asparagus officinalis*	Asparagus, saparu keras	>100
21	*Solanum tuberosum*	ubi benggala, ubi kentang, ubi gendang	>100

Table 4.4 continued

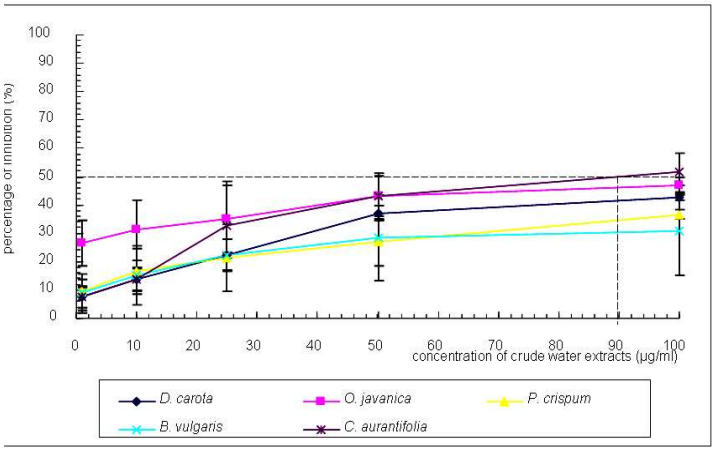
22	*Cucumis sativus*	timun, timun China, mentimun	>100
23	*Cucurbita moschata*	labu merah, labu perang,	>100
24	*Lagenaria sceraria*	labu ayer putih, labu botol	>100
25	*Momordica charantia*	peria	>100
26	*Luffa acutangula*	petola segi, ketola segi	>100
27	*Lactuca sativa*	salad, selada	>100
28	*Neptunia prostrata*	tangki,	>100
29	*Pachyrrhizus erosus*	sengkuwang,	>100
30	*Parkia speciosa*	petai	>100
31	*Pisum sativum*	kacang puteh	>100
32	*Phaseolus vulgaris*	kacang buncis	>100
33	*Amaranthus gangeticus*	bayam merah	>100
34	*Apium graveolens*	selderi	>100
35	*Centella asiatica*	pegaga	>100
36	*Daucus carota*	lobak merah	>100
37	*Beta vulgaris*	bit	>100
38	*Citrus aurantifolia*	limau	>100
39	*Brassica oleracea* var. *alboglabra*	Kai lan	>100



40	*Petroselium crispum*	parsley	>100

The *in vitro* growth inhibition of HeLa cells by crude water extracts of the 40 selected vegetables are shown as dose-response curves in Figures 4.4(a) to 4.4(h). The inhibition rates of HeLa cells were 2.00-48.47% at 1 µg/ml, 7.57-51.47 at 10 µg/ml, 11.33-64.44% at 25 µg/ml, 11.63-67.63% at 50 µg/ml and 12.57-72.15% at 100 µg/ml. Only 2 out of 40 crude water extracts of the selected vegetables studied namely, *C. annum* and *L. sceraria* exhibited IC_{50} values less than 20 µg/ml and therefore considered actively cytotoxic against HeLa cells (Table 4.5). When compared *L. sceraria* were the more active. The IC_{50} value for crude water extracts of *L. sceraria* was 6 µg/ml while the IC_{50} value for crude water extracts of *C. annum* was 17 µg/ml. On the other hand, *C. frutescens*, *L. esculentum, C. moschata, C. caudatus* and *V. sinensis, A . fistulosum, S. grandiflora, A. graveolens* and *C. aurantifolia* exhibited no cytotoxic rates against HeLa cells. Their IC_{50} values ranged from 39.5 µg/ml to 90 µg/ml. The remainder of water extracts exhibited IC_{50} values greater than 100 µg/ml against HeLa cells.

Generally, from 80 crude methanol and water extracts of selected vegetables studied, only 9 of them exerted active cytotoxic effect against HeLa cells. Both crude methanol and water extracts of *C. annum* exhibited the active cytotoxic effects against HeLa cells with IC_{50} values of 13 µg/ml and 17 µg/ml, respectively. The crude methanol extracts of *I. reptans* and the crude water extracts of *L. sceraria* appeared to be more potent as compared to the others due to their abilities to retard more than 50% of cell growth at concentrations lower than as 10wer than 10 µg/ml.



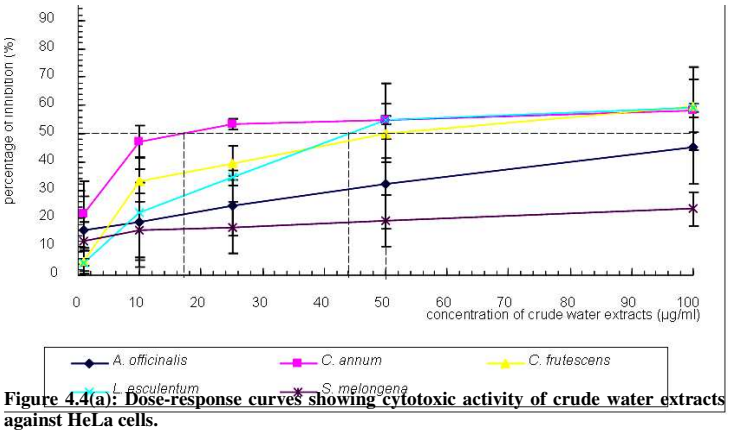


Figure 4.4(a): Dose-response curves showing cytotoxic activity of crude water extracts of *A. officinalis*, *C. annum*, *C. frutescens*, *L. esculentum* and *S. melongena* against HeLa cells.

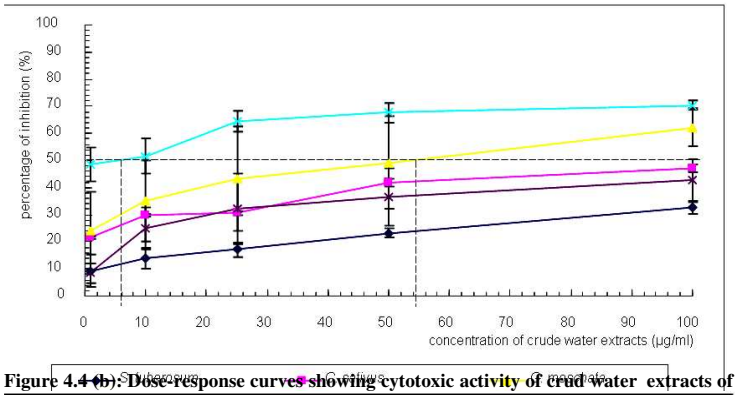


Figure 4.4 (b): Dose-response curves showing cytotoxic activity of crud water extracts of *S. tuberosum, C. sativus, C. moschata, L. sceraria* and *M. charantia* against HeLa cells.

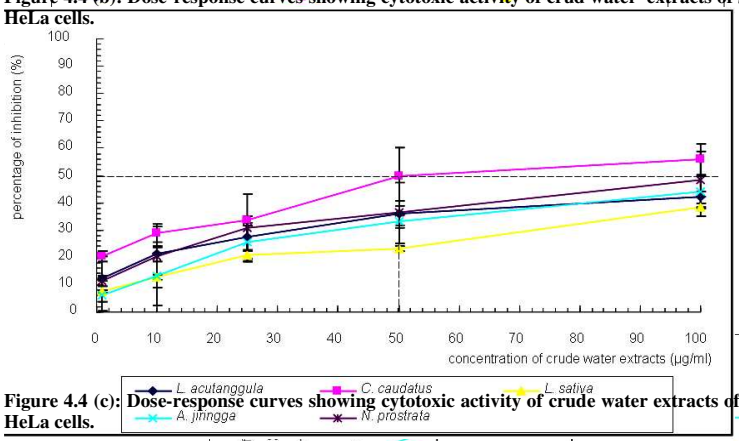


Figure 4.4 (c): Dose-response curves showing cytotoxic activity of crude water extracts of *L. acutanggula, C. caudatus, L. sativa, A. jiringga* and *N. prostrate* against HeLa cells.

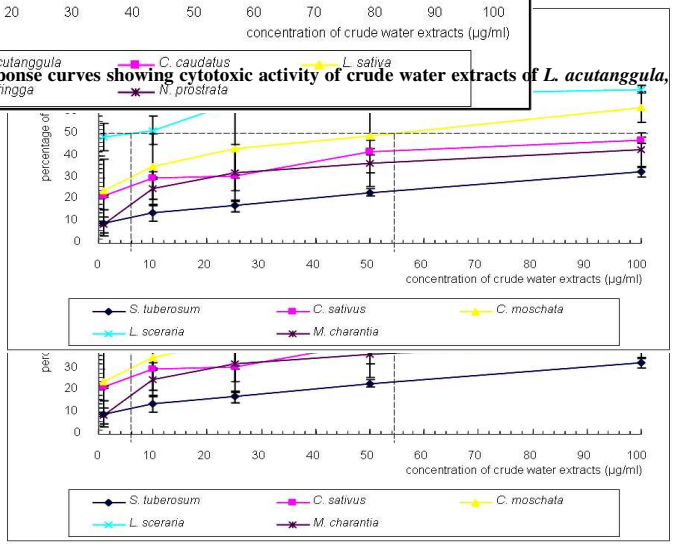


Figure 4.4 (d): Dose-response curves showing cytotoxic activity of crude water extracts of *P. erosus, P. tetragonolobus, P. speciosa, S. grandiflora* and *V. sinensis* against HeLa cells.

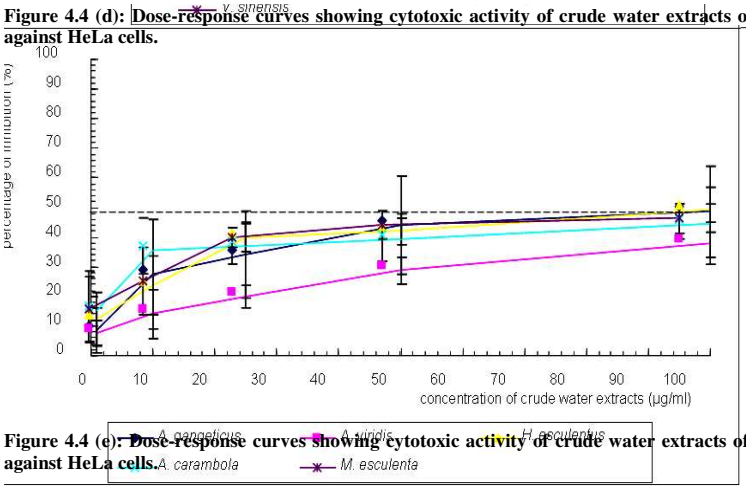


Figure 4.4 (e): Dose-response curves showing cytotoxic activity of crude water extracts of *A. gangeticus, A. viridis, H. esculenthus, A. carambola* and M. esculenta against HeLa cells.

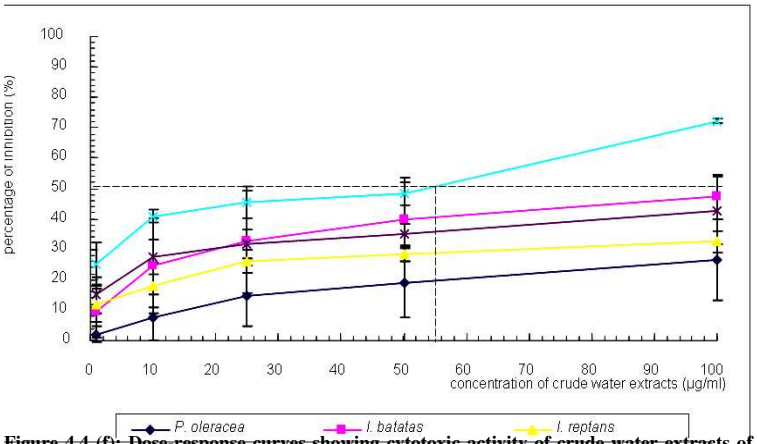


Figure 4.4 (f): Dose-response curves showing cytotoxic activity of crude water extracts of *P. oleracea, I. batatas, I. reptans, A. graveolens* and *C. asiatica* against HeLa cells.

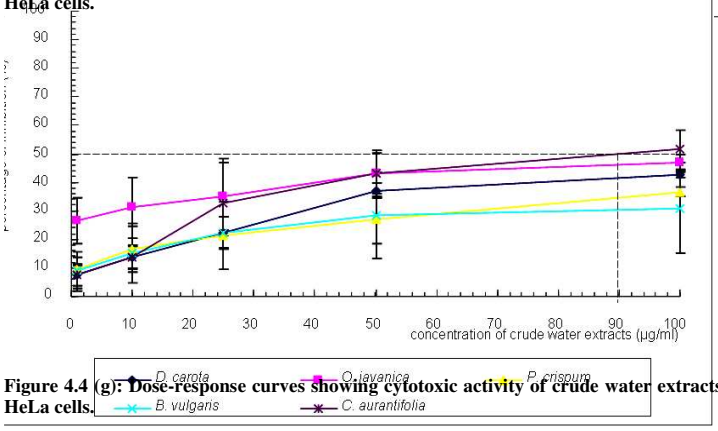


Figure 4.4 (g): Dose-response curves showing cytotoxic activity of crude water extracts of *D. carota, O. javanica, P. crispum, B. vulgaris* and *C. aurantifolia* against HeLa cells.

