APOPTOTIC EFFECTS OF α-MANGOSTIN ON CERVICAL CANCER CELL LINES

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DEPARTMENT OF PHARMACY, FACULTY OF MEDICINE,
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Abstract

α-Mangostin (AM) is a xanthone type of compound which exhibits a promising and diverse pharmacological effects. Several *in vitro* studies have shown that AM induces apoptosis and cell death in breast cancer cell lines MCF-7 and MDA-MB-231. In this study, the apoptotic and antitumor effects of AM on human cervical cancer cell lines HeLa and Ca Ski were investigated. The cytotoxic properties of AM were evaluated on HeLa (HPV18-containing) and Ca Ski (HPV16-containing) cell lines, as well as on human normal ovarian cell line (SV40), by using MTT assay.

The apoptogenic effects of AM on HeLa and Ca Ski cells were assessed using fluorescence microscopy analysis (AO/PI double staining and Hoechst dye). The effect of AM on cell proliferation was also studied through clonogenic assay. ROS production evaluation, flow cytometry (cell cycle) analysis, and multiple cytotoxicity assays were also conducted to determine the mechanism of cell apoptosis involving caspases 3/7, 8, and 9.

The cytotoxic effect of AM on cancer cells was higher than normal cells wherein it exhibited low IC_{50} values on HeLa and Ca Ski cells. Cell shrinkage, membrane blebbing, chromatin condensation, and apoptotic body formation were observed on HeLa and Ca Ski cells. Furthermore, AM induced mitochondrial apoptosis and cell cycle arrest in the G2/M phase in HeLa cells and enhanced S-phase accumulation in Ca Ski cells. The mitochondrial apoptosis was confirmed based on significant increase in the levels of caspases 3/7 and 9 in a dose-dependent manner. By contrast, unaltered caspase 8 levels in both cell lines indicated the non-involvement of an extrinsic pathway in cell death mechanism.

Moreover, cytochrome c release from the mitochondria to the cytosol and morphological changes in matrix metalloproteinase-2 (MMP) in HeLa and Ca Ski cells provided

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evidences that AM can induce apoptosis via mitochondrial-dependent pathway and cell cycle arrest. AM exerted a remarkable antitumour effect and induced characteristic apoptogenic morphological changes, which indicated the occurrence of cell death. This study reveals that AM could be a potential anticancer compound for cervical cancer.

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Abstrak

α-Mangostin (AM) adalah suatu sebatian jenis xanthone yang mempamerkan kesan farmakologi yang pelbagai dan mempunyai potensi untuk dibangunkan. Beberapa kajian *in vitro* menunjukkan bahawa AM telah mengaruh apoptosis dan kematian sel dalam titisan sel kanser payudara MCF-7 dan MDA-MB-231. Dalam kajian ini, kesan apoptotik dan antitumor oleh AM ke atas titisan sel kanser serviks manusia HeLa dan Ca Ski telah dikaji. Ciri-ciri sitotoksik AM telah dinilai ke atas sel-sel HeLa (mengandungi HPV-18) dan Ca Ski (mengandungi HPV-16), begitu juga pada titisan sel normal ovari (SV4) melalui asei MTT.

Kesan-kesan apoptogenik oleh AM ke atas sel-sel HeLa dan Ca Ski telah dinilai menggunakan analisis mikroskop pendarfluor (pewarnaan berganda AO/PI dan pencelup Hoechst). Kesan AM ke atas penggandaan sel telah juga dikaji melalui asei klonogenik. Penilaian penghasilan ROS, analisis aliran sitometri (kitar sel), dan asei gandaan sitotoksik juga telah dijalankan untuk menentukan mekanisme apoptosis sel melibatkan caspase 3/7, 8 dan 9.

Kesan sitotoksik oleh AM ke atas sel-sel kanser adalah lebih tinggi berbanding sel-sel normal dimana ia mempamerkan nilai IC_{50} yang rendah di dalam sel-sel HeLa dan Ca Ski. Pengecutan sel, benjolan membran, kondensasi kromatin, dan pembentukan jasad apoptotik telah diperhatikan di dalam sel-sel HeLa dan Ca Ski. Tambahan pula, AM merangsang apoptosis mitokondria dan penahanan kitar sel pada fasa G2/M pada sel-sel HeLa dan peningkatan penggumpulan fasa S pada sel-sel Ca Ski. Apoptosis mitolondria telah disahkan berpandukan peningkatan yang signifikan bagi aras caspase 3/7 dan 9 secara penggantungan dos. Berbeza bagi caspase 8 bagi ketindaan perubahan aras di dalam kedua-dua titisan sel menunjukkan ketidak-penglibatan tapak laluan ekstrinsik dalam mekanisme kematian sel.

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Pelepasan sitokrom c daripada mitokondria ke sitosol dan perubahan morfologi dalam matriks metalloproteinase-2 (MMP) di dalam sel-sel HeLa dan Ca Ski membuktikan pembuktian bahawa AM boleh merangsang apoptosis melalui tapak jalan pengantungan mitokondria dan penahanan kitar sel. AM mempamerkan kesan antitumor yang jelas dan merangsang perubahan apoptogenistik dan morfologi, yang menandakan berlakunya kematian sel.

Kajian ini mendedahkan bahawa AM boleh menjadi sebatian antikanser yang berpotensi bagi kanser serviks.

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List of Symbols and Abbreviations

Abbreviation	Description
AM	α-Mangostin
MTT	[3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]
Hsp70	70 kilo Dalton heat shock proteins
AO	Acridine Orange
AP-1	Activator protein 1
ATCC	American Type Culture Collection
ANOVA	Analysis of Variance
AST	Aspartate aminotransferase
Bcl-2	B-cell lymphoma 2
Bax	Bcl-2–associated X protein
BRCA1	Breast cancer susceptibility gene 1
BRCA2	Breast cancer susceptibility gene 2
Ca Ski	Human cervical cancer cell line
CO_2	Carbon dioxide
cm	Centimeter
CIN	Cervical Intraepithelial Neoplasia
JNK1/2	c-Jun N-terminal kinases1/2
cDNA	Complementary DNA
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
DNA	Deoxyribonucleic acid

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DMSO	Dimethyl sulphoxide
EDTA	Disodium ethylene diaminetetracetate
FAS, (EC 2.3.1.85)	Type I fatty-acid synthase
c-Fos	FBJ murine osteosarcoma viral (V-Fos) oncogene homolog
h	Hour
HeLa	Human cervical cancer cell line
iNOS	Inducible nitric oxide synthase
IC_{50}	Inhibitory concentration (50%)
IL4	Interleukin 4
0.05	Level of significance (Type 1 error)
LDL	Low-density lipoprotein
mRNA	Messenger ribonucleic acid
Mg	Microgram
µL	Microliter
Min	Minute
MAPK	Mitogen-activated protein kinases
RAW 264.7	Murine macrophage cell line
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
%	Percentage
PBS	Phosphate buffer saline
PI	Propidium iodide
PGE2	Prostaglandin E2

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Rpm	Revolution per minute
RNA	Ribonucleic acid
SEM	Scanning Electron Microscopy
STAT1	Signal transducer and activator of transcription 1
S.D.	Standard deviation
S.E.M.	Standard Error of the Mean
3T3-L1	A cell line derived from mouse
TNFα	Tumor necrosis factor alpha
TP53	Tumor protein p53
200X	Two hundred times

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List of Appendices

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CHAPTER 1: INTRODUCTION

1.1 Background

Cancer is a group of related diseases which is initiated by rapid division of abnormal cells beyond the normal boundaries; these cells exhibit potential to invade and/or spread to other parts of the body. Cancer is a leading cause of high mortality and morbidity rates worldwide. A total of 15.2 million new cancer cases were diagnosed and more than 8.8 million cancer deaths occurred in 2015 (Bray F, 2017). Annual incidence of cancer is predicted to increase to 22 million within the next two decades. In Malaysia, the number of newly diagnosed cancer cases increased to 41,236 in 2015 and more than 24,000 cancer-related deaths occurred according to the GLOBOCAN 2015 report (Ferlay et al., 2015).