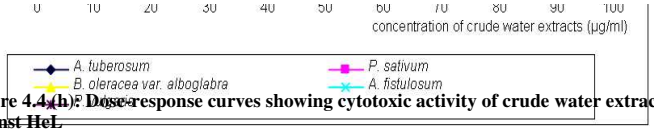


Figure 4.4(h): Dose response curves showing cytotoxic activity of crude water extracts of *A. tuberosum, P. sativum, B. oleracea var. alboglabra* and *P. vulgaris* against HeL

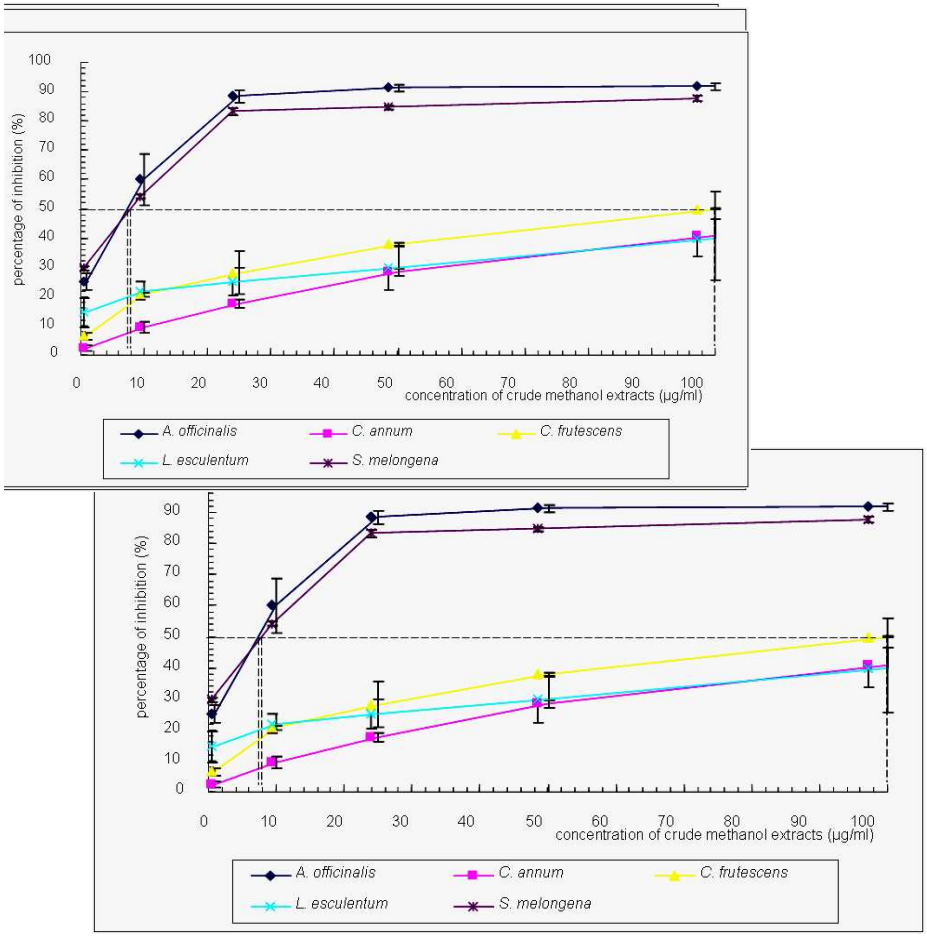


Table 4.5: IC_{50} values of crude water extracts of selected vegetables against HeLa cells

No	Plant	Local name	IC_{50} (µg/ml)
1	*Lagenaria sceraria*	labu ayer putih, labu botol	**6.00**
2	*Capsicum annum*	lada merah,	**17.00**
3	*Vigna sinensis*	kacang panjang	39.50
4	*Lycopersicum esculentum*	tomato	44.00
5	*Capsicum frutescens*	chabai, lada api, lada kerawit	50.00
6	*Cosmos caudatus*	ulam rajah	50.00
7	*Cucurbita moschata*	labu merah, labu perang,	54.50
8	*Apium graveolens*	selderi	55.00
9	*Allium fistulosum*	Daun bawang	80.00
10	*Sesbania grandiflora*	turi	90.00
11	*Citrus aurantifolia*	limau	90.00
12	*Asparagus officinalis*	Asparagus, saparu keras	>100
13	*Allium tuberosum*	Ku chai	>100
14	*Solanum melongena*	terong	>100
15	*Solanum tuberosum*	ubi benggala, ubi kentang, ubi gendang	>100
16	*Cucumis sativus*	timun, timun China, mentimun	>100
17	*Momordica charantia*	peria	>100
18	*Luffa acutangula*	petola segi, ketola segi	>100
19	*Lactuca sativa*	salad, selada	>100
20	*Archidendron jiringga*	jering	>100
21	*Neptunia prostrata*	tangki,	>100

Table 4.5 continued

22	*Pachyrrhizus erosus*	sengkuwang,	>100
23	*Psophocarpus tetragonolobus*	kacang botor,	>100
24	*Parkia speciosa*	petai	>100
25	*Pisum sativum*	kacang puteh	>100
26	*Phaseolus vulgaris*	kacang buncis	>100
27	*Amaranthus gangeticus*	bayam merah	>100
28	*Amaranthus viridis*	bayam pasir	>100
29	*Hibiscus esculentus*	bendi	>100
30	*Averrhoa carambola*	belimbing besi	>100
31	*Manihot esculenta*	ubi kayu	>100
32	*Portulaca oleracea*	beremi	>100
33	*Ipomoea batatas*	keledek	>100
34	*Ipomoea reptans*	kangkung	>100
35	*Centella asiatica*	pegaga	>100
36	*Daucus carota*	lobak merah	>100
37	*Oenanthe javanica*	shelum	>100
38	*Beta vulgaris*	bit	>100
39	*Brassica oleracea* var. *alboglabra*	Kai lan	>100
40	*Petroselium crispum*	parsley	>100

4.2.3 *In vitro* Cytotoxic Activity of Selected Vegetable Extracts Against CaSki Cells

As illustrated in Figures 4.5-4.6, CaSki cells showed different cytotoxic profiles when treated with different crude extracts from selected vegetables. The *in vitro* growth inhibition of CaSki cells by crude methanol extracts of the 40 vegetables are shown as dose-response curves in Figure 4.5(a) to 4.5(h). The IC_{50} values were extrapolated from the dose-response curves and summarized in Table 4.6. The inhibition activities ranged from 1.39-44.70% at 1 µg/ml, 6.38-70.59% at 10 µg/ml, 14.62-88.94% at 25 µg/ml, 28.36-92.49% at 50 µg/ml, 39.89-93.95% at 100 µg/ml.

Out of 40 vegetables evaluated only 19 vegetables, namely *A. officinalis, A. fistulosum, S. melongena, S. tuberosum, Cucurbita moschata, L. sceraria, A. jiringga, N. prostrata, V. sinensis, P. sativum, P. vulgaris, A. gangeticus, A. viridis, H. esculentus, A. carambola, P. oleracea, I. reptans, B. vulgaris and B. oleracea* var. *alboglabra* were active against CaSki cells with IC_{50} values less than 20 µg/ml. The IC_{50} values ranged from 4.9 µg/ml to 19.8 µg/ml.

Crude methanol extracts of *Capsicum annum, Lycopersicum esculentum,Manihot esculenta* and *Petroselum crispum* inhibited CaSki cells with percentage of inhibition less than 50% at all concentrations tested in the present study. Therefore, IC_{50} values could not be determined from the existing dose-response curves.

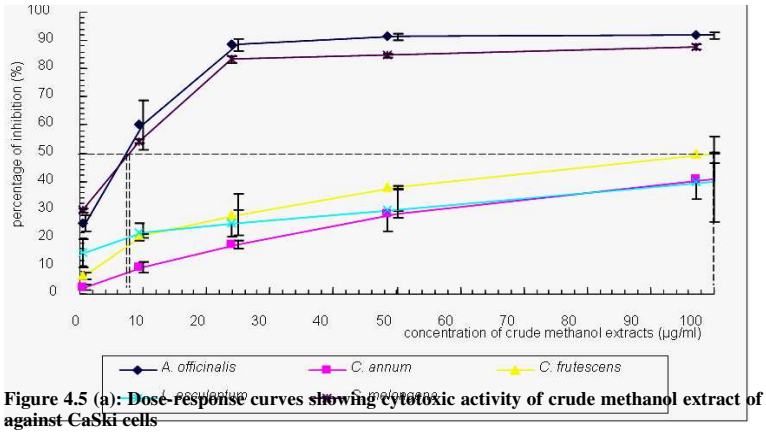


Figure 4.5 (a): Dose-response curves showing cytotoxic activity of crude methanol extract of *A. officinalis*, *C. annum*, *C. frutescens*, *L. esculentum* and *S. melongena* against CaSki cells

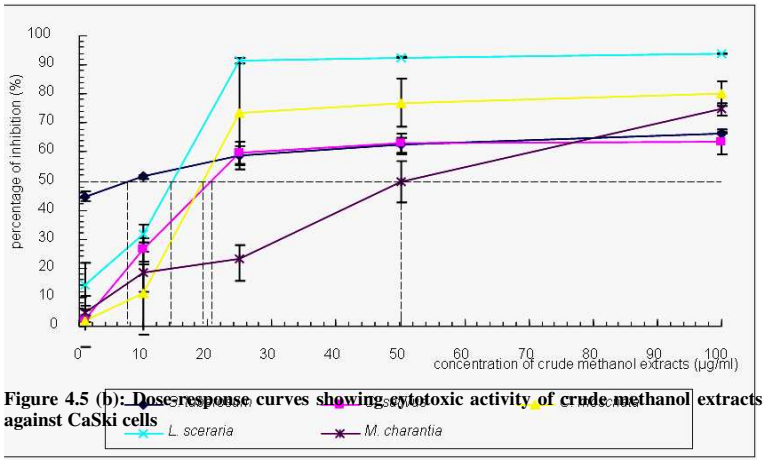
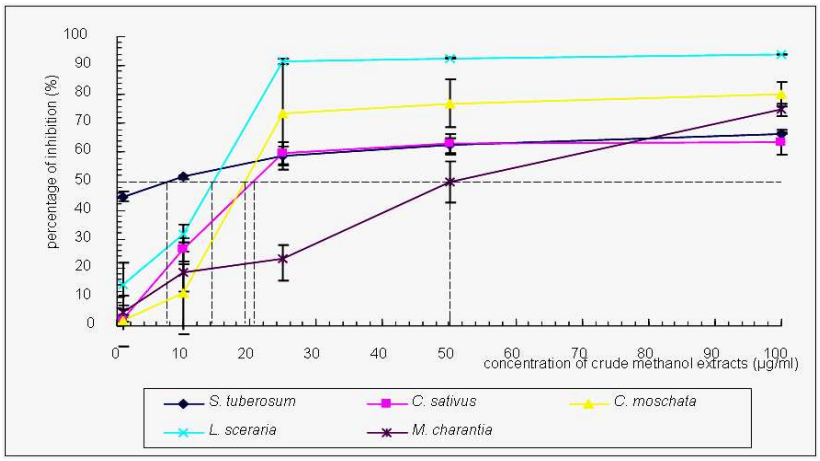


Figure 4.5 (b): Dose-response curves showing cytotoxic activity of crude methanol extracts of *S. tuberosum, C. sativus, C. moschata, L. sceraria* and *M. charantia* against CaSki cells



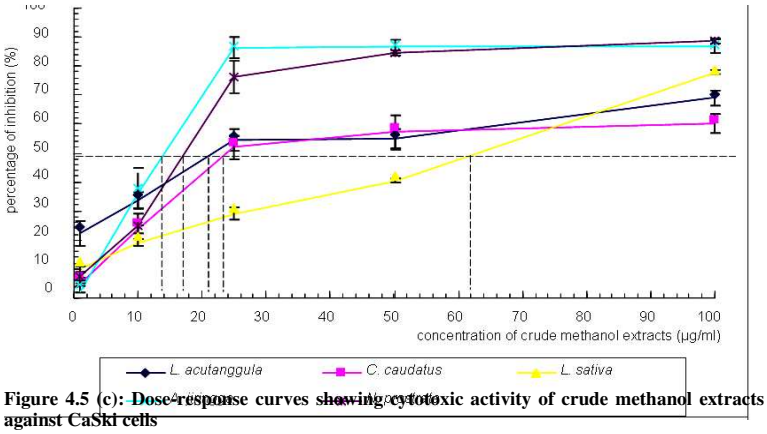


Figure 4.5 (c): Dose-response curves showing cytotoxic activity of crude methanol extracts of *L. acutanggula, C. caudatus, L. sativa, A. jiringga* and *N. prostrata* against CaSki cells

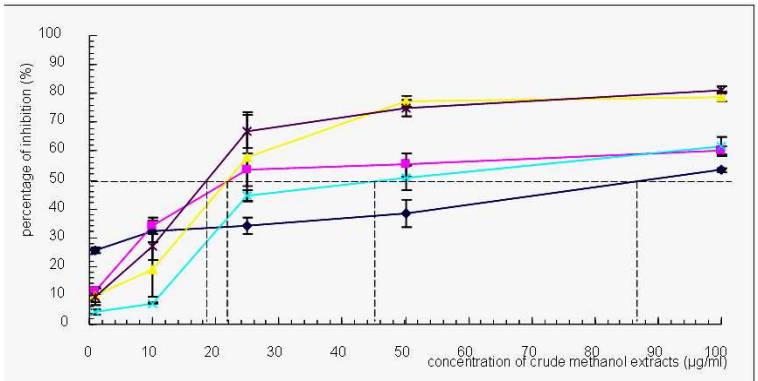


Figure 4.5 (d): Dose-response curves showing cytotoxic activity of crude methanol extracts of *P. erosus, P. tetragonolobus, P. speciosa, S. grandiflora* and *V. sinensis* against CaSki cells

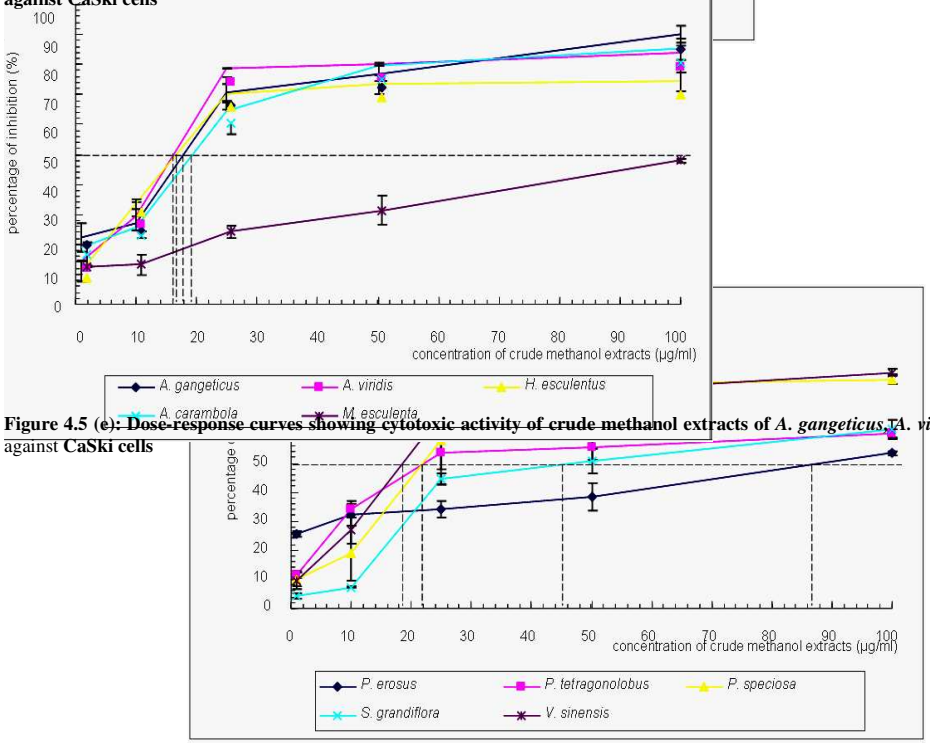
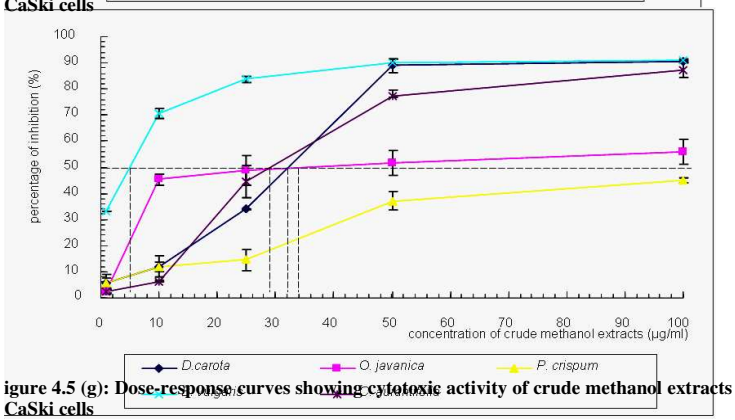


Figure 4.5 (e): Dose-response curves showing cytotoxic activity of crude methanol extracts of *A. gangeticus, A. viridis, H. esculentus, A. carambola* and *M. esculenta* against **CaSki cells**

Figure 4.5 (f): Dose-response curves showing cytotoxic activity of crude methanol extracts of *P. oleracea, I. batatas, I. reptans, A. graveolens* and *C. asiatica* against CaSki cells



igure 4.5 (g): Dose-response curves showing cytotoxic activity of crude methanol extracts of *D. carota, O. javanica, P. crispum, B. vulgaris* and *C. aurantifolia* against CaSki cells

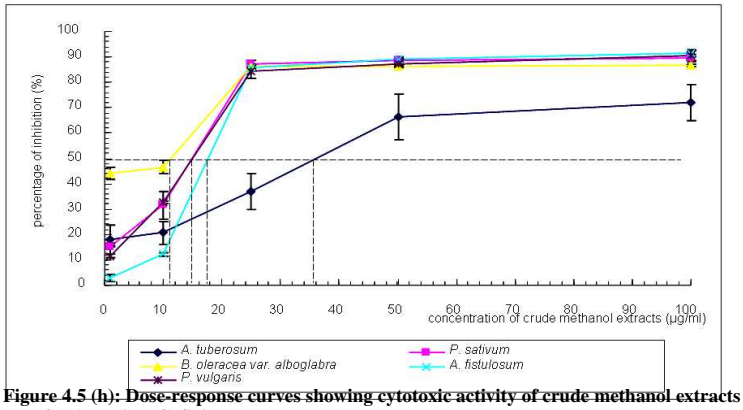


Figure 4.5 (h): Dose-response curves showing cytotoxic activity of crude methanol extracts of *A.tuberosum, P. sativum, B. oleracea* var. *alboglabra, a. fistulosum,* and *P. vulgaris* against CaSki cells

Table 4.6: IC_{50} values of crude methanol of selected vegetables against CaSki cells

No	Plant	Local name	IC_{50} (µg/ml)
1	*Beta vulgaris*	bit	**4.90**
2	*Asparagus officinalis*	Asparagus, saparu keras	**7.80**
3	*Solanum tuberosum*	ubi benggala, ubi kentang, ubi gendang	**7.80**
4	*Solanum melongena*	terong	**9.20**
5	*Brassica oleracea* var. *alboglabra*	Kai lan	**11.50**
6	*Archidendron jiringga*	jering	**13.90**
7	*Lagenaria sceraria*	labu ayer putih, labu botol	**14.10**
8	*Phaseolus vulgaris*	kacang buncis	**14.80**
9	*Pisum sativum*	kacang puteh	**14.80**
10	*Amaranthus gangeticus*	bayam merah	**16.00**
11	*Amaranthus viridis*	bayam pasir	**16.00**
12	*Portulaca oleracea*	beremi	**16.00**
13	*Hibiscus esculentus*	bendi	**17.00**
14	*Neptunia prostrata*	tangki,	**17.00**
15	*Allium fistulosum*	Daun bawang	**17.80**
16	*Ipomoea reptans*	kangkung	**17.80**
17	*Averrhoa carambola*	belimbing besi	**19.50**
18	*Cucurbita moschata*	labu merah, labu perang,	**19.80**
19	*Vigna sinensis*	kacang panjang	**19.80**
20	*Luffa acutangula*	petola segi, ketola segi	21.00
21	*Cucumis sativus*	timun, timun China, mentimun	22.00

Table 4.6 continued

22	*Parkia speciosa*	petai	22.00
23	*Psophocarpus tetragonolobus*	kacang botor,	22.00
24	*Cosmos caudatus*	ulam rajah	23.50
25	*Apium graveolens*	selderi	24.00
26	*Citrus aurantifolia*	limau	29.00
27	*Ipomoea batatas*	keledek	31.30
28	*Centella asiatica*	pegaga	32.50
29	*Daucus carota*	lobak merah	32.50
30	*Oenanthe javanica*	shelum	33.00
31	*Allium tuberosum*	Ku chai	35.80
32	*Sesbania grandiflora*	turi	45.00
33	*Momordica charantia*	peria	50.50
34	*Lactuca sativa*	salad, selada	62.00
35	*Pachyrrhizus erosus*	sengkuwang,	86.90
36	*Capsicum annum*	lada merah,	>100
37	*Capsicum frutescens*	chabai, lada api, lada kerawit	100
38	*Lycopersicum esculentum*	tomato	>100

39	*Manihot esculenta*	ubi kayu	>100
40	*Petroselium crispum*	parsley	>100

The *in vitro* growth inhibition of CaSki cells by crude water extracts of the 40 selected vegetables are shown as dose-response curves in Figures 4.6(a) to 4.6(h). The IC_{50} values were extrapolated from the dose-response curves and summarized in Table 4.7. The results reveal that the water extracts to be cytotoxic of CaSki cells with killing percentages of 4.57-41.36% at 1µg/ml, 9.7-65.66% at 10 µg/ml, 14.95-81.93% at 25 µg/ml, 23.65-84.92 at 50 µg/ml and 28.17-87.78 at 100 µg/ml.

Out of the 40 vegetables evaluated only 11 vegetables water extracts, namely *Asparagus officinalis*, *Capsicum annum, Lagenaria sceraria, Cosmos caudatus, Neptunia prostrata, psophocarpus tetragonolobus, Parkia speciosa, Vigna sinensis, phaseolus vulgaris, Hibiscus esculentus, Portulaca oleracea, and Beta vulgaris* were actively cytotoxic against CaSki cells with IC_{50} values less than 20 µg/ml. The IC_{50} values ranged were 5.9 µg/ml to 15 µg/ml.

Crude water extracts of *Pachyrrhizus erosus*, *Amaranthus gangeticus, Solanum melongena, Averrhoa carambola, Manihot esculenta, Apium graveolens, Oenanthe javanica, and Citrus aurantifolia* inhibited CaSki cells with percentage of inhibition less than 50% at all concentrations tested in this study. Therefore, IC_{50} values could not be determined from the existing dose-response curves.

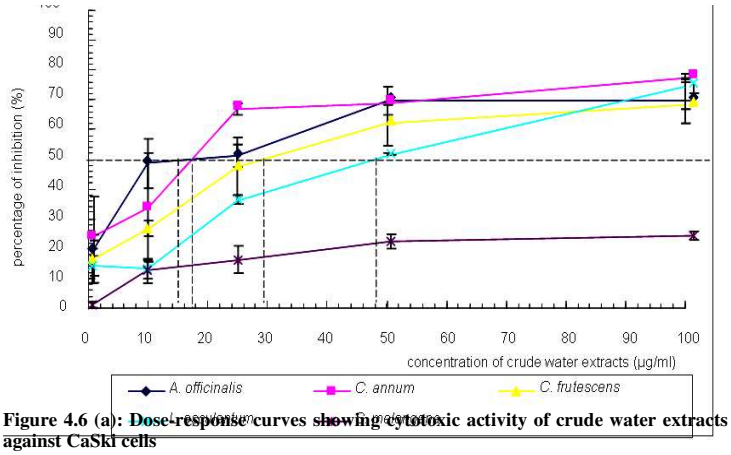


Figure 4.6 (a): Dose-response curves showing cytotoxic activity of crude water extracts of *A. officinalis*, *C. annum*, *C. frutescens*, *L. esculentum* and *S. melongena* against CaSki cells

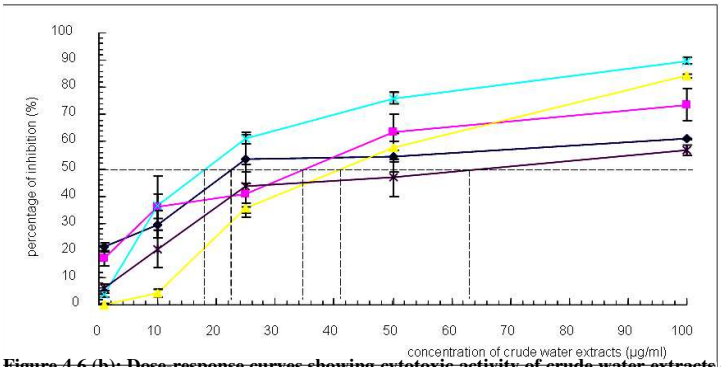


Figure 4.6 (b): Dose-response curves showing cytotoxic activity of crude water extracts of *S. tuberosum, C. sativus, C. moschata, L. sceraria* and *M. charantia* against CaSki cells

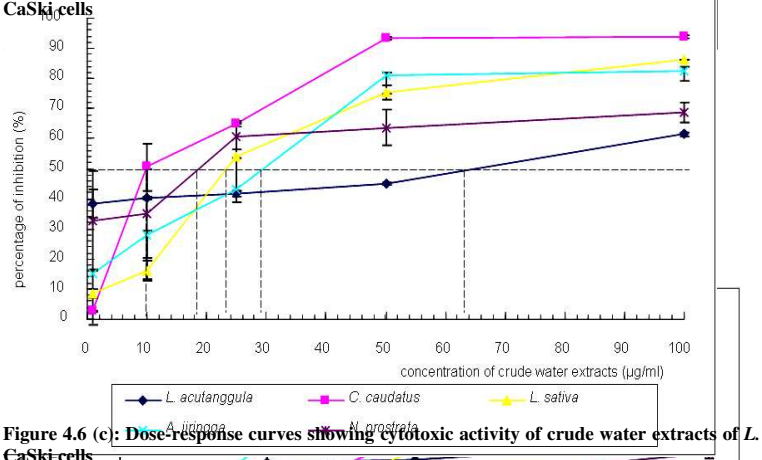


Figure 4.6 (c): Dose-response curves showing cytotoxic activity of crude water extracts of *L. acutanggula, C. caudatus, L. sativa, A. jiringga* and *N. prostrata* against CaSki cells

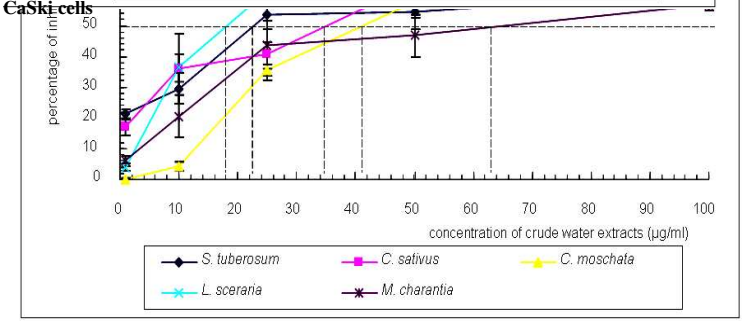


Figure 4.6 (d): Dose-response curves showing cytotoxic activity of crude water extracts of *P.erosus, P. tetragonolobus, P. speciosa, S. grandiflora* and *V. sinensis* against CaSki cells

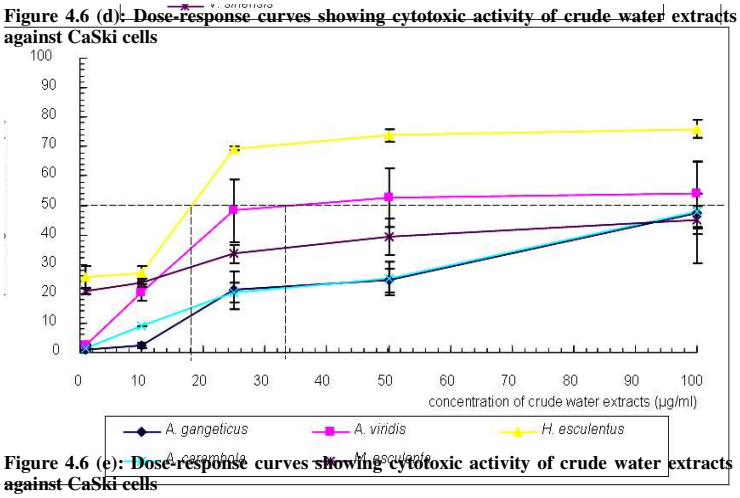


Figure 4.6 (e): Dose-response curves showing cytotoxic activity of crude water extracts of *A. gangeticus, A. viridis, H. esculentus, A. carambola* and *M. esculenta* against CaSki cells

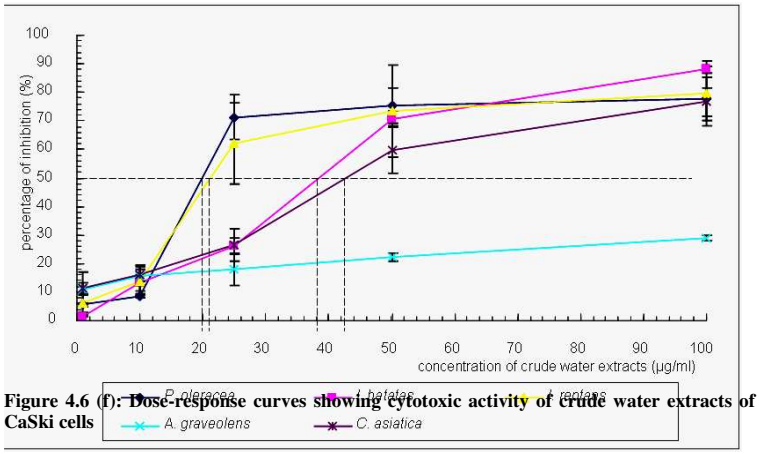


Figure 4.6 (f): Dose-response curves showing cytotoxic activity of crude water extracts of *P. oleracea, I. batatas, I. reptans, A. graveolens* and *C. asiatica* against CaSki cells

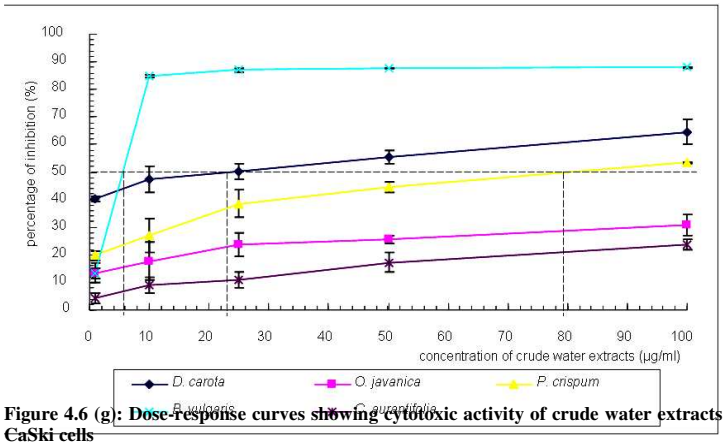


Figure 4.6 (g): Dose-response curves showing cytotoxic activity of crude water extracts of *D. carota, O. javanica, P. crispum, B. vulgaris* and *C. aurantifolia* against CaSki cells