Cervical cancer is the fourth most common cancer in women worldwide; about 530,000 new cases of cervical cancer were diagnosed annually (7.9% of female total cases), and more than 280,000 deaths were recorded (7.5% of female total cancer deaths) (Bray F, 2017). In less developed regions cervical cancer is the second most common cancer in women, with an estimated 445,000 (84% of the total cases) new cases diagnosed in 2012 (Bray F, 2017). In Malaysia, cervical cancer is the second most commonly diagnosed cancer in women, after breast cancer (Bruni et al., 2015). According to the Ministry of Health in Malaysia, 2398 new diagnosed cases and 695 cervical cancer-related deaths were reported in 2015 (Bray F, 2017).

Despite immunization and introduction of screening programs, the mortality rate of cervical cancer has not reduced to the desired level because of poor access to screening and treatment services. In addition, most of cervical cancer deaths are recorded in low-

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and middle-income countries. Viral infection, such as hepatitis B virus (HBV), hepatitis C virus (HCV), and human papillomavirus (HPV), have been identified as strong risk factors for specific cancers and are responsible for up to 25% of cancer cases in low- and middle-income countries (Plummer et al., 2016).

Cancer often takes years to develop after infection with high-risk HPV, thereby providing opportunities to detect and treat precancerous lesions before they develop into cancer. Cervical cancer is the easiest to control and effectively prevent among all malignant tumors (Nour, 2009). This cancer type can be prevented through regular screening (Bosch et al., 2012; Kane, 2012). Two screening tests, namely Papanicolaou (or Pap smear) and HPV tests are recommended for early detection and prevention of cervical cancer (Hall et al., 2018; Miller et al., 2014). Cervical cancer incidence can be reduced to about 80% through cytological screening at the population level for every 3 to 5 years (Ferlay J, 2013). Reduction in cancer incidence can only be attained if the quality of the screening process is optimal. Even with immunization against cervical cancer and introduction of screening programs, the mortality rate of this cancer has not declined to the required level (McGraw & Ferrante, 2014).

In Malaysia, preventive efforts to decrease the incidence of cervical cancer to the desired level are still low; cervical cancer was the third most common female cancer in 2007 (Shafei et al., 2014), and became the second most common female cancer in 2012 after breast cancer (Bray F, 2017).

Up to date, the treatment strategies for most cancers which have been applied for over three decades are surgery with chemotherapy and/or radiation therapy, However, most cancer patients seem to have relapsed and developed resistance to the available chemotherapy agents.

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Based on previous studies and considering the limited available therapies for cancer, natural compounds may serve as supportive agents for potent and safe cancer treatment with few side effects (Yin et al., 2013). The use of natural bioactive compounds as alternative therapeutic agents for different cancer types has been reported in many regions worldwide, mainly in Asia (Hasima et al., 2010; Itharat et al., 2004; Yin et al., 2013) .

Various tropical plants exhibit interesting biological activities for therapeutic applications. Several new biologically active compounds were found to exert a synergistic anticarcinogenic effect when used with standard drugs (Pan et al., 2012; Pedraza-Chaverri et al., 2008). Malaysia is one of the richest countries with abundant plant coverage. Plants from tropical forests are considered as key targets for screening of anticancer agents (Pan et al., 2012). Therefore, in this study, a potential bioactive compound namely α-mangostin (AM) was selected based on its previous reported biological activities including antibacterial, antifungal, antiinflammatory, antioxidant, antiviral, and cytotoxic activities (Ibrahim et al., 2016; Matsumoto et al., 2005).

AM is an effective anticarcinogen agent and has attracted considerable research attention as reported by scientific studies on the effect of this compound on many cancer types. AM showed excellent anticancer effects on breast cancer (Ibrahim et al., 2016), colorectal cancer (Nakagawa et al., 2007; Yoo et al., 2011), leukemia (J. J. Chen et al., 2014) and head and neck squamous carcinoma cells (Kaomongkolgit et al., 2011). Various studies have discussed the effect of AM on different kinds of cancer; however, to the best of our knowledge, the anticancer effect of AM on cervical cancer and its mechanism of action have not been reported, despite it being the second most common female cancer in Malaysia.

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1.2 Problem statement

Cancer treatments through radiation, surgery, and available chemotherapeutic agents do not significantly reduce the high incidence and mortality rates of many cancer types. Moreover, treatments such as radiotherapy and/or chemotherapy will result in severe side effects such as fatigue, hair loss, anemia, nausea, vomiting, appetite changes, constipation, diarrhoea, mouth, tongue, and throat ulcers, nerve and muscle problems, kidney problems, weight changes, chemo brain that affects concentration and focus, changes in sexual function and fertility problems (Monsuez et al., 2010; Savard et al., 2015; Sitzia & Huggins, 1998). Hence, new therapeutic approaches must be developed for cervical cancer.

As a justification of this study, even though a good advance in the treatment of some cancer types has been achieved, cervical cancer treatment has not significantly progressed during the last 80 years; and radiation therapy and surgery remain as the main standard treatments for this cancer. In this regard, researchers are encouraged to discover and develop new alternative treatments against cervical cancer by studying the effect of recently discovered compounds with remarkable biological activities, specifically those with proven anticancer and antiviral activities.

1.3 Aim and objectives

This study mainly aims to assess the antitumor effect of AM on cervical cancer cells. The main goal of this research will be achieved by the following objectives:

i. To study the cytotoxic effect of α-mangostin (AM) on two different cervical cancer cell lines, namely the HPV 18-containing human cervical adenocarcinoma cell (HeLa), HPV 16-containing human epidermoid carcinoma cell (Ca Ski) and on human ovarian epithelial cell (SV40).

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ii. To evaluate the effects of α-mangostin (AM) on the ability of HeLa and Ca Ski cells to form colonies.

iii. To evaluate apoptotic morphological changes in HeLa and Ca Ski cells after treatment with α-mangostin (AM) using propidium iodide and acridine orange double staining (AO/PI) and Hoechst 33258 dye.

iv. To investigate the possible apoptotic mechanism and cell death induced by α-mangostin (AM) on HeLa and Ca Ski cells.

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CHAPTER 2: LITERATURE REVIEW

2.1 General overview of cancer

Cancer is a group of diseases characterized by uncontrollable cell growth with the aptitude to invade or spread to other body parts. The spread of cancer from a particular organ of the body to another is called metastasis. Cancer is also known as a malignant tumor or neoplasm. More than 100 different types of cancer exist, and cancer type is classified based on the type of cell affected (Bray F, 2017). Lung, prostate, colorectal, and stomach cancers are the most prevalent cancer types in males, whereas breast, colorectal, cervical, and lung cancers are the most prevalent in females. Overall, the most prevalent types are breast, prostate, lung cancer, colorectal, and cervical cancers (Figure 2.1) (Ferlay et al., 2015). In 2015, the number of cancer death worldwide increases to 8.8 million, and about 70% of these cases were recorded from Africa, Asia, and Central and South America (Bray F, 2017).

Most cancer cases (90–95%) occur because of environmental factors, whereas other cases occur as a result of inheritance defect of genes (Figure 2.2A) (Kane, 2012). Environmental factors and lifestyle which increase the incidence and mortality of cancer; these factors include tobacco use, unhealthy diet, obesity, infection, exposure to pollutants, alcohol use, stress, lack of physical activity, and exposure to radiation (both ionizing and non-ionizing) (Figure 2.2C) (Kane, 2012). Annually, millions of people are at risk of developing cancers due to environmental carcinogens. These cancers include lung cancer from inhaling pollutants, such as asbestos fiber and leukemia from exposure to benzene (Kushi et al., 2012).

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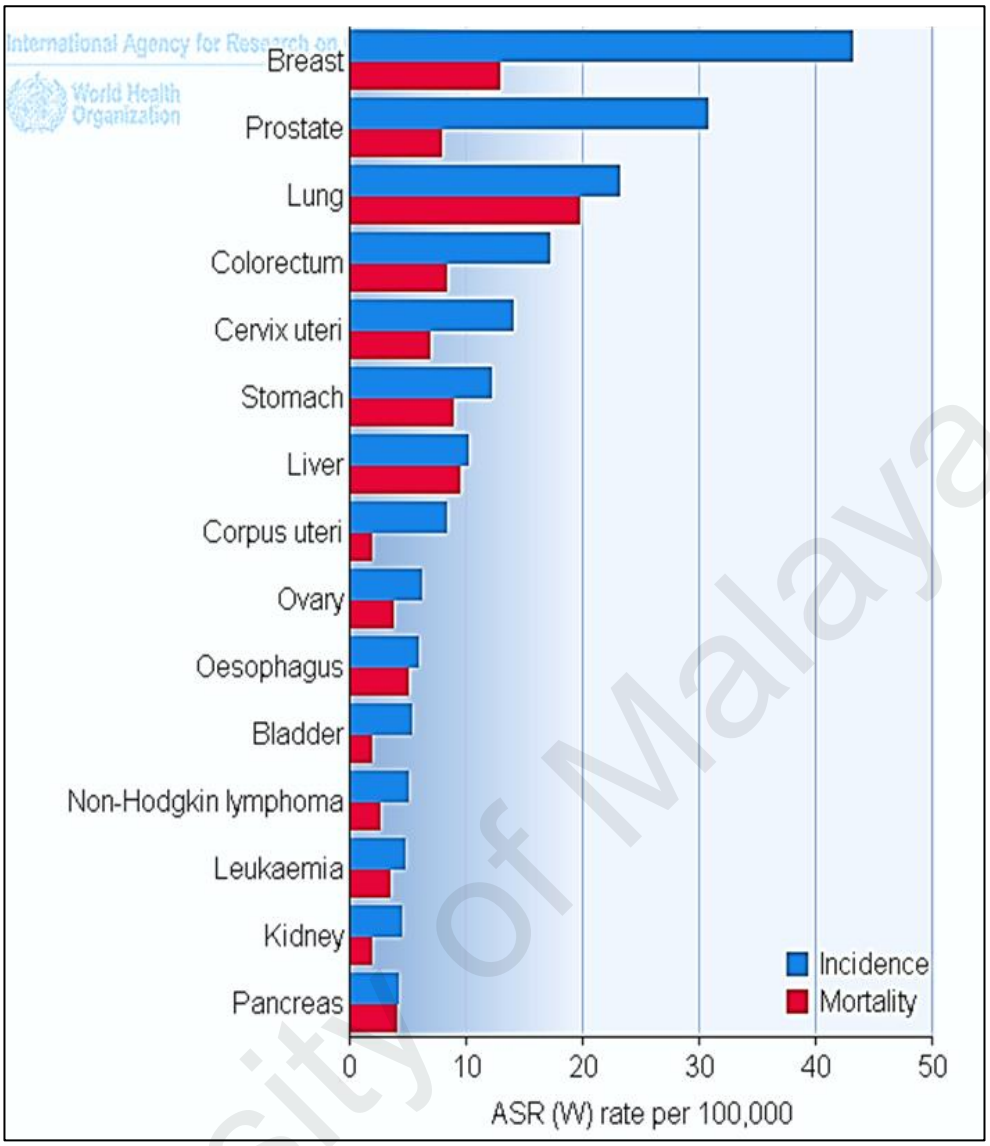

Figure 2.1: Incidence and mortality rates of different cancer types in both sexes worldwide (Ferlay et al., 2015)

Several chemical substances (carcinogens) are related to specific cancer types; for example, tobacco contains over 60 different carcinogens, including polycyclic aromatic hydrocarbons and nitrosamines.

These carcinogens in tobacco smoke are the main risk factors for most lung cancer cases (Pleasance et al., 2009), as well as for other cancer types, such as cancers of head, neck, larynx, esophagus, stomach, kidney, bladder, and pancreas (Pleasance et al., 2009).

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Food may serve as carcinogens and is associated with specific cancers. For example, high salt diet is associated with gastric cancer (Kelley & Duggan, 2003), low vegetable and fruit intake is linked to breast and lung cancers, high caloric diet increases the risk of breast cancer (Mahabir, 2013; Michels & Ekbom, 2004), and excessive alcohol intake increases the risk of colorectal and prostate cancers (Wyre & Thrasher, 2016).

Low physical activity may also increase the risk of developing cancer not only because it leads to body weight gain but it may also inhibit the functions of immune and endocrine systems and obesity. Low physical activity contribute to 30%–35% of cancer-related deaths (Figure 2.2C) (Kane, 2012).

Approximately 15%–20% of cancers are caused by bacteria, parasites, and viral infections (Kane, 2012). Viral infections are the most prevalent infection that may develop to cancer; for example, HPV is a known cause of cervical carcinoma, whereas hepatitis B and C viruses are the leading causes of hepatocellular cancer (Samaras et al., 2010).

Radiation (ionizing and non-ionizing) is another factor that initiates almost 10% of invasive cancers (Kane, 2012). Moreover, using ionizing radiation in treatment of specific cancer types could cause another cancer type (Ng & Shuryak, 2015; Patel & Jackson, 2018).

Hereditary cancers are initiated by inherited genetic defects. Less than 0.3% individuals have genetic mutation which significantly increases cancer risk and causes about 5%–10% of all cancer cases (Figure 2.2A) (Kane, 2012). knowing the history of a specific hereditary cancer can guide physicians to decide for the appropriate treatment and management strategy (Emery et al., 2001). The key steps for the application and evaluation of clinical genomics for cancer treatment are illustrated in Figure 2.3.

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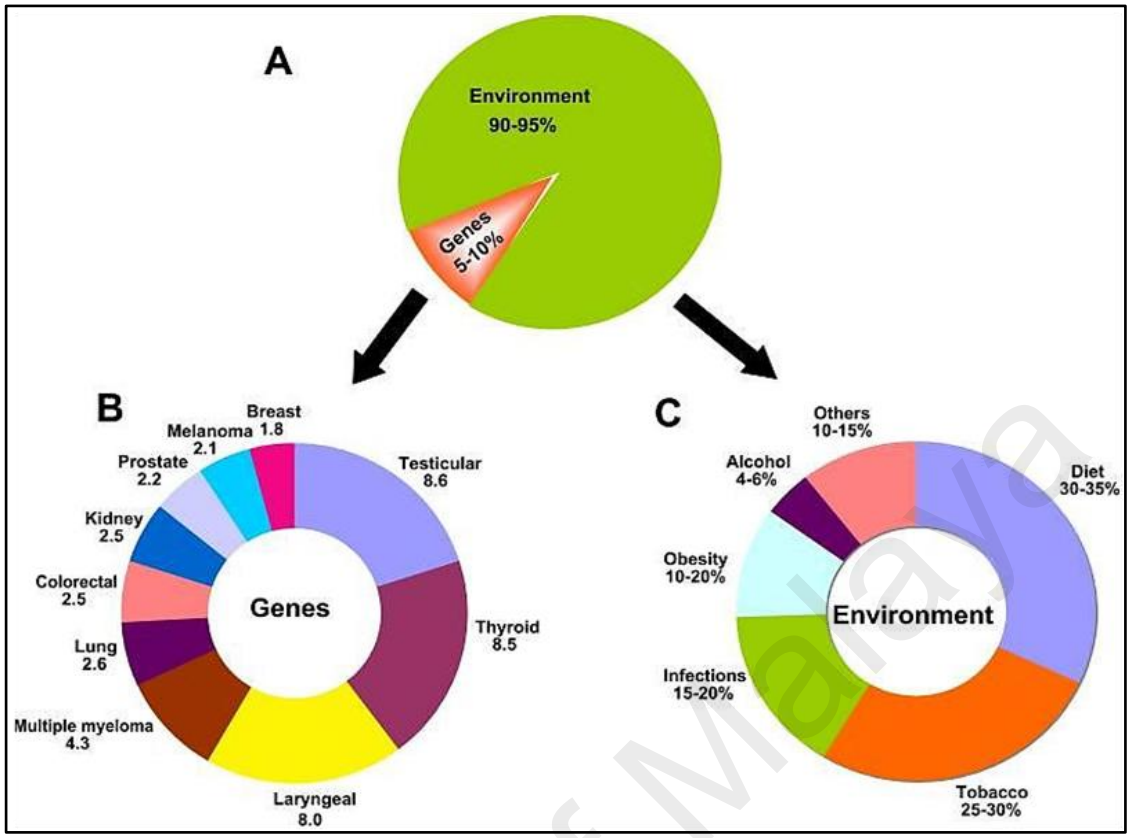

Figure 2.2: Environmental and genetic contribution in cancer initiation. (A) The contribution percentages of environmental and genetic factors to cancer. (B) Family risk ratios for certain cancers (C) The percentage of some environmental factor contribution to cancer (Kane, 2012)

Cells can undergo uncontrolled growth due to DNA mutations, which can cause cell inability to correct DNA damage by DNA repair genes or to undergo apoptosis. Gene mutations can also lead to uncontrollable cell division because of defects in proto-oncogenes and/or tumor suppressor genes; for example, BRCA1 and BRCA2 are tumor recognition suppressor genes linked to breast and ovarian cancer predisposition, respectively (Lynch et al., 2013). Mutations in the oncosuppressor gene TP53 are one of the most common genetic mutations in cervical cancer (Tornesello et al., 2013), breast cancer (Arcand et al., 2008), and other cancer types (Petitjean et al., 2007).