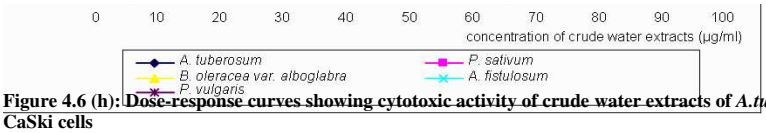


Figure 4.6 (h): Dose-response curves showing cytotoxic activity of crude water extracts of *A.tuberosum, P. sativum, B. oleracea* var. *alboglabra, a. fistulosum* against CaSki cells

Table 4.7:IC_{50} values of crude water extracts of selected vegetables against CaSki cells

No	Plant	Local name	IC_{50} (µg/ml)
1	*Beta vulgaris*	bit	**5.90**
2	*Phaseolus vulgaris*	kacang buncis	**5.90**
3	*Cosmos caudatus*	ulam rajah	**10.00**
4	*Psophocarpus tetragonolobus*	kacang botor,	**10.00**
5	*Parkia speciosa*	petai	**11.50**
6	*Asparagus officinalis*	Asparagus, saparu keras	**15.00**
7	*Capsicum annum*	lada merah,	**17.50**
8	*Hibiscus esculentus*	bendi	**18.00**
9	*Lagenaria sceraria*	labu ayer putih, labu botol	**18.00**
10	*Vigna sinensis*	kacang panjang	**18.00**
11	*Neptunia prostrata*	tangki,	**18.20**
12	*Portulaca oleracea*	beremi	**20.00**
13	*Ipomoea reptans*	kangkung	22.00
14	*Solanum tuberosum*	ubi benggala, ubi kentang, ubi gendang	22.50
15	*Daucus carota*	lobak merah	23.00
16	*Lactuca sativa*	salad, selada	23.50
17	*Brassica oleracea* var. *alboglabra*	Kai lan	24.00
18	*Archidendron jiringga*	jering	29.00
19	*Capsicum frutescens*	chabai, lada api, lada kerawit	29.50
20	*Allium tuberosum*	Ku chai	30.00
21	*Pisum sativum*	kacang puteh	30.00

Table 4.7 continued

22	*Amaranthus viridis*	bayam pasir	33.50
23	*Cucumis sativus*	timun, timun China, mentimun	34.50
24	*Ipomoea batatas*	keledek	38.00
25	*Cucurbita moschata*	labu merah, labu perang,	41.50
26	*Centella asiatica*	pegaga	42.50
27	*Lycopersicum esculentum*	tomato	48.50
28	*Sesbania grandiflora*	turi	49.50
29	*Luffa acutangula*	petola segi, ketola segi	63.00
30	*Momordica charantia*	peria	63.00
31	*Allium fistulosum*	Daun bawang	65.00
32	*Petroselium crispum*	parsley	79.00
33	*Amaranthus gangeticus*	bayam merah	>100
34	*Apium graveolens*	selderi	>100
35	*Averrhoa carambola*	belimbing besi	>100
36	*Citrus aurantifolia*	limau	>100
37	*Manihot esculenta*	ubi kayu	>100
38	*Oenanthe javanica*	shelum	>100
39	*Pachyrrhizus erosus*	sengkuwang,	>100
40	*Solanum melongena*	terong	>100

4.2.4 *In vitro* Cytotoxic Activity of Crude Methanol and Water Extracts of Selected Vegetable Against MRC5 Cells

The *in vitro* cytotoxicity activities of crude methanol and water extracts against MRC5 cells are illustrated as dose-response curves in Figures 4.7(a-h) and 4.8(a-h). As before the IC_{50} values for all crude extracts were extrapolated from the dose-response curves and summarized in Tables 4.8 and 4.9.

Generally, all crude methanol extracts from the 40 selected vegetables demonstrated no cytotoxic activities against MRC5 cells. The killing percentages produced by the methanol extracts ranged from 2.74-32.33 at 1 µg/ml, 8.2-40.20% at 10 µg/ml, 12.46-44.53% at 25 µg/ml, 20.39-50.35% at 50 µg/ml, 25.00-52.98% at 100 µg/ml. Only 8 out of 40 selected vegetable extracts, have IC_{50} values which could be extrapolated from dose-respone curves. The IC_{50} values extrapolated were between 48 µg/ml to 96 µg/ml produced by *Asparagus officinalis*, *Allium tuberosum, A. Centella asiatica*, *Vigna sinensis*, *Capsicum annum*, *Psophocarpus tetragonolobus*, *Solanum melongena* and *Citrus aurantifolia* (Table 4.8). The IC_{50} values for the other vegetable extracts could not be determined from the existing killing-curves.

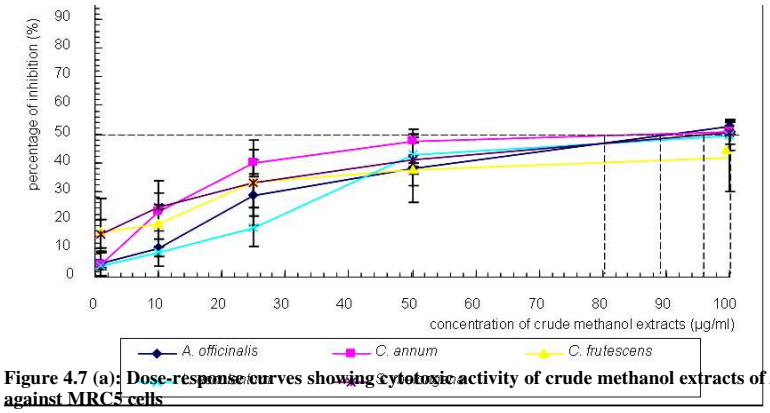


Figure 4.7 (a): Dose-response curves showing cytotoxic activity of crude methanol extracts of *A. officinalis*, *C. annum*, *C. frutescens*, *L. esculentum* and *S. melongena* against MRC5 cells

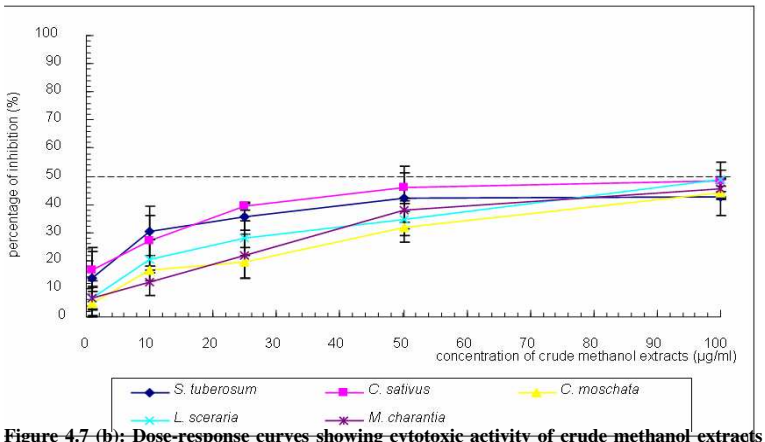


Figure 4.7 (b): Dose-response curves showing cytotoxic activity of crude methanol extracts of *S. tuberosum, C. sativus, C. moschata, L. sceraria* and *M. charantia* against MRC5 cells

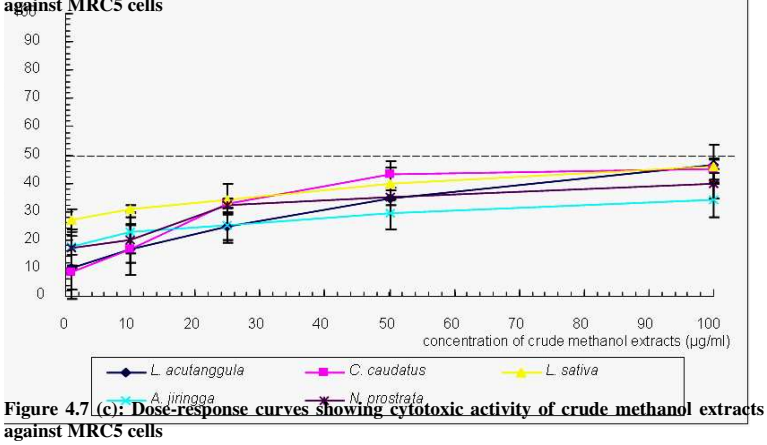


Figure 4.7 (c): Dose-response curves showing cytotoxic activity of crude methanol extracts of *L. acutanggula, C. caudatus, L. sativa, A. jiringga* and *N. prostrata* against MRC5 cells

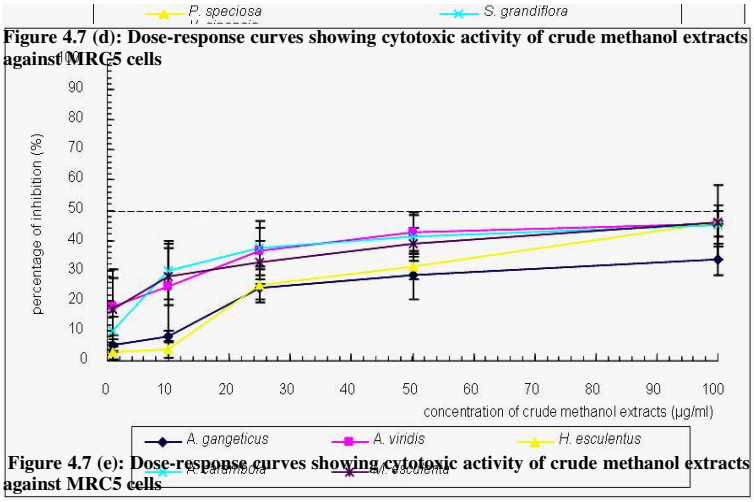


Figure 4.7 (d): Dose-response curves showing cytotoxic activity of crude methanol extracts of *P. erosus, P. tetragonolobus, P. speciosa, S. grandiflora* and *V. sinensis* against MRC-5 cells

Figure 4.7 (e): Dose-response curves showing cytotoxic activity of crude methanol extracts of *A. gangeticus, A. viridis, H. esculentus, A. carambola* and *M. esculenta* against MRC5 cells

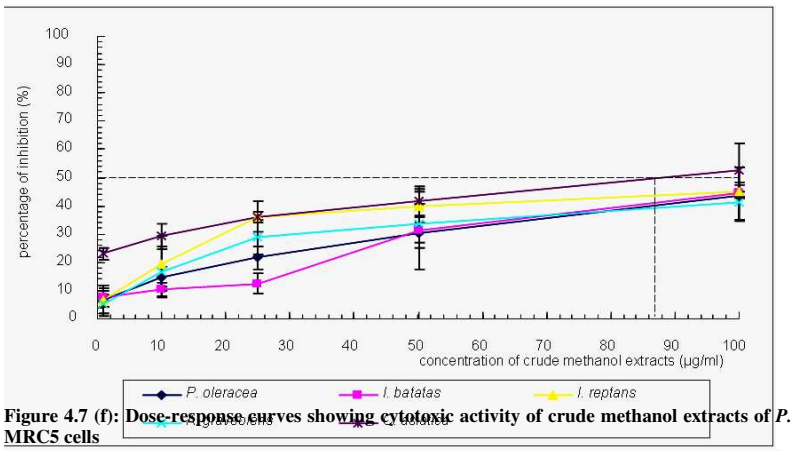


Figure 4.7 (f): Dose-response curves showing cytotoxic activity of crude methanol extracts of *P. oleracea, I. batatas, I. reptans, A. graveolens* and *C. asiatica* against MRC5 cells

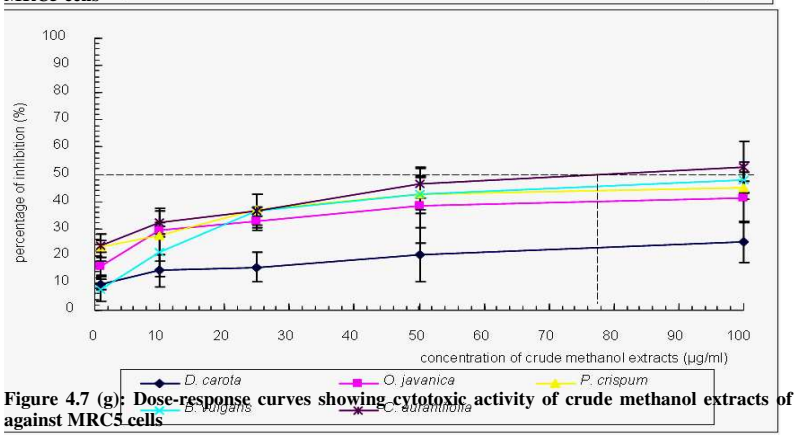


Figure 4.7 (g): Dose-response curves showing cytotoxic activity of crude methanol extracts of *D. carota, O. javanica, P. crispum, B. vulgaris* and *C. aurantifolia* against MRC5 cells

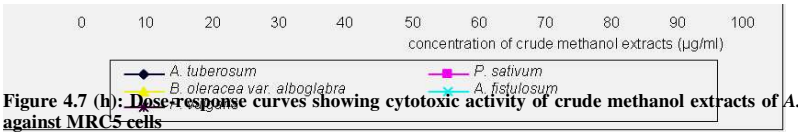


Figure 4.7 (h): Dose response curves showing cytotoxic activity of crude methanol extracts of *A. tuberosum, P. sativum, B. oleracea var. alboglabra* and *P. vulgaris* against MRC5 cells

Table 4.8: IC_{50} values of crude methanol extracts of selected vegetables against MRC5 cells

No	Plant	Local name	IC_{50} (µg/ml)
1	*Psophocarpus tetragonolobus*	kacang botor	48.00
2	*Vigna sinensis*	kacang panjang	68.500
3	*Citrus aurantifolia*	limau	77.80
4	*Capsicum annum*	lada merah	80.00
5	*Centella asiatica*	Pegaga	87.00
6	*Asparagus officinalis*	Asparagus, saparu keras	89.00
7	*Allium tuberosum*	Ku chai	95.00
8	*Solanum melongena*	terong	96.00
9	*Allium fistulosum*	Daun bawang	>100
10	*Amaranthus gangeticus*	bayam merah	>100
11	*Amaranthus viridis*	bayam pasir	>100
12	*Apium graveolens*	Selderi	>100
13	*Archidendron jiringga*	jering	>100
14	*Averrhoa carambola*	belimbing besi	>100
15	*Beta vulgaris*	Bit	>100
16	*Brassica oleracea* var. *alboglabra*	Kai lan	>100
17	*Capsicum frutescens*	chabai, lada api, lada kerawit	>100
18	*Cosmos caudatus*	ulam rajah	>100
19	*Cucumis sativus*	timun, timun China, mentimun	>100
20	*Cucurbita moschata*	labu merah, labu perang,	>100
21	*Daucus carota*	lobak merah	>100

Table 4.8 continued

22	*Hibiscus esculentus*	Bendi	>100
23	*Ipomoea batatas*	keledek	>100
24	*Ipomoea reptans*	kangkung	>100
25	*Lactuca sativa*	salad, selada	>100
26	*Lagenaria sceraria*	labu ayer putih, labu botol	>100
27	*Luffa acutangula*	petola segi, ketola segi	>100
28	*Lycopersicum esculentum*	tomato	>100
29	*Manihot esculenta*	ubi kayu	>100
30	*Momordica charantia*	peria	>100
31	*Neptunia prostrata*	tangki	>100
32	*Oenanthe javanica*	shelum	>100
33	*Pachyrrhizus erosus*	sengkuwang	>100
34	*Parkia speciosa*	petai	>100
35	*Petroselium crispum*	parsley	>100
36	*Phaseolus vulgaris*	kacang buncis	>100
37	*Pisum sativum*	kacang puteh	>100
38	*Portulaca oleracea*	beremi	>100
39	*Sesbania grandiflora*	turi	>100

40	*Solanum tuberosum*	ubi benggala, ubi kentang, ubi gendang	>100

The *in vitro* cytotoxicity activities of crude water extracts against MRC 5 cells are illustrated as dose-response curves in Figures 4.8(a)-4.8(h). The IC_{50} values were extrapolated from the dose-response curves and summarized in Table 4.9. Only 7 out of 40 selected vegetable extracts, have IC_{50} values which could be extrapolated from dose-respone curves. The IC_{50} values extrapolated which were between 59.5 µg/ml-97.5 µg/ml was produced by *Allium tuberosum*, *A. fistulosum, Centella asiatica*, *Vigna sinensis*, *Luffa acutanggula, Hibiscus esculentus, Averrhoa carambola, Apium graveolen and Citrus aurantifolia*. The IC_{50} value for the other vegetable extracts could not be determined from the existing killing-curves.