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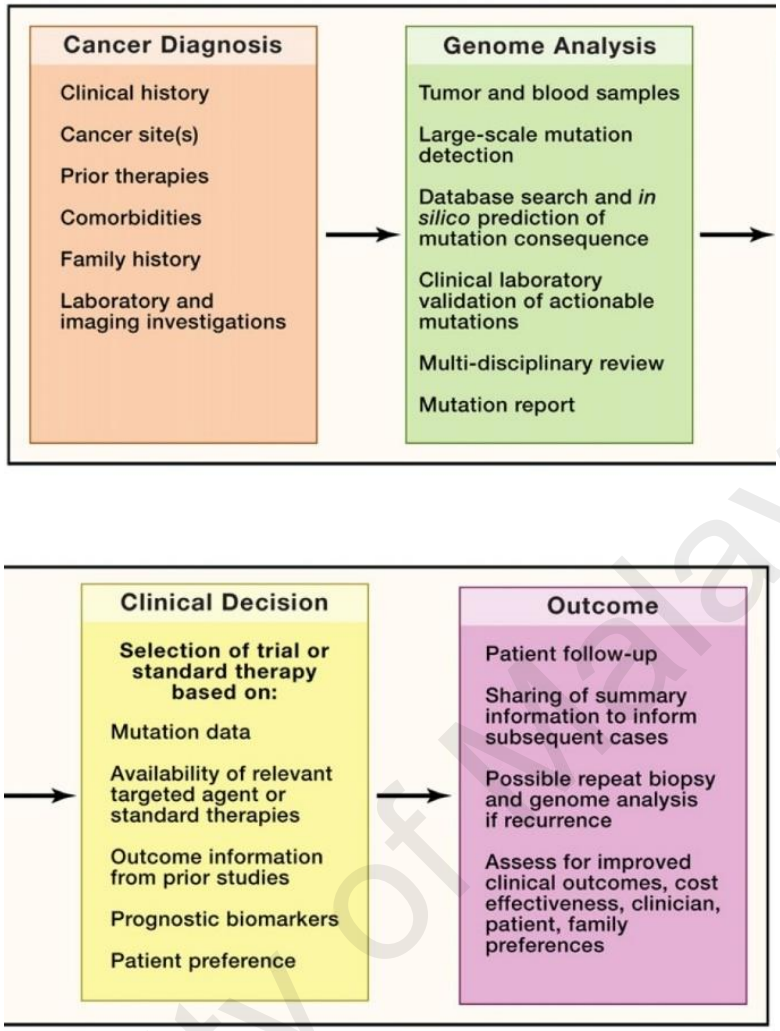

Figure 2.3: The Genetic Basis for Cancer Treatment

Hormones play a pivotal role in sex-related cancers, such as breast, ovarian, prostate, testis, and thyroid cancers, and osteosarcoma. Some hormones can enhance cell proliferation and thus increase the chance of random genetic defect accumulation, which is a main cancer stimulus. (Althuis et al., 2004; Brown & Hankinson, 2015; Grodstein et al., 1998; Nanda et al., 1999; Riman et al., 2002). For example, estrogen which is considered the main risk factor for different gynaecological cancers such as breast, ovarian and endometrial cancers (Brown & Hankinson, 2015; Häring et al., 2012; Shang, 2007).

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2.2 Apoptosis

Apoptosis or “programmed cell death” is a leading mode of cell death involving biochemical events leading to characteristic cell changes and death on scheduled time (Elmore, 2007). Programmed cell death is a common phenomenon in developing processes or in normal physiological situations to eliminate the old or damaged cells. Furthermore, apoptosis also protects the cells from immune reactions, diseases and harmful agents through their defense mechanism.

There are some characteristic morphological features of apoptotic cells such as blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation (Elmore, 2007). Determination of cell death through apoptosis or necrosis can be examined from the type and degree of some stimuli. In contrast to necrosis, which is a traumatic cell death that results from acute cellular injury, apoptosis is a highly regulated and controlled process. The differences between apoptosis and necrosis are well illustrated in Table 2.1.

Apoptosis causes cells to shrink, form blebs on the cell membrane, develop nuclear materials fragmentation and condensation and break down mitochondria leading to cytochrome c release. The fragments then packaged into apoptotic bodies and consumed by macrophages in a process called phagocytosis, the macrophages in turn release cytokines that inhibit inflammatory responses. In contrast, necrosis causes cell to swell, form vacuoles on their surface, distend or shrink rapidly and destroy the cell's chemical structures, leading to uncontrolled release of cytochrome c. Unlike apoptosis, necrotic cells are not targeted by macrophages, so the effects of the cell rupture can spread quickly and throughout the body and initiate inflammation.

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Table 2.1: Differences between Apoptosis and Necrosis (Majno & Joris, 1995)

	Types of Cell Death	
	Apoptosis	**Necrosis**
Cause	Programmed	Damage or Trauma
Stimuli (Physiological or Pathological)	Both	Pathological only
Nucleus	Fragmentation and Condensation	Nuclear Dissolution
Cell size	Reduced (shrinkage)	Enlarged (swelling)
Plasma membrane	Intact	Disrupted
Cellular contents	Packaged into apoptotic bodies and consumed by phagocytes	Enzymatic digestion, leak out into tissue
Intrinsic or Extrinsic	Both	Extrinsic only
Inflammatory Response	No	Frequent

Up to now, there are two main apoptotic pathways including the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. The extrinsic and intrinsic pathways converge on the same terminal, or completing pathway. This pathway is initiated by the cleavage of caspase-3 and results in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, cross-linking of proteins, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors and finally uptake by phagocytic cells (Elmore, 2007).

The intrinsic signaling pathway or mitochondrial pathway that trigger apoptosis comprise various arrays of non-receptor mediated stimuli which produce intracellular signals that directly act on targets within the cell and mitochondrial initiated events. In respond to numerous apoptotic stimuli, alterations occur in the inner mitochondrial membrane that results in opening of the mitochondrial permeability transition (MPT) pore, loss of the

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mitochondrial transmembrane potential that implicate outer mitochondrial membrane permeabilization and release of pro-apoptotic proteins from the intermembrane space into the cytosol (Elmore, 2007).

2.3 Cervical cancer

2.3.1 Anatomy of the cervix

The cervix, the lower part of the human female reproductive system, is a cylinder-shaped tube that connects the vagina to the uterus. The cervix has cartilaginous structure, which is covered by smooth moist tissues and has a length of 2–3 cm. Figure 2.3 shows the three main parts of the cervix.

- The ectocervix part is the vaginal portion of the cervix, which can be observed from the interior vagina; this part is lined by flat, scale-like squamous epithelium. The opening at the center of the endocervix into the vagina is called external os.

- The endocervix part is the endocervical duct, which is a channel from the external os to the uterus; this part is lined by the rectangular columnar epithelium.

- The transformation zone is the overlapping contact area between the endocervix and ectocervix, where cells are changed, and the most common part for the development of abnormal cells in the cervix, as well as the preferred position of infection, regeneration, squamous metaplasia, and neoplasia (Figure 2.4) (Abdul-Karim et al., 1982; Reid, 1983).