Overall results indicated that, the crude methanol and water extracts from the 40 selected vegetables were found to be not cytotoxic against MRC5 cell lines.

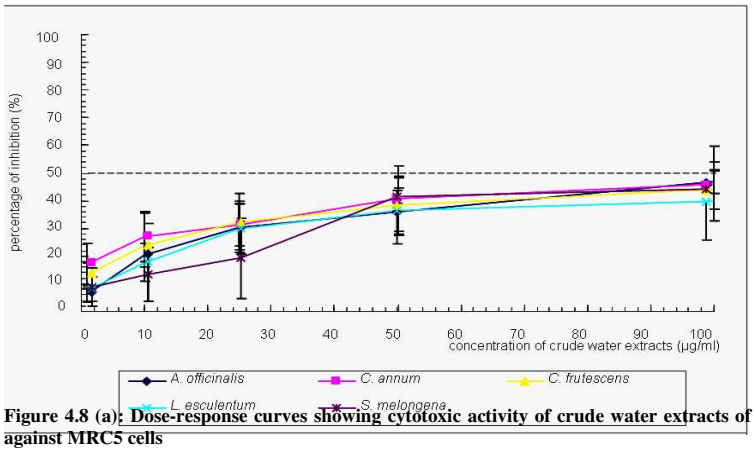


Figure 4.8 (a): Dose-response curves showing cytotoxic activity of crude water extracts of *A. officinalis*, *C. annum*, *C. frutescens*, *L. esculentum* and *S. melongena* against MRC5 cells

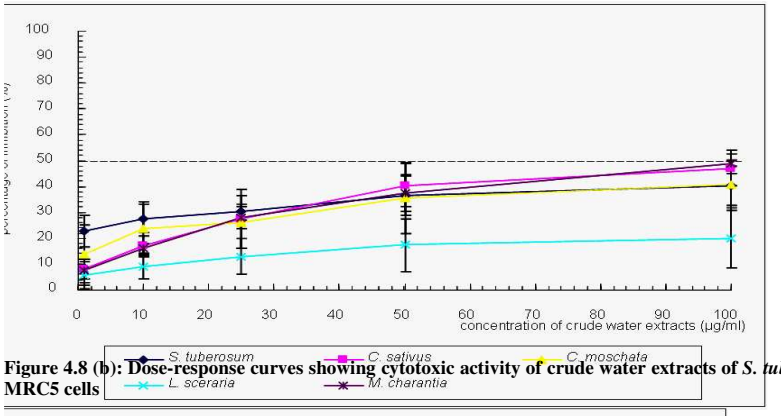


Figure 4.8 (b): Dose-response curves showing cytotoxic activity of crude water extracts of *S. tuberosum, C. sativus, C. moschata, L. sceraria* and *M. charantia* against MRC5 cells

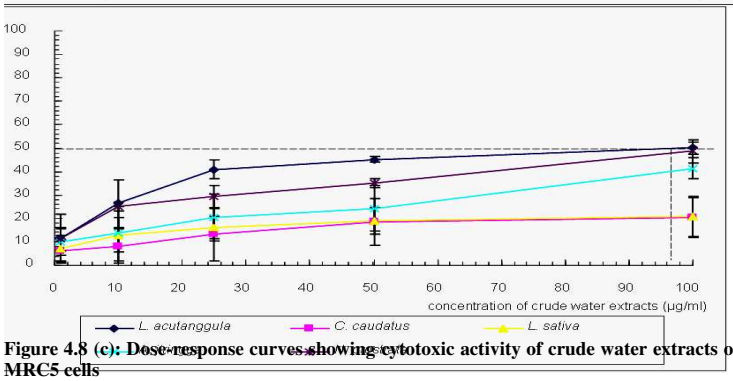


Figure 4.8 (c): Dose-response curves showing cytotoxic activity of crude water extracts of *L. acutanggula, C. caudatus, L. sativa, A. jiringga* and *N. prostrata* against MRC5 cells

Figure 4.8 (d): Dose-response curves showing cytotoxic activity of crude water extracts of *P.erosus, P. tetragonolobus, P. speciosa, S. grandiflora* and *V. sinensis*

against MRC5 cells

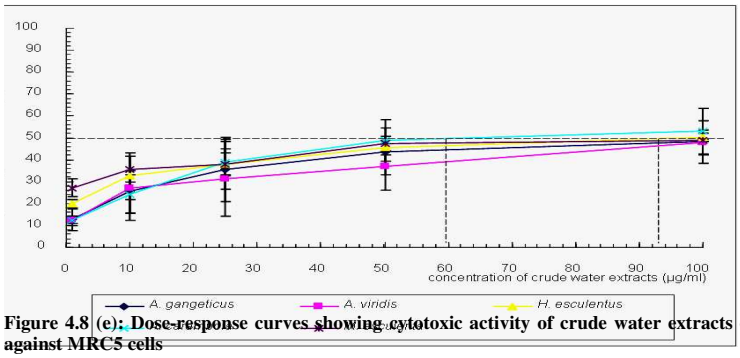


Figure 4.8 (e): Dose-response curves showing cytotoxic activity of crude water extracts of *A. gangeticus, A. viridis, H. esculentus, A. carambola* and *M. esculenta* against MRC5 cells

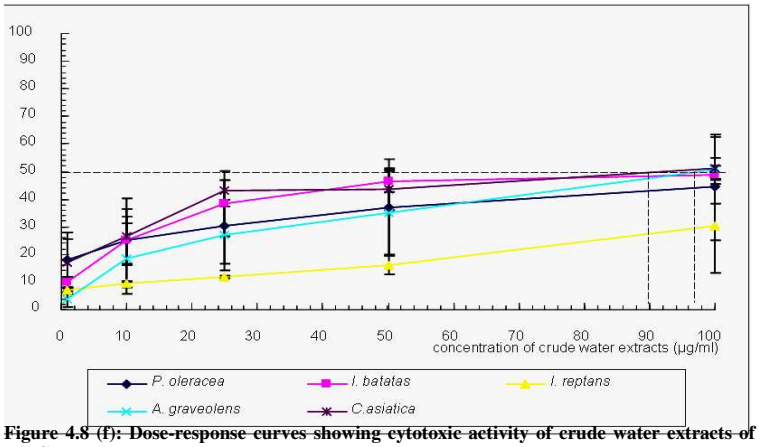


Figure 4.8 (f): Dose-response curves showing cytotoxic activity of crude water extracts of *P. oleracea, I. batatas, I. reptans, A. graveolens* and *C. asiatica* against MRC5 cells

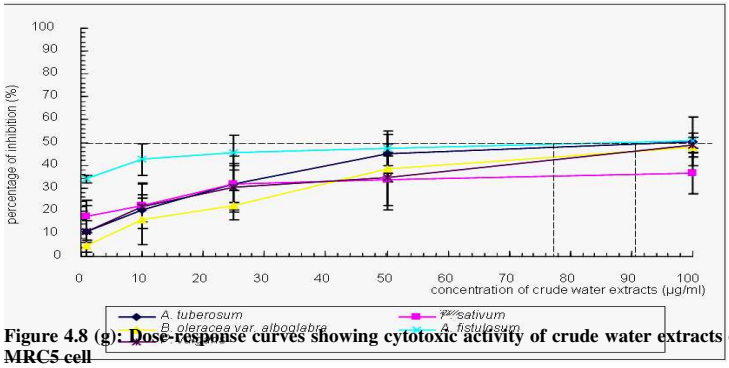


Figure 4.8 (g): Dose-response curves showing cytotoxic activity of crude water extracts of *D. carota, O. javanica, P. crispum, B. vulgaris* and *C. aurantifolia* against MRC5 cell

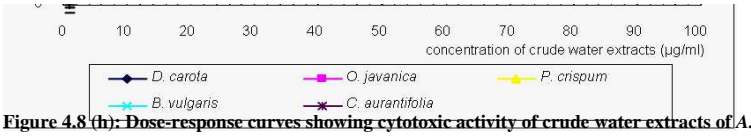


Figure 4.8 (h): Dose-response curves showing cytotoxic activity of crude water extracts of *A.tuberosum, P. sativum, B. oleracea* var. *alboglabra, A. fistulosum,* and *P. vulgaris* against MRC5 c

Table 4.9: IC_{50} values of crude water extracts of selected vegetables against MRC5 cells

No	Plant	Local name	IC_{50} (µg/ml)
1	*Averrhoa carambola*	belimbing besi	59.50
2	*Allium fistulosum*	Daun bawang	77.50
3	*Vigna sinensis*	kacang panjang	83.50
4	*Centella asiatica*	pegaga	90.00
5	*Allium tuberosum*	Ku chai	90.80
6	*Hibiscus esculentus*	bendi	93.00
7	*Citrus aurantifolia*	limau	96.00
8	*Luffa acutangula*	petola segi, ketola segi	96.80
9	*Apium graveolens*	selderi	97.50
10	*Amaranthus gangeticus*	bayam merah	>100
11	*Amaranthus viridis*	bayam pasir	>100
12	*Archidendron jiringga*	jering	>100
13	*Asparagus officinalis*	Asparagus, saparu keras	>100
14	*Beta vulgaris*	bit	>100
15	*Brassica oleracea* var. *alboglabra*	Kai lan	>100
16	*Capsicum annum*	lada merah,	>100
17	*Capsicum frutescens*	chabai, lada api, lada kerawit	>100
18	*Cosmos caudatus*	ulam rajah	>100
19	*Cucumis sativus*	timun, timun China, mentimun	>100
20	*Cucurbita moschata*	labu merah, labu perang,	>100
21	*Daucus carota*	lobak merah	>100

Table 4.9 continued

22	*Ipomoea batatas*	keledek	>100
23	*Ipomoea reptans*	kangkung	>100
24	*Lactuca sativa*	salad, selada	>100
25	*Lagenaria sceraria*	labu ayer putih, labu botol	>100
26	*Lycopersicum esculentum*	tomato	>100
27	*Manihot esculenta*	ubi kayu	>100
28	*Momordica charantia*	peria	>100
29	*Neptunia prostrata*	tangki,	>100
30	*Oenanthe javanica*	shelum	>100
31	*Pachyrrhizus erosus*	sengkuwang,	>100
32	*Parkia speciosa*	petai	>100
33	*Petroselium crispum*	parsley	>100
34	*Phaseolus vulgaris*	kacang buncis	>100
35	*Pisum sativum*	kacang puteh	>100
36	*Portulaca oleracea*	beremi	>100
37	*Psophocarpus tetragonolobus*	kacang botor,	>100
38	*Sesbania grandiflora*	turi	>100
39	*Solanum melongena*	terong	>100
40	*Solanum tuberosum*	ubi benggala, ubi kentang, ubi gendang	>100

4.3 Anti-HPV 16 E6 Oncoprotein Activity in Selected Vegetable

The crude methanol and water extracts from 10 selected vegetables were analyzed qualitatively for their anti-HPV 16 E6 protein activity in HPV 16-containing cervical cancer-derived cell line, CaSki. The CaSki cells were treated with the vegetable extracts at various concentrations (1, 10, 25, 50 and 100 µg/ml) for 3 days. The immunocytochemistry technique, 3-step Indirect Avidin-Biotin Immunoperoxidase with the anti-HPV 16 monoclonal antibody were successfully applied in this study to analyse the expression of E6 HPV 16 oncoprotein in treated and untreated CaSki cells.

Presence or reddish-brownish coloured products in the cytoplasmic and/or nuclear regions indicate expression/ presence of the HPV 16 E6 oncoprotein. The higher the intensity of the coloured products, the higher the amounts of the protein. On the other hand, the lower the amount of E6 simultaneously suggested evidence of suppression by the vegetable extracts. The staining intensity was classified as no stain (-), very weak (+), weak (2+), moderate (3+), strong (4+) and very strong (5+) as illustrated in Figure 4.9.

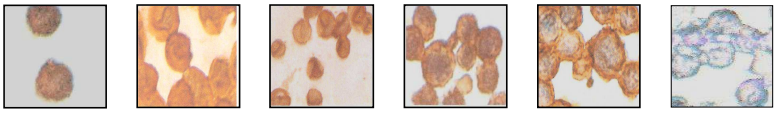
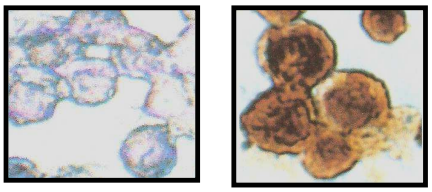


Figure 4.9: Staining intensities of HPV 16 E6 oncoprotein in CaSki cells using the 3-step Indiret Avidin-Biotin Immunoperoxidase methods.