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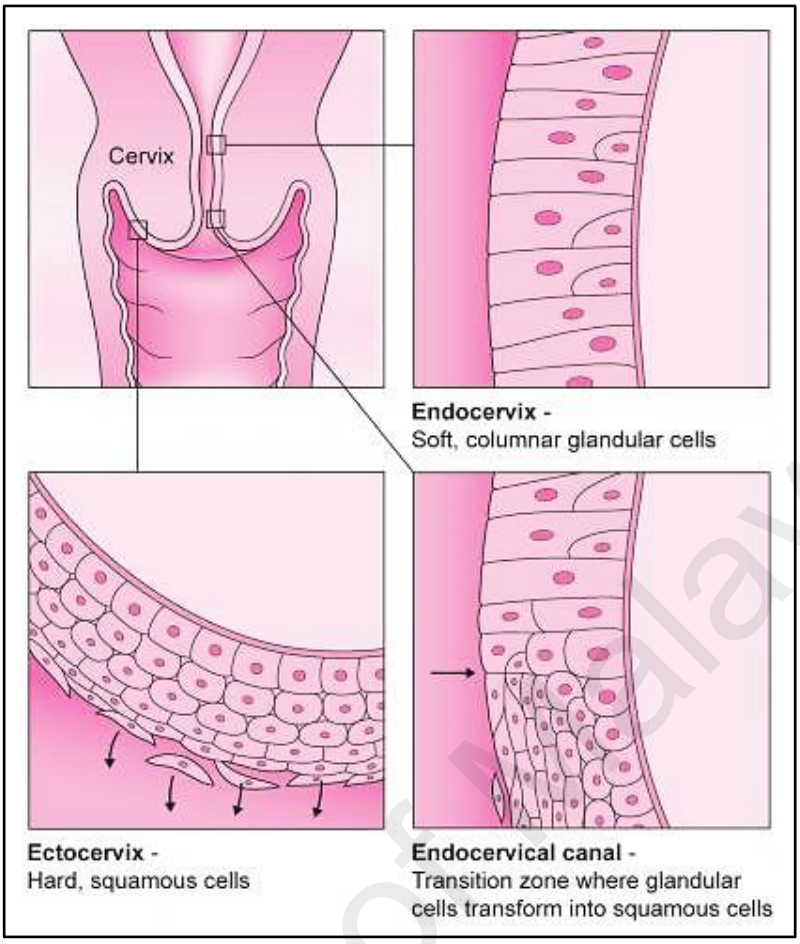

Figure 2.4: Cervix and transformation zone (Jostrust, 2015)

2.3.2 Definition and epidemiology

Cervical cancer is a malignant neoplasm that initiates mostly in the transformation zone between the cervix and uterus (Figure 2.5).

The incidence of cervical cancer increases within the age range of 30–39 years and significantly increases to the maximum at age of more than 50 years (Table 2.2) . Recent estimations mention that more than 527,600 women are diagnosed with cervical cancer each year, and over 265,600 (7.5% of total female malignancy deaths) women die because of this cancer (Figure 2.6) (Bruni et al., 2015).

In less developed countries, more than 2.6 million women aged 15 years and older have high risk to develop cervical cancer and it contributes about 11.6% of the total new cancer

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cases, and more than one tenth (230,158) of total female cancer-related deaths in 2012 (Figure 2.6).

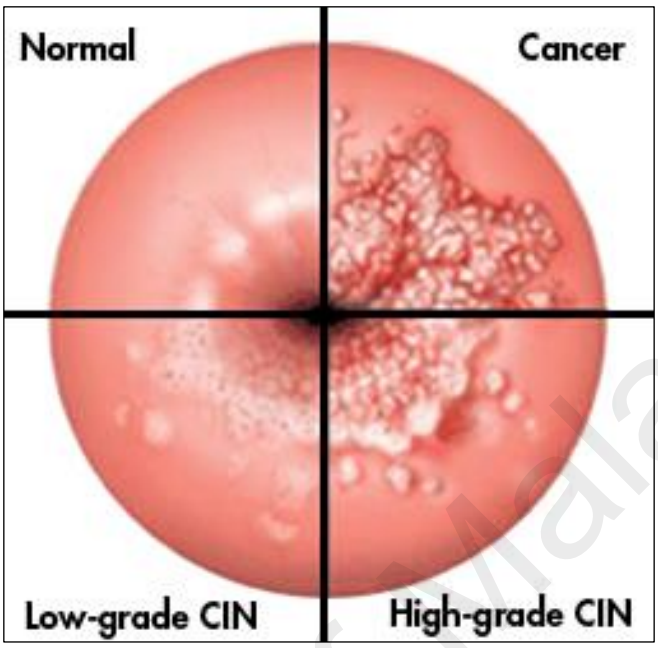

Figure 2.5: Transformation zone between the cervix and uterus, which is the area of cervical cancer progression (Islaslab, 2015)

Table 2.2: Estimated number of cervical cancer new cases in 2014, worldwide, females, all ages (Ferlay et al., 2015)

Age (years)	0-14	15-29	30-44	45-59	60^{+}
Number of cases	166	23,111	153,081	221,762	171,727

The high prevalence of cervical cancer in less developed countries could be due to the deficiency or incompetent screening programs; therefore, large variations in morbidity and mortality rates of cervical cancer are recorded among different countries according to population awareness and submitted efforts to achieve the satisfactory rates.

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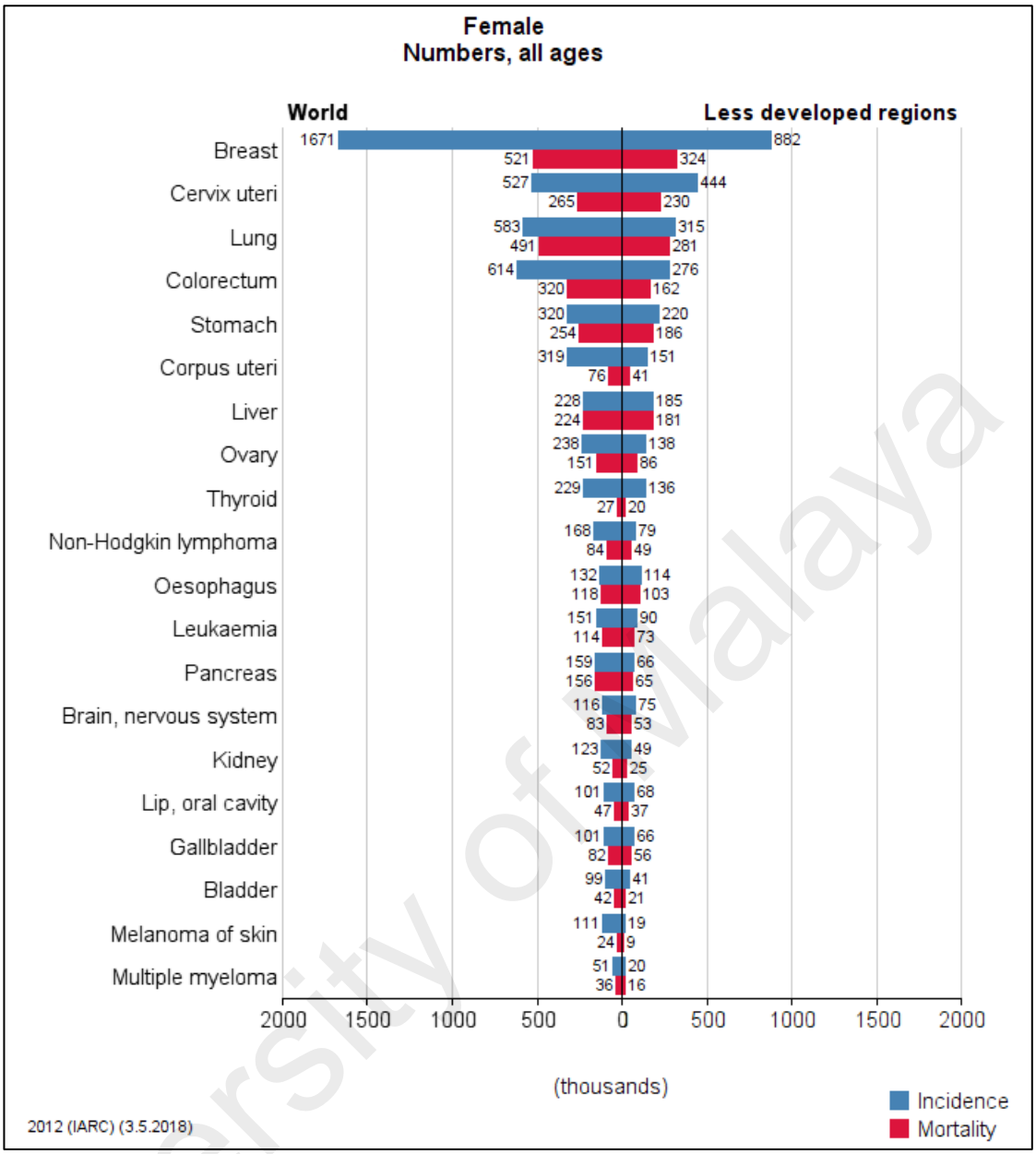