In this present study, two types of negative controls were used. The CaSki cells not treated with the extracts and incubated with anti-HPV 16 E6 monoclonal antibody and CaSki cells not treated with the extracts and not incubated with anti-HPV 16 E6 monoclonal antibody. The staining results for untreated CaSki cells with and without anti-HPV monoclonal antibody are shown in Figures 4.10(a) and 4.10(b). All untreated CaSki cells appeared to be morphologically intact. The negative control incubated with anti-HPV monoclonal antibody exerted very strong reddish-brown stain (5+) due to the detection of high expression of E6 protein while no stain (-) was observed for untreated CaSki cells without incubation with anti-HPV 16 E6 monoclonal antibody



(a) (b)

Figure 4.10 (a): Untreted CaSki cells incubated with anti-HPV 16 E6 monoclonal antibody. Very strong staining (5+) was observed in the nuclear and cytoplasmic regions (400X)

Figure 4.10 (b): Untreated without incubation with anti-HPV 16 E6 monoclonal antibody. No staining (-) was observed (400X)

Expressions of HPV 16 E6 protein after treatment with varying concentrations of vegetable extracts are shown in Figures 4.11-4.20. The staining intensities of the CaSki cell treated with different extracts at various concentrations were compared with each other and with the negative control. The morphology of the cells and the intensity of the coloured products were analysed.

4.3.1 *Asparagus officinalis*

As seen in Figure 4.11, the intensity of reddish-brown stain in CaSki cells treated with methanol and water extracts of *Asparagus officinalis* decreased with increasing concentrations of the vegetable extracts. This suggest suppression of HPV 16 E6 oncoprotein in CaSki cells treated with *A. officinalis* extracts at concentration as low as 1 µg/ml. Suppression increased with increasing concentration of the methanol and water extracts of *A. officinalis*.

The methanol extracts of *A. officinalis* produced just a weak suppression of HPV 16 E6 oncoprotein at 1 µg/ml and 10 µg/ml. The suppression increased then on to moderate at 25 µg/ml, strong at 50 µg/ml and became very strong at 100 µg/ml. In contrast, the weak suppression of HPV 16 E6 oncoprotein was observed in CaSki cells treated with water extracts of *A.officinalis* at 1 µg/ml, 10 µg/ml, 25 µg/ml and 50 µg/ml butbecame very strong at 100 µg/ml.

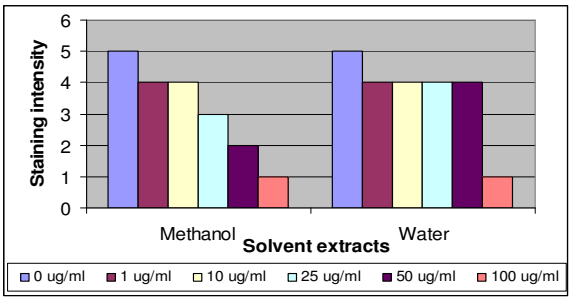


Figure 4.11: Staining intensities of Caski cells treated with *Asparagus officinalis*

Classification for staining intensity as: no stain (0), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

4.3.2 *Beta vulgaris*

Figure 4.12, illustrates a reduction in intensity of the reddish-brown stain in CaSki cells treated with methanol and water extracts of *Beta vulgaris*. The expression of HPV 16 E6 oncprotein decreased with increasing concentrations of the *B. vulgaris*. The methanol extracts of *B. vulgaris* exhibited moderate suppression of HPV 16 E6 oncprotein at 1 µg/ml and 10 µg/ml. However from then suppression of HPV 16 E6 in Caski cells treated with methanol extracts of *B. vulgaris* became very strong at 25 µg/ml, 50 µg/ml and 100 µg/ml.

Overall indicated that the water extracts of *Beta vulgaris* was slightly more suppressive against HPV 16 E6 oncoprotein at 10 µg/ml as compared to methanol counterparts. In general, water extracts of *B. vulgaris* exhibited moderate suppression at 1 µg/ml, and stronger at 10 µg/ml and became very strong at 25 µg/ml, 50 µg/ml and 100 µg/ml. CaSki cells treated with *B. vulgaris* extracts remain morphologically intact at all concentrations.

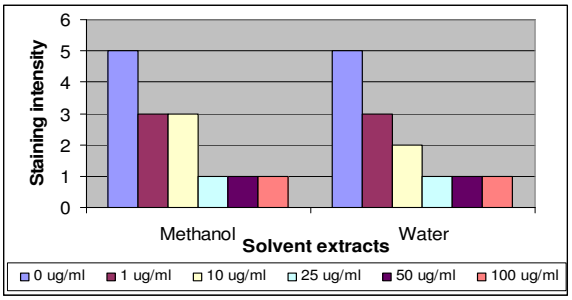


Figure 4.12: Staining intensities of Caski cells treated with *Beta vulgaris*

Classification for staining intensity as: no stain (0), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

4.3.3 *Ipomea batatas*

Figure 4.13 shows that, a reduction in intensity of the reddish-brown stain in CaSki cells treated with methanol and water extracts of *I. batatas*. Expression of HPV 16 E6 oncoprotein decreased with increasing concentrations of *I. batatas* extracts. The methanol extracts of *I. batatas* exhibited very strong suppression of HPV 16 E6 oncoprotein at all concentrations tested.

In contrast, the water extracts of *I. batatas* inhibited weak suppression of HPV 16 E6 oncoprotein at 1 µg/ml and 10 µg/ml but became strong at 25 µg/ml, 50 µg/ml and 100 µg/ml. In general, CaSki cells treated with *I. batatas* extracts remained morphologically intact at all concentrations.

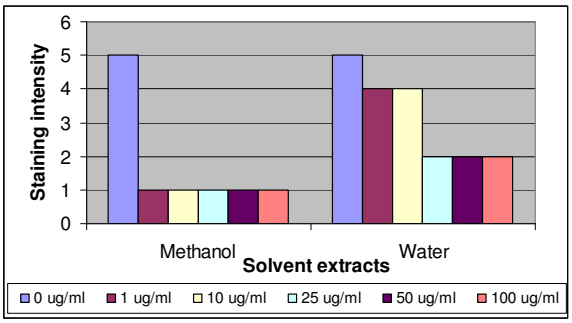


Figure 4.13: Staining intensities of Caski cells treated with *Ipomea batatas*

Classification for staining intensity as: no stain (0), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

4.3.4. *Manihot esculentus*

Figure 4.14, shows that methanol and water extracts of the *M. esculentus* exerted inhibition activity against the HPV-16 E6 oncoprotein. All CaSki cells treated with *M. esculentus* extracts remained morphologically intact at all concentrations used.

In general, the *M. esculenta* extracts demonstrated good inhibition activities against HPV16 E6 oncoprotein in CaSki cells. The methanol extracts of *M. esculenta* exhibited strong suppression of HPV 16 E6 oncoprotein at concentrations 1 µg/ml and 10 µg/ml. Suppression of HPV 16 E6 became very strong at concentrations 25 µg/ml, 50µg/ml and 100 µg/ml. In contrast, the water extract of *M. esculentus* exhibited strong suppression of HPV 16 E6 oncoprotein at all concentrations tested.

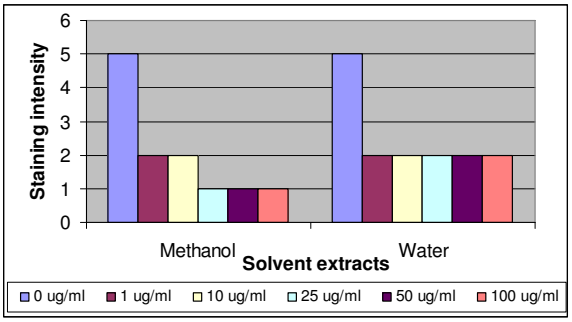


Figure 4.14: Staining intensities of Caski cells treated with *Manihot esculentus*

Classification for staining intensity as: no stain (0), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

4.3.5 *Pachyrrizus erosus*

Figure 4.15 show that, the intensities of reddish-brown stain decreased with increasing concentrations of methanol and water extracts of *P. erosus*. This shows that the expression of HPV-16 E6 oncoprotein in CaSki cells reduced when the concentration of *P. erosus* extracts increased. Overall results showed that the reddish brown stain was seen in both nuclear and cytoplasmic regions of CaSki cells. The methanol extract of *P. erosus* produced no suppression of HPV 16 E6 at 1 µg/ml, but produced weak suppression at 10 µg/ml. The suppression increased then on to moderate at 25 µg/ml and became strong at 50 µg/ml and 100 µg/ml.

In contrast, the water extract of *P. erosus* exhibited moderate suppression inhibition of HPV 16 E6 oncoprotein at concentrations as low as 1 µg/ml. Suppression of HPV 16 E6 became strong at 10 µg/ml and 25 µg/ml. The inhibition became very strong then on at concentrations 50 µg/ml and 100 µg/ml. The CaSki cells treated with methanol extract started to lysie at concentrations 25 µg/ml to 100 µg/ml. In contrast, the CaSki cells remained morphological intact when treated with water extracts at all of concentrations used.

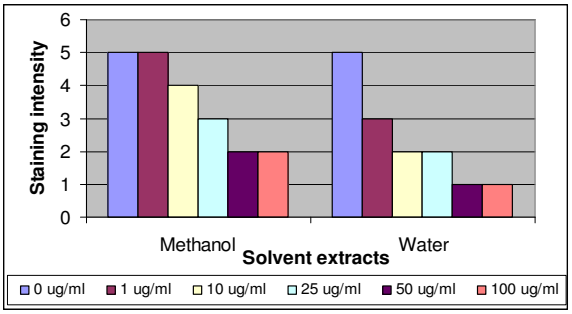


Figure 4.15: Staining intensities of Caski cells treated with *Pachyrrizus erosus*

Classification for staining intensity as: no stain (0), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

4.3.6 *Pertroselium crispum*

As seen in Figure 4.16, there is reduction in intensity of the reddish-brown stain at concentrations as low as 1 µg/ml for methanol and water extracts of *P. crispum*. Very weak suppression of HPV 16 E6 oncoprotein was observed in CaSki cells treated with methanol extracts at all concentrations tested and in CaSki cells treated with water

extracts at concentrations 1 µg/ml, 25 µg/ml and 50 µg/ml. The suppression of HPV 16 E6 protein became moderate in cells treated with 100 µg/ml of the water extract. The integrity of CaSki cells treated with *P. crispum* extracts were negatively affected where the cells lysed at concentrations as low as 1 µg/ml (for methanol and water extract) and 10 µg/ml. The distribution of stain was observed either in the cell cytoplasm only or both nucleus and cytoplasm.

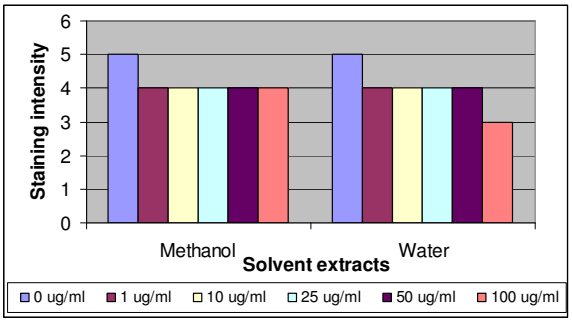


Figure 4.16: Staining intensities of Caski cells treated with *Petroselium crispum*

Classification for staining intensity as: no stain (0), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

4.3.7 *Phaseolus vulgaris*

Figure 4.17, illustrates that the methanol extract of *P. vulgaris* exhibited very strong suppression of HPV 16 E6 at 1 µg/ml and 10 µg/ml. However, the suppression weakened from then on resulting in the reddish-brown stain to become stronger with increasing concentrations of the *P. vulgaris* methanol extract.

In contrast, the water extracts did not exhibit any suppression of HPV 16 E6 oncoprotein at 1 µg/ml and 10 µg/ml. Weak suppression was seen at 25 µg/ml, suppression of HPV 16 E6 become strong at 50µg/ml and very strong at 100 µg/ml. Cell lysis was observed at 1 µg/ml for water extracts and at concentration as low as 25 µg/ml for methanol extracts.

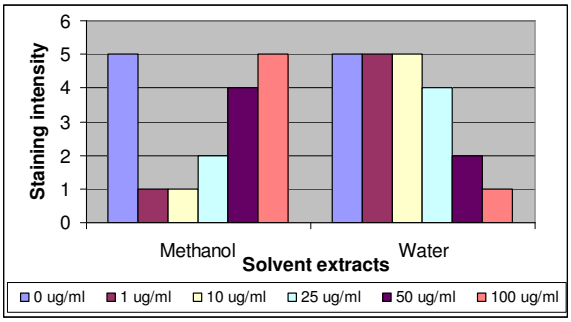


Figure 4.17: Staining intensities of Caski cells treated with *Phaseolus vulgaris*

Classification for staining intensity as: no stain (0), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

4.3.8 *Portulaca oleracea*

The resulting staining intensity of CaSki cells treated with *Portulaca oleracea* extracts are shown in Figure 4.18. In general, the *P. oleracea* extracts demonstrated good inhibition activities against HPV16 E6 oncoprotein in CaSki cell. The methanol extract of *P. oleracea* very strongly suppressed HPV 16 E6 oncoprotein at concentrations 1 µg/ml, 10 µg/ml and 25 µg/ml. However, the inhibition weakened a little (though still strong) at concentrations 50 µg/ml and 100 µg/ml.

In contrast, the water extract of *P. oleracea* produced strong inhibition of HPV 16 E6 oncoprotein at concentrations as low as 1 µg/ml and 10 µg/ml. The inhibition then became very strong at higher concentrations. All the CaSki cells treated with *P. oleracea* extracts appeared to be morphologically intact. Overall results showed that the reddish brown stain was seen in both nuclear and cytoplasmic regions.

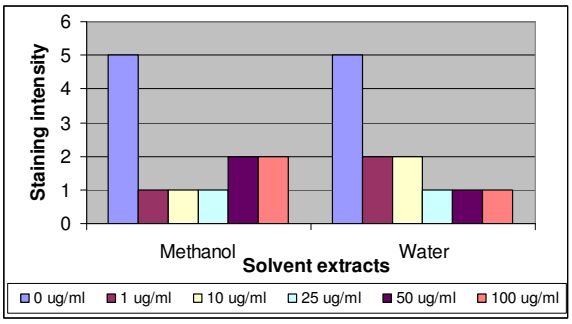


Figure 4.18: Staining intensities of Caski cells treated with *Portulaca oleracea*

Classification for staining intensity as: no stain (0), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

4.3.9 *Sesbania grandiflora*

As denoted by the reduction of reddish-brown stain in CaSki cells, the inhibition activities of HPV 16-E6 oncoprotein by *S. grandiflora* extracts increased with the increasing concentrations of the extracts (Figure 4.19). The methanol extract of *S. grandiflora* produced a strong inhibition of HPV 16 E6 oncoprotein at concentrations as low as 1 µg/ml and remained consistent at concentrations 10 µg/ml and 25 µg/ml. The inhibition became very strong from then on at concentrations 50 µg/ml and 100 µg/ml.