Figure 2.6: Worldwide incidence and mortality rates of cervical cancer in 2012 (Ferlay et al., 2015)

In Malaysia, despite the available cervical cancer vaccination and screening programs, the number of cancer deaths has not decreased to the desired level (Figures 2.5et al., 2010). Cervical cancer remains the second most prevalent cancer in women in Malaysia with more than 2 000 newly diagnosed cases annually (Figure 2.7); also, this cancer type is the third most prevalent cancer in women worldwide and ranks fourth in female mortality (Figure 2.7) (Ferlay et al., 2015).

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Cervical cancer is a potentially preventable cancer because of the available HPV vaccination and precancerous lesion screening. Early detection of precancerous lesions using the available screening techniques and treatment before developing to cancer can decrease the mortality rates of cervical cancer. Studies reported that the overall awareness of HPV infection, prevention, and cervical cancer in Malaysia is substandard (Aljunid et al., 2010), hence the necessity to increase women's knowledge and awareness, and promote their perspective toward HPV prevention and screening. Cervical cancer morbidity and mortality rates have significantly decreased in many countries by applying cervical cancer prevention programs, such as repeated Pap smear screening and early treatment.

2.3.3 Etiology and risk factors

HPV isolation from cervical lesions is a remarkable accomplishment in defining the etiology of cervical carcinogenesis (Della et al., 1978; Meisels & Fortin, 1977). There are more than 110 different types of HPV, 14 of these types (HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) are considered high risk for the development of cervical cancer and its precursor lesions, while other HPV types such as 26, 53, 67, 70, 73, 82 are considered to be low risk types (Table 2.3) (Alejo et al., 2018; de Sanjose et al., 2010).

Discovery of high-risk HPV types 16 and 18 isolated from cervical cancer cell lines and biopsies led to great advance in determining cervical cancer etiology (Dürst et al., 1983). The involvement of HPV infection in the two main cervical cancer types, namely, squamous cell carcinoma and adenocarcinoma, is evenly potent. Most HPV infections cannot cause cervical intraepithelial neoplasia (CIN) or cervical cancer because HPV infections are commonly cleaned and eliminated by the immune system.

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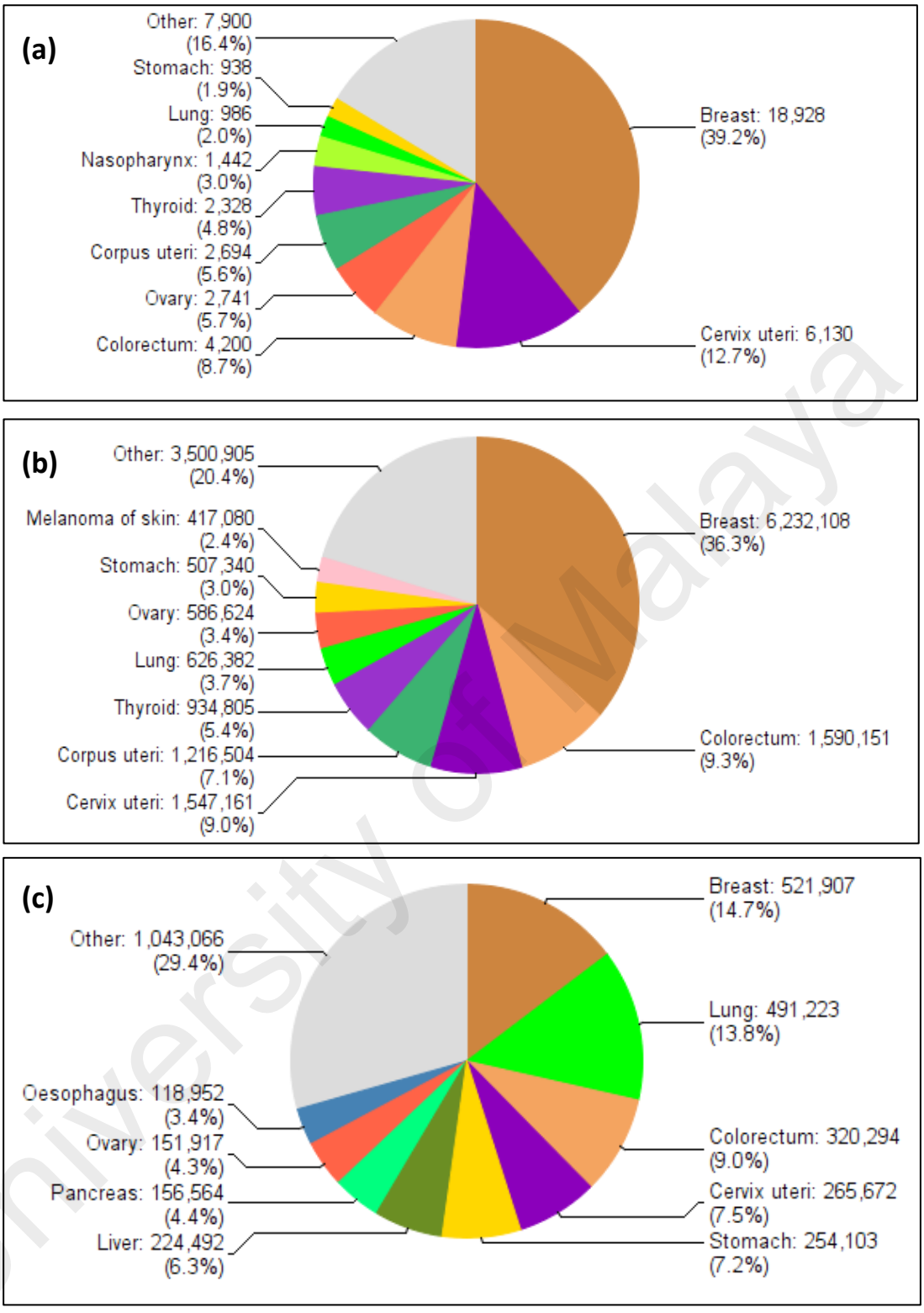

Figure 2.7: The prevalence and mortality rates of cervical cancer in 2012, (a) estimated female 5-year prevalent cancer cases in Malaysia and (b) estimated female 5-year prevalent cancer cases worldwide and (c) estimated number of female cancer deaths worldwide (Ferlay et al., 2015)

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Persistent infection with HPV-16 and -18 genotypes is the main cause of cervical cancer and are responsible for 70% of cervical cancer cases worldwide (Bosch et al., 2008). (Faridi et al., 2011). HPV-16 has been identified in almost all cervical precancerous and cancer lesions and is the leading risk factor that contributes to 50%–55% of invasive cervical cancer cases; moreover, HPV-18 is the second leading risk factor that contributes to 10%–15%, with few regional changes (Bosch et al., 2008).

HIV-infected women showed a high risk of HPV co-infection, HPV 16 and HPV 56 were the most prevalent genotypes (Badial et al., 2018). Long-term oral contraceptives contributes an important risk factor for cervical cancer (Roura et al., 2016).

Women exposed to diethylstilbestrol (DES) in utero are at increased risk for cervical cancer and its precancerous lesions (Huo et al., 2017). Furthermore tobacco smoking increases the probability of high risk HPV infection which is the main cause of cervical cancer. As well as, passive smoking among non-smoking women is associated with the risk of CIN 1 (Chatzistamatiou et al., 2018; Min et al., 2018). These risk factors will act not independently, but in combination with HPV infection for the progression of cervical cancer. Some of these co-factors are listed in Table 2.3.