In contrast the water extract of *S. grandiflora* produced a weak suppression of HPV 16 E6 at 1 µg/ml. The suppression increased then on to moderate at 10 µg/ml, strong at 25 µg/ml and 50 µg/ml and became very strong at 100 µg/ml. Evident here that methanol extract produced a greater inhibition effects as compared to water extract. The integrity of CaSki cells were negatively affected where the cells lysed after treated with methanol at 25 µg/ml and 50 µg/ml.

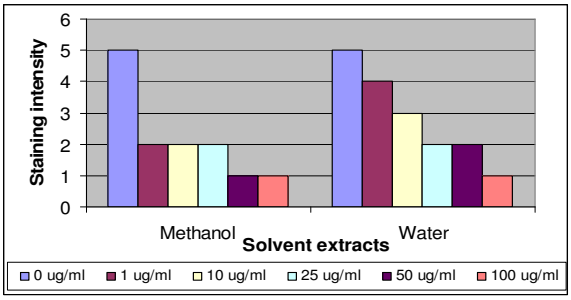


Figure 4.19: Staining intensities of Caski cells treated with *Sesbania grandiflora*

Classification for staining intensity as: no stain (0), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

4.3.10 *Solanum tuberosum*

The resulting staining intensity of the CaSki cells treated with *Solanum tuberosum* extracts are shown in Figure 4.20. The decrease in staining intensity was observed in CaSki cells treated with increasing concentration of the extracts. This indicates that the *S. tuberosum* extracts inhibited the expression of HPV 16 E6 oncoprotein in a dose-dependent manner, where the suppressing effects increased with increasing concentration of the extracts.

The water extracts of *S. tuberosum* exerted a more suppressive effect against HPV-16 E6 oncoprotein as compared to methanol extracts. Both extracts exerted weak suppressing effects against HPV 16 E6 oncoprotein at concentrations 1 µg/ml and 10 µg/ml. However, from then on CaSki cells treated with water extracts exhibited very weak reddish brown stain, indicating very strong suppression at concentrations, 25 µg/ml, 50 µg/ml and 100 µg/ml. Methanol extracts of *S. tuberosum* on the other hand produced just moderate suppression of the oncoprotein at concentrations, 25 µg/ml, 50 µg/ml and 100 µg/ml. The integrity of CaSki cells treated with *S. tuberosum* extracts were negatively affected where the cells lysed at concentration as low as 25 µg/ml (for methanol extract).

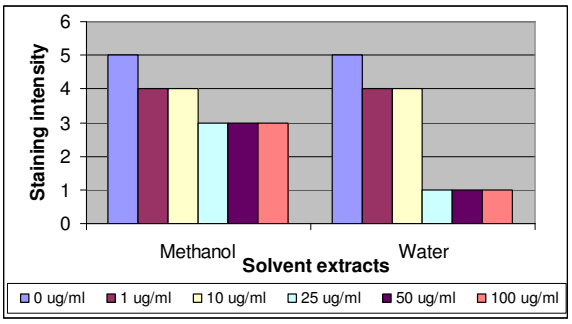


Figure 4.20: Staining intensities of Caski cells treated with *Solanum tuberosum*

Classification for staining intensity as: no stain (0), very weak (1+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

4.4 Comparison of Anti-HPV 16 E6 Oncoprotein Activity in Selected Vegetable

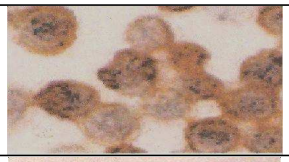
Twenty crude extracts (methanol and water) from ten selected vegetables were analyzed qualitatively for possible anti-HPV 16 E6 oncoprotein activity. The staining intensity of coloured products was observed in CaSki cells treated with the selected vegetable crude extracts indicated that each vegetable extracts possess different ability in suppressing the expression of HPV 16 E6 oncoprotein (Figure 4.21 and Figure 4.22).

Overall results indicate that not all of the vegetable extracts suppressed the HPV 16 E6 oncoprotein in a dose-dependent manner where greater inhibition activity against E6 oncoprotein was observed in higher extract concentration.

Ten out of 20 extracts (50%) were found effective in suppressing the HPV 16 E6 oncoprotein at the lowest extract concentration (25 µg/ml and below) used in this study. They were both methanol and water extracts for *P. oleracea*, *I. batatas*, *M. esculentus*, methanol extract for *P. erosus*, *B. vulgaris*, *S. grandiflora* and water extracts for *A. officinalis*.

Overall results indicated that *P. oleracea* extracts was the most effective in suppressing the HPV 16 E6 oncoprotein. When compared, water extracts was better than methanol extracts at suppressing the HPV 16 E6 oncoprotein.

Appearance of CaSki cells after treatment with methanol extract of *Asparagus officinalis*

	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	2+

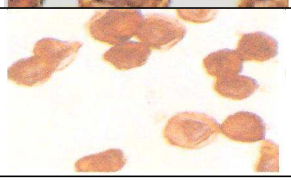
			
	10	Intact	4+
	1	Intact	4+

Figure 4.21: CaSki cells treated with methanol extracts of *Asparagus officinalis* (400x)
Note: Classification for the intensity of staining as no stain (-), very weak (+), weak (2+), moderate (3+), strong (4+) and very strong (5+

Appearance of CaSki cells after treatment with water extract of *Solanum tuberosum*

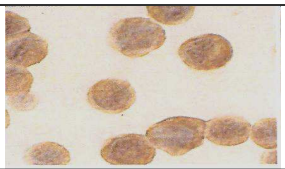
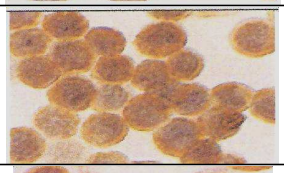
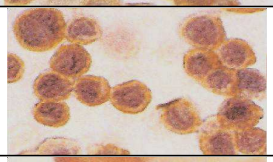
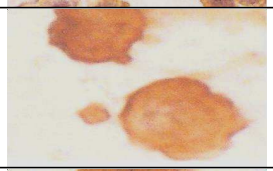
	Concentration of extracts (µg/ml)	**Morphology of cells**	**Intensity of reddish-brown stain**
	100	Intact	+
	50	Intact	+
	25	Intact	+
	10	Intact	4+
	1	Intact	4+

Figure 4.22: CaSki cells treated with water extracts of *Solanum tuberosum* (400x)
Note: Classification for the intensity of staining as no stain (-), very weak (+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

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 (28ºC 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, 40ºC-60ºC 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 120ºC 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 180ºC 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 º C and culture media were kept at 4ºC.

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)-2,5-diphenyltetrazolium 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).

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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 IC_{50} value less than 20 µg/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 perfomed 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 pre-incubated 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 IC_{50} values 7.8 µg/ml and 16 µg/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 IC_{50} value 6 µg/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 IC_{50} values 19.8 µg/ml and 14.1 µg/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 IC_{50} values more than 20 µg/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 IC_{50} values were reported to be 93.8 µg/ml and 98.8 µg/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 µg/ml, and the IC_{50} value was found to be 281.15 µg/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 IC_{50} at 2.7 µM and 3 µM 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 8ºC. LSAB peroxidase kit was stored in freezer (4ºC). 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 µg/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 µg/ml and 10 µg/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 µg/ml and 10 µg/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 µm filter membrane (Orange Scientific) and stored at 4ºC 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 4ºC 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 µg/ml) of streptomycin and (100 IU/ml) penicillin (PAA Lab., Austria) were added followed by filteration of the media using, 0.22 µm filter membrane and stored at 4ºC 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 µm filter membrane and stored at 4ºC 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 4ºC.

Preparation of Phosphate Buffered saline (PBS)

Phosphate Buffered Saline (PBS) was prepared using 1.52 g of Sodium phosphate anhydrous ($NaHPO_4$, Merck), 0.58 g of Potassium dihydrogen orthophosphate (KH_2PO_4. 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 µm pore size filters and stored at -20ºC.

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 dH_20 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 4ºC until required.

Neutral Red Medium for Cytotoxic Assay

12 ml of 10% supplemented RPMI medium or EMEM medium were mixed with 150 µl 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 ($CaCl_2$, Sigma) was dissolved in 500 µl formaldehyde (Sigma) and 99.5 ml of distilled water and kept at 4ºC.

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 4ºC.

Preparation of Phosphate Buffered saline PBS (pH 7.6)

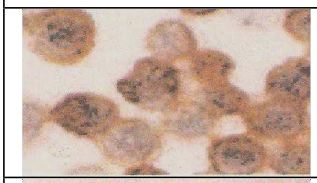
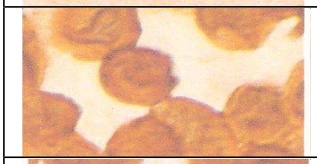
Phosphate Buffered saline (pH 7.6) was prepared using 1.5 g Sodium phosphate anhydrous (Na_2HPO_4, Merck), 0.2 g of potassium dihydrogen orthophosphate (KH_2PO_4, Merck) and 7.75 g of Sodium chloride (NaCl, BDH Analar) in 1 liter of distilled water. The pH was adjusted to 7.6 using a pH meter (Hanna Instrument 84171, USA). The buffer was then filtered using Whatman filter paper and stored at room temperature until required.

3% hydrogen peroxidase (H_2O_2)

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

Appendix C

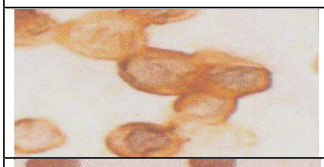
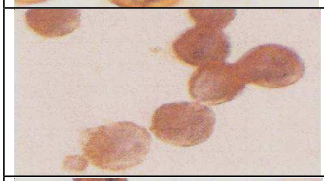
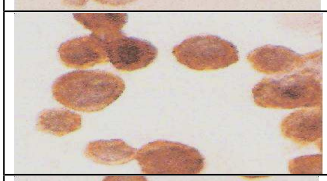
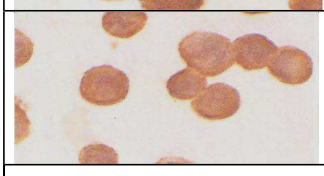
Appearanxce of CaSki cells after treatment with methanol of *Asparagus officinalis*

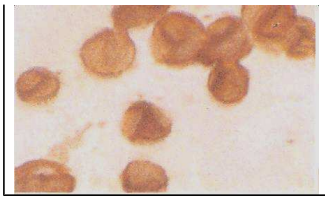
	Concentration of extracts (µg/ml)	Morphology of cells	Intensity of reddish-brown stain
	100	Intact	+
	50	Majority intact but some showing lysis	2+
	25	Majority intact but some showing lysis	3+
	10	Intact	4+
	1	Intact	4+

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

Note: Classification for the intensity of staining as no stain (-), very weak (+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

Appearance of CaSki cells after treatment with water of *Asparagus officinalis*

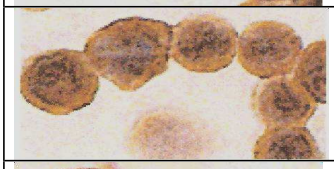
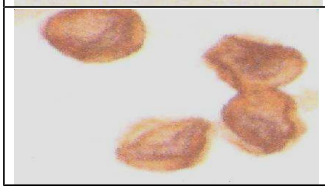
	Concentration of extracts (µg/ml)	Morphology of cells	Intensity of reddish-brown stain
	100	Lysis	3+
	50	Intact	3+
	25	Intact	3+
	10	Intact	3+

	1	Intact	3+

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

Note: Classification for the intensity of staining as no stain (-), very weak (+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

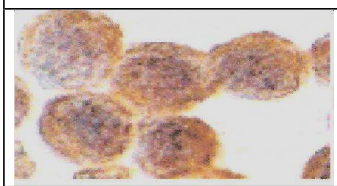
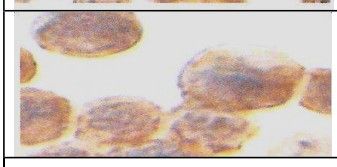
Appearance of CaSki cells after treatment with methanol of *Beta vulgaris*

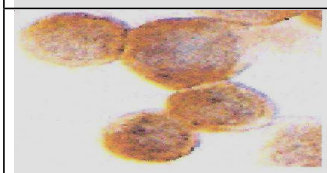
	Concentration of extracts (µg/ml)	Morphology of cells	Intensity of reddish-brown stain
	100	Intact	+
	50	Intact	+
	25	Intact	+
	10	Intact	3+
	1	Intact	3+

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

Note: Classification for the intensity of staining as no stain (-), very weak (+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

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

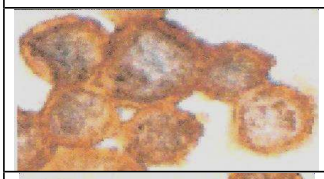
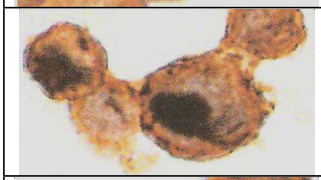
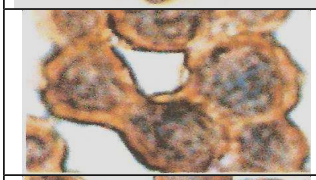
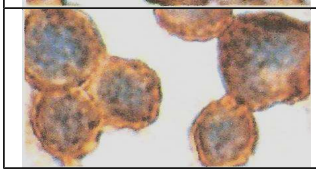
	Concentration of extracts (µg/ml)	Morphology of cells	Intensity of reddish-brown stain
	100	Intact	+
	50	Intact	+

	25	Intact	+
	10	Intact	2+
	1	Intact	2+

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

Note: Classification for the intensity of staining as no stain (-), very weak (+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

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

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

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

Note: Classification for the intensity of staining as no stain (-), very weak (+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

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

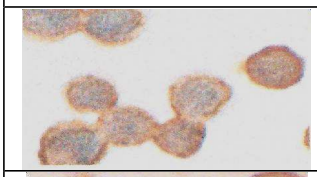
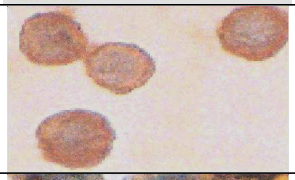
	Concentration of extracts (µg/ml)	**Morphology of cells**	**Intensity of reddish-brown stain**

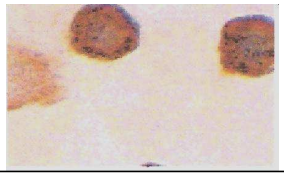
	100	Intact	2+
	50	Intact	2+
	25	Intact	2+
	10	Intact	4+
	1	Intact	4+

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

Note: Classification for the intensity of staining as no stain (-), very weak (+), weak (++), moderate (+++), strong (++++) and very strong (+++++)