Many studies recognized a group of other risk cofactors that have a role in the progression of cervical carcinogenesis; these factors include sexual behavior, such as sexual intercourse at early age and multiple sexual partners; multi-parity; low socioeconomic status; *Chlamydia trachomatis* infection (Daniel et al., 2011), herpes simplex virus type 2 (HSV-2) infection (Baldauf et al., 1995) and micronutrient deficiency such as retinol, carotene and other carotenoids (Gariglio et al., 2009; X. Zhang et al., 2012).

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Table 2.3: Most common risk factors contributing to cervical cancer initiation and progression (Castellsagué et al., 2006)

Increases risk with sufficient evidence	May increase risk with probable evidence
• Human papillomavirus (HPV) types 16,18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 • Human immunodeficiency virus (HIV) and immunosuppression • Estrogen-progestogen contraceptives • Diethylstilbestrol (DES) • Tobacco smoking	• Human papillomavirus types 26, 53, 67, 70, 73, 82 • Tetrachloroethylene

Number of full-term pregnancies are associated with cervical cancer risk. Cervical cancer risk in women who have had one full-term pregnancy is 15% higher than that in women who have had none; the risk increases in parallel with the number of full-term pregnancies and is higher in women who had a full-term pregnancy at a younger age than those at old ages. This parity is linked only with squamous cell carcinoma and has no association with adenocarcinoma (Sogukpınar et al., 2013).

2.3.4 Human Papillomavirus (HPV)

HPV is a common infection transmitted sexually. More than 110 types of HPV exist, and at least 13 oncogenic HPV types can cause cancer. Most studies focused on few types that can cause diseases, such as genital warts and cancers. HPV-16 and -18 (high-risk types) are the two most common types of HPVs and are responsible for more than 70% of invasive cervical cancer cases and its precancerous lesions (Plummer et al., 2016).

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HPV is a small, non-enveloped, epitheliotropic, double-stranded DNA virus with a genome of approximately 8000 base pairs that can infect the basal layer of stratified squamous epithelia in keratinocytes of the skin or mucous membranes, where it replicates in the nucleus. HPV infection is not cytolytic; degeneration of cells leads to the release of viral particles, which can survive even at low temperatures and without a host for several months.

Nearly all sexually active individuals (men and women) are infected with HPV at least once in their lifetime (Weinstock et al., 2004). About 70% and 90% of clinical HPV infections can clear and regress to subclinical type with no symptoms within 1 and 2 years, respectively. However, 5%–10% of subclinical female infections could develop to clinical and form benign papilloma, such as warts (Chelimo et al., 2013) or precancerous lesions of the cervix and lead to invasive cancer (Goldstein et al., 2009) or other cancers in different body organs, such as the vulva, vagina, penis, anus, and oropharynx (Stanley et al., 2012). Available information about HPV remains limited; HPV infection incidence, transmission and risk factors are generally indefinite. Many reports indicated that multiple sexual activity partners are the main transmission method. HPV transmission occurs primarily via skin-to-skin contact through sexual intercourse, including vaginal–penile, anal–penile, oral–penile, and hand–genital of the same person and sexual partners (Hernandez et al., 2008). Although oral–penile contact may explain oral HPV infection, which may progress to oral cancer; this infection is uncommon and not evidently related to oral–penile contact (Winer et al., 2003). Genital HPV infections are rarely detected in virgins, and the non-penetrative sexual contact increases the risk of genital HPV infection in virgin women (Winer et al., 2010).

In a separate study on HPV sexual transmission, approximately 1% of virgin women without sexual activity are positive for HPV, and 10% of virgin women with sexual contact are positive (Winer et al., 2003).

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Non-sexual hand contact is proven to be weakly associated with HPV transmission (Winer et al., 2010). Transmission of HPV between hands and genitals among sexual partners or even of the same person is not the main source of HPV transmission but it is considerable (Hernandez et al., 2008). HPV infection can be rarely transmitted during delivery from the mother to her baby. Perinatal transmission of HPV-6 and -11 may cause juvenile-onset recurrent respiratory papillomatosis, which is very rare and occurs in 2 of 100,000 children in the United States (Sinal & Woods, 2005).

HPV-6 and -11 are the main genotypes that cause benign papilloma, HPV-16 and -18 genotypes are the main genotypes that cause cervical cancer cases (Figure 2.8) (Chelimo et al., 2013).

In Malaysia, 11.34 million women are at risk of developing cervical cancer in 2014, and about 1.0% of them are supposed to have HPV-16/18 infection at any time; this finding indicates that HPV-16 or -18 infection cause about 88.6% of invasive cervical cancer cases (Bruni et al., 2015).

Several studies indicate the presence of HPV DNA in blood. However whether or not the virus itself is present in the blood of infected remains unknown (Cocuzza et al., 2017; Foresta et al., 2013). This condition suggests that HPV may be transmitted via blood. However, as the transmission of HPV through non-sexual means is uncommon, blood donations are not currently screened for HPV, and HPV-positive individuals are still not prohibited from donating blood (Bodaghi et al., 2005).

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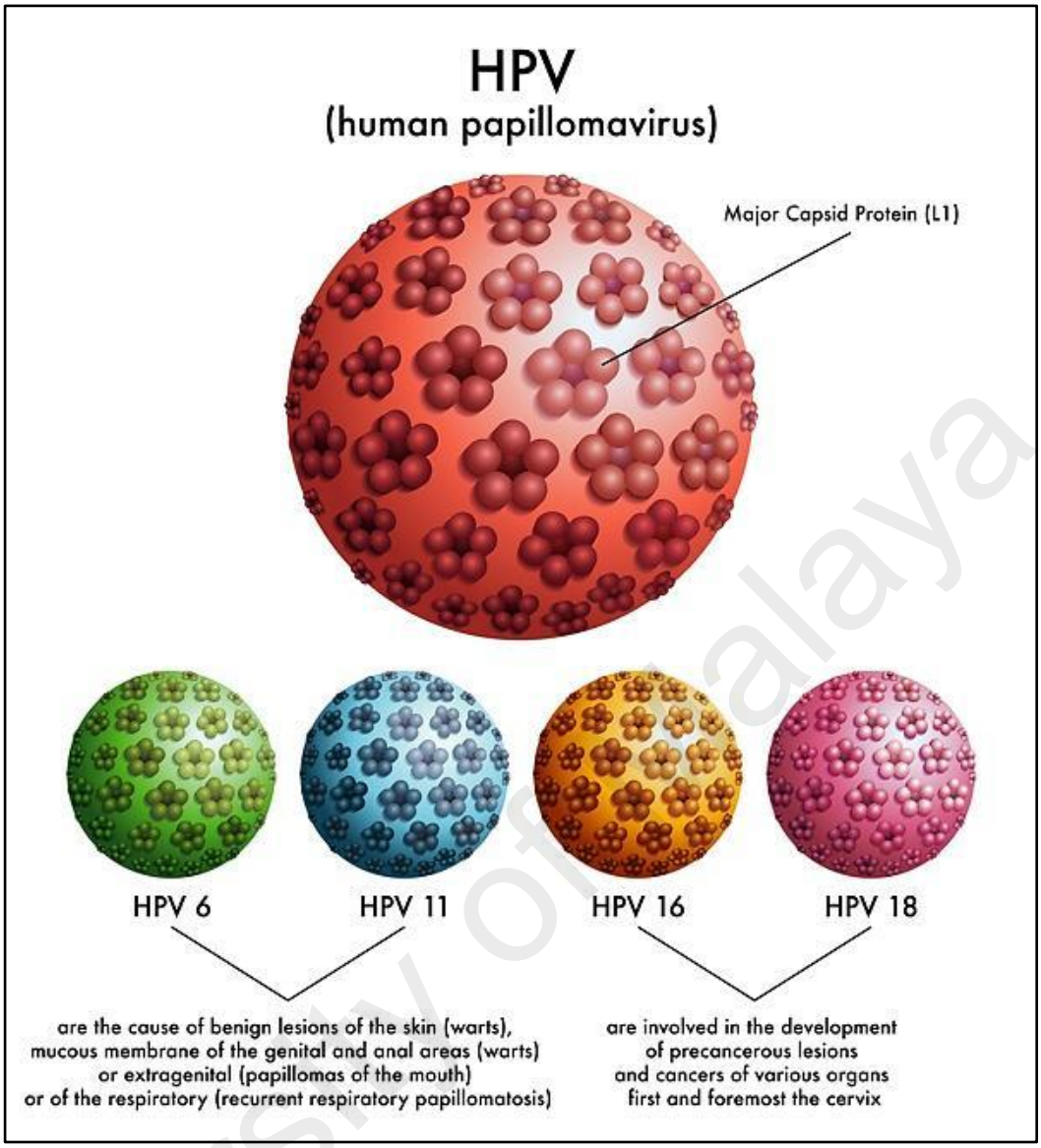