Appearance of CaSki cells after treatment with methanol of *Manihot esculenta*

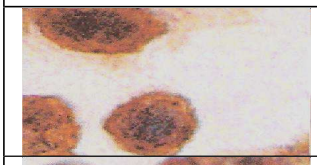
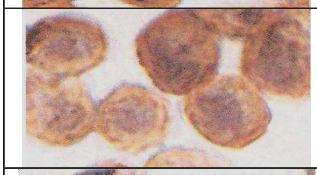
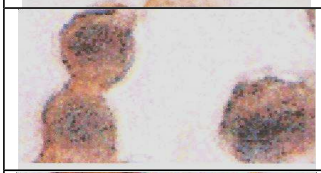
	Concentration of extracts (µg/ml)	**Morphology of cells**	**Intensity of reddish-brown stain**
	100	Intact	+
	50	Intact	+
	25	Intact	+
	10	Intact	2+
	1	Intact	2+

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

Note: Classification for the intensity of staining as no stain (-), very weak (+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

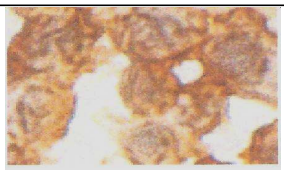
Appearance of CaSki cells after treatment with water of *Manihot esculenta*

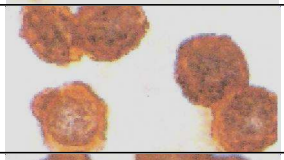
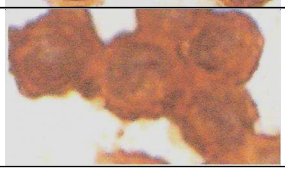
	Concentration of extracts (µg/ml)	Morphology of cells	Intensity of reddish-brown stain
	100	Intact	2+
	50	Intact	2+
	25	Intact	2+
	10	Intact	2+
	1	Intact	2+

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

Note: Classification for the intensity of staining as no stain (-), very weak (+), weak (2+, moderate (3+, strong (4+ and very strong (5+

Appearance of CaSki cells after treatment with methanol of *Pachyrizus erosus*

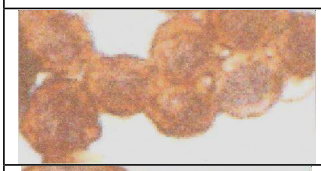
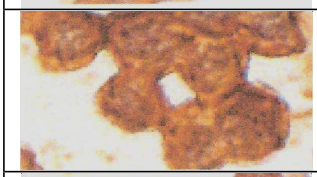
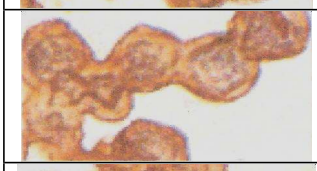
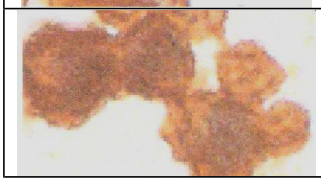
	Concentration of extracts (µg/ml)	Morphology of cells	Intensity of reddish-brown stain
	100	Lysis	2+
	50	Lysis	2+
		Majority intact	

	25	but some showing lysis	3+
	10	Intact	4+
	1	Intact	5+

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

Note: Classification for the intensity of staining as no stain (-), very weak (+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

Appearance of CaSki cells after treatment with water of *Pachyrizus erosus*

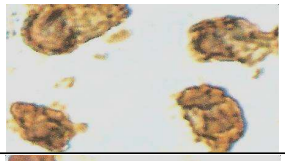
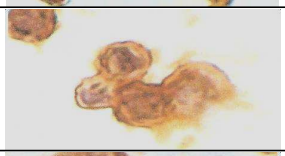
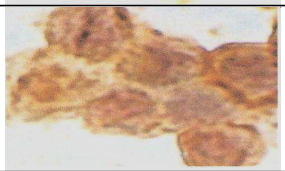
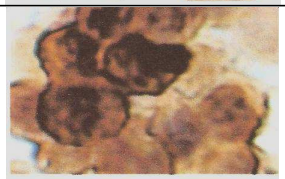
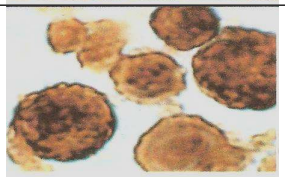
	Concentration of extracts (µg/ml)	**Morphology of cells**	**Intensity of reddish-brown stain**
	100	Intact	+
	50	Intact	+
	25	Intact	2+
	10	Intact	2+
	1	Majority intact but some showing lysis	3+

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

Note: Classification for the intensity of staining as no stain (-), very weak (+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

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

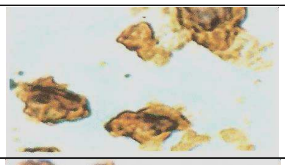
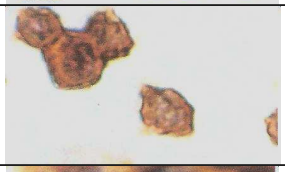
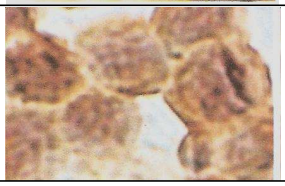
	Concentration of extracts (µg/ml)	**Morphology of cells**	**Intensity of reddish-brown stain**

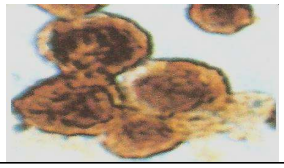
	100	Lysis	3+
	50	Majority intact but some showing lysis	3+
	25	Majority intact but some showing lysis	4+
	10	Majority intact but some showing lysis	4+
	1	Majority intact but some showing lysis	5+

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

Note: Classification for the intensity of staining as no stain (-), very weak (+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

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

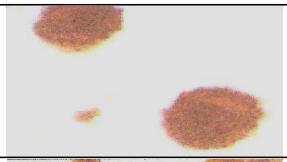
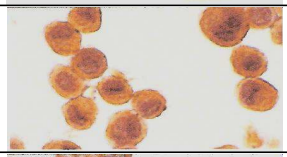
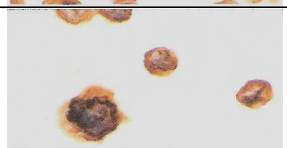
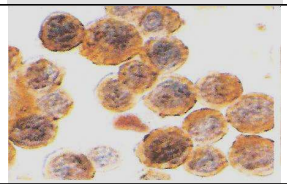
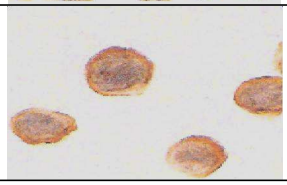
	Concentration of extracts (µg/ml)	**Morphology of cells**	**Intensity of reddish-brown stain**
	100	Lysis	3+
	50	Majority intact but some showing lysis	3+
	25	Lysis	4+
	10	Lysis	4+
	1	Majority intact but some showing lysis	5+

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

Note: Classification for the intensity of staining as no stain (-), very weak (+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

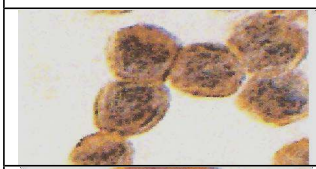
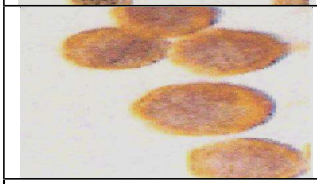
Appearance of CaSki cells after treatment with methanol of *Phaseolus vulgaris*

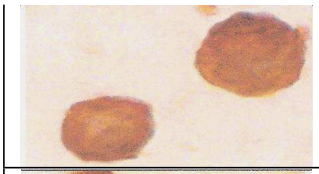
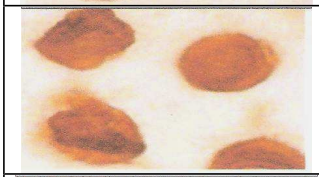
	Concentration of extracts (µg/ml)	Morphology of cells	Intensity of reddish-brown stain
	100	Intact	5+
	50	Intact	4+
	25	Intact	2+
	10	Intact	+
	1	Intact	+

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

Note: Classification for the intensity of staining as no stain (-), very weak (+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

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

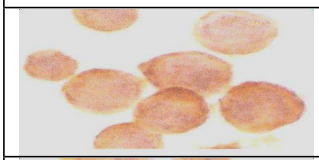
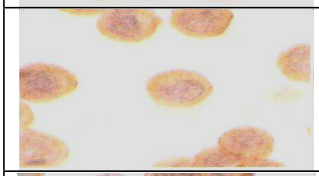
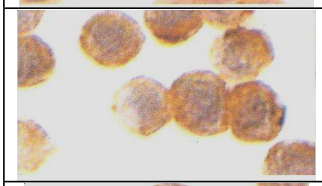
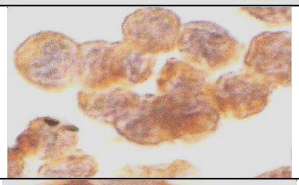
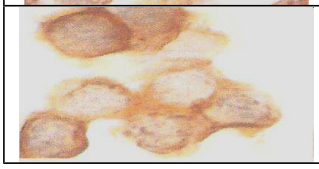
	Concentration of extracts (µg/ml)	Morphology of cells	Intensity of reddish-brown stain
	100	Lysis	+
	50	Lysis	2+

	25	Lysis	4+
	10	Lysis	5+
	1	Intact	5+

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

Note: Classification for the intensity of staining as no stain (-), very weak (+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

Appearance of CaSki cells after treatment with methanol of *Portulaca oleracea*

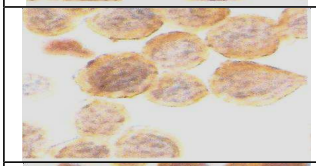
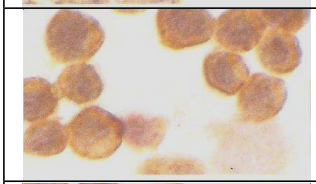
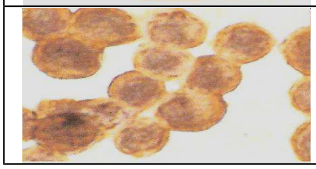
	Concentration of extracts (µg/ml)	Morphology of cells	Intensity of reddish-brown stain
	100	Intact	2+
	50	Intact	2+
	25	Intact	+
	10	Intact	+
	1	Intact	+

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

Note: Classification for the intensity of staining as no stain (-), very weak (+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

Appearance of CaSki cells after treatment with water of *Portulaca oleracea*

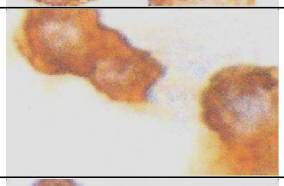
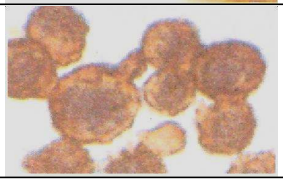
	Concentration of extracts (µg/ml)	Morphology of cells	Intensity of reddish-brown stain

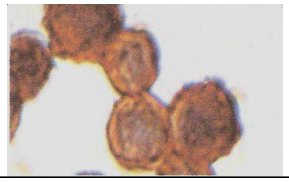
	100	Intact	+
	50	Intact	+
	25	Intact	+
	10	Intact	2+
	1	Intact	2+

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

Note: Classification for the intensity of staining as no stain (-), very weak (+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

Appearance of CaSki cells after treatment with methanol of *Sesbania grandiflora*

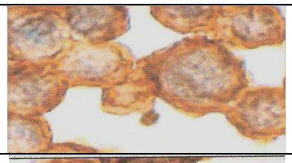
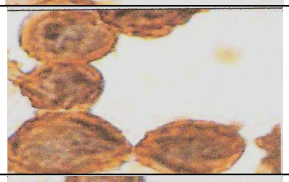
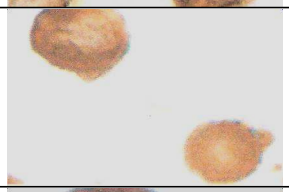
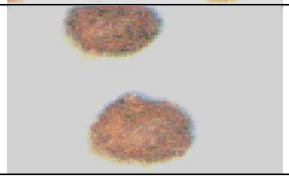
	Concentration of extracts (µg/ml)	**Morphology of cells**	**Intensity of reddish-brown stain**
	100	Intact	+
	50	Lysis	+
	25	Lysis	2+
	10	Intact	2+
	1	Intact	2+

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

Note: Classification for the intensity of staining as no stain (-), very weak (+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

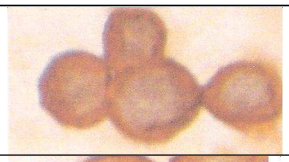
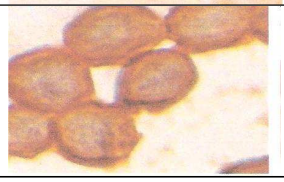
Appearance of CaSki cells after treatment with water of *Sesbania grandiflora*

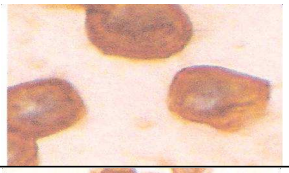
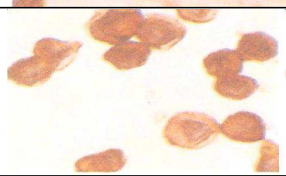
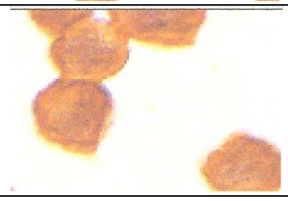
	Concentration of extracts (µg/ml)	Morphology of cells	Intensity of reddish-brown stain
	100	Intact	+
	50	Intact	+
	25	Intact	2+
	10	Intact	3+
	1	Intact	5+

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

Note: Classification for the intensity of staining as no stain (-), very weak (+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

Appearance of CaSki cells after treatment with methanol of *Solanum tuberosum*

	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+

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

Note: Classification for the intensity of staining as no stain (-), very weak (+), weak (2+), moderate (3+), strong (4+) and very strong (5+)

Appearance of CaSki cells after treatment with water of *Solanum tuberosum*

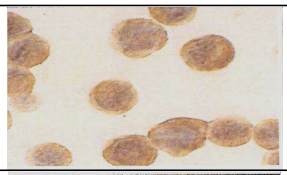
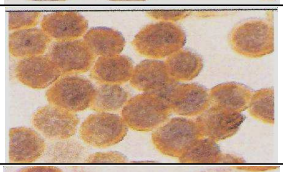
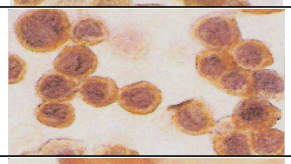
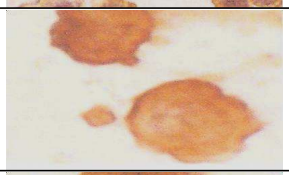
	Concentration of extracts (µg/ml)	Morphology of cells	Intensity of reddish-brown stain
	100	Intact	+
	50	Intact	+
	25	Intact	+
	10	Intact	4+
	1	Intact	4+

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

Note: Classification for the intensity of staining as no stain (-), very weak (+), weak (2+), moderate (3+), strong (4+) and very strong (5+)