Figure 2.8: Most prevalent types of HPV (CDC, 2014)

2.3.5 Diagnosis and prevention

Cervical cancer is a potentially preventable cancer. Hence, to investigate why the morbidity and mortality rates of this easily prevented cancer are still high, specifically in less developed countries, is necessary. The three cervical cancer prevention strategies include prevention of HPV infection through HPV vaccination; detection and treatment of precancerous and pre-invasive lesions; and detection and treatment of cancer at the early stage.

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Primary prevention through vaccination against high-risk HPV oncogenes, which is the main cause of cervical carcinogenesis, is a promising tool to prevent cervical cancer. HPV vaccines are available, but they are relatively expensive. Moreover, several challenges are involved in the widespread implementation of HPV vaccines worldwide, specifically in less developed countries.

In Malaysia, the prophylactic HPV vaccine was licensed in November 2006, and HPV vaccination program was approved by the Malaysian government with three doses given freely to all 13-year-old girls on the 21st of February 2008; this dosage is the same to that recommended by the World Health Organization in National Immunization programs (Zaridah, 2014). Nevertheless, the eventual success in reducing cervical cancer incidence by HPV vaccine program in Malaysia will depend on its continuous application and affordability because no health insurance covers HPV vaccination programs.

Currently available HPV vaccines are monovalent (HPV-16), bivalent (HPV-16 and -18) (Cervarix®, GlaxoSmithKline Vaccines), and quadrivalent (HPV-6, -11, -16, and -18) (Gardasil®, Merck/Sanofi–Pasteur).

HPV vaccination could decrease the incidence rate of cervical cancer and its precancerous lesions worldwide, specifically in highly developed countries (Van Kriekinge et al., 2014). HPV vaccines are safe and provide high degree of protection against (HPV-16 and -18) infection in fully vaccinated women. However, no decrease in the incidence rate of cervical cancer has been noted up to now in Malaysia. Therefore, the overall awareness and knowledge about HPV infection, vaccination, and cervical cancer remains insufficient to encourage women to undergo vaccination program. Hence, more efforts are needed to increase women's knowledge, awareness, and attitude regarding HPV infection, vaccination, and cervical cancer.

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The most frequent method for cervical cancer screening is Pap smear, also known as conventional cytology. Visual inspection with acetic acid (VIA) and HPV DNA tests are alternatives to cytology-based screening. Both HPV DNA test and VIA are not being introduced into the Malaysian screening program (Bruni et al., 2015). Cervical cancer screening program was first implemented in 1940. In Malaysia, the Ministry of Health began conducting the screening programs of cervical cancer using Pap smear test in 1969 for early cervical cancer detection among women aged 20–65 years (Wong et al., 2009).

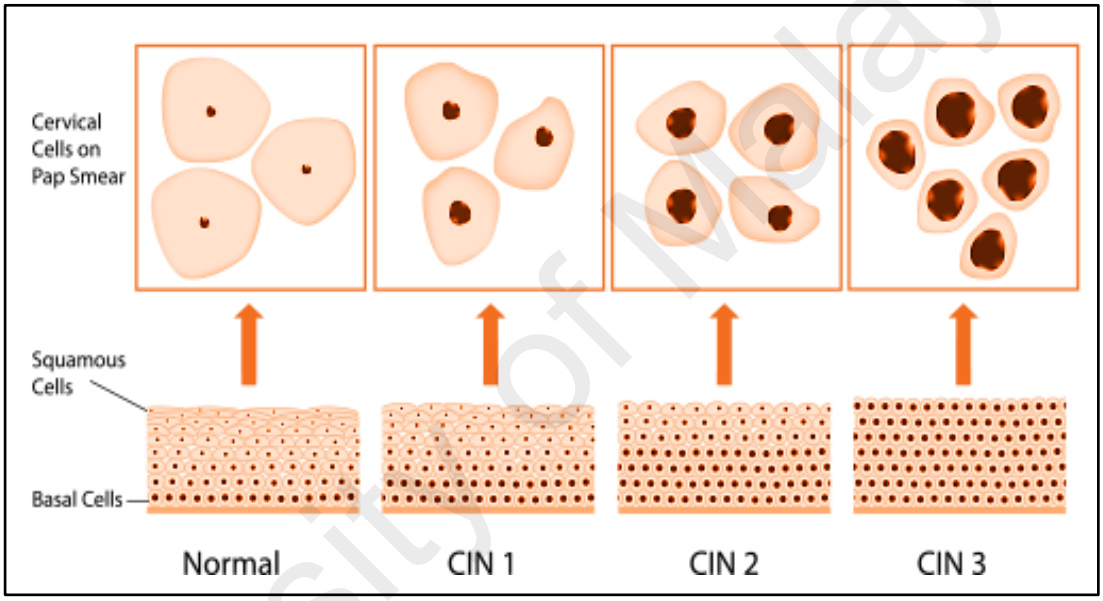

Figure 2.9: Different cervical cancer stages with Papanicolaou test (Pap smear screening) (Olaitan, 2014). Note: CIN 1: cervical intraepithelial neoplasia 1, CIN 2: cervical intraepithelial neoplasia 2, CIN 3: cervical intraepithelial neoplasia 3

Pap smear is a screening tool used to examine unusual variations in the transformation zone cells of the cervix (Figure 2.9) and aims to recognize the precancerous lesions of the cervix and thus prevent these lesions from progressing to cancer. Women's knowledge about HPV infection and other risk factors of cervical cancer is linked with the acceptance of Pap smear test. Pap smear test exhibits certain challenges, with high false negative rates (20%–30%) and incorrect sampling, where 8% of the collected specimens are incorrect (Burd, 2003). Another issue with Pap smear is the inaccurate evaluation of the

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specimen because of inconsistent cell distribution onto the microscopic slide, and contamination with yeast, bacteria, blood, and inflammatory cells. These contaminants can affect the detection of abnormal cells (Bolick & Hellman, 1998).

The most recent screening recommendations for prevention and early detection of cervical cancer are that all women aged 21-29 years should have a Pap smear screening every 3 years, and no need to be tested for HPV unless they have abnormal Pap smear result. Women aged 30-65 years should have both a Pap smear screening and a HPV test every 5 years. Women aged over 65 years and used to have a normal screening results should not be screened for cervical cancer (Saslow et al., 2012).

In several western countries such as United State, Portugal and England, where screening programs have long been established, morbidity and mortality rates of cervical cancer have been successfully reduced. Cervical cancer rates have decreased by as much as 65% over the past four decades and cervical cancer deaths have decreased by as much as 90% in these countries (Akinlotan et al., 2017; Mendes et al., 2018).

The earliest stage of cervical cancer is carcinoma *in situ* (CIS), a precancerous stage where the cancer is confined only on the surface layer of the cervix and has not penetrated into deeper layers of the cervical tissue. Cancers produced in the neck of the cervix, stage 1A (micro-invasive) or 1B (gross tumor), and have started to spread into the top of the vagina (stage 2A) are considered early cervical cancer.

Cases with cancer stromal overrun of less than 3 mm and without spreading to the lymph nodes are generally treated with simple hysterectomy (Lu & Burke, 2000; Mathevet et al., 2003). Radical hysterectomy, radiotherapy and pelvic lymphadenectomy are the most common therapies for patients with more than 3 mm cancer stromal overrun or have a risk of pelvic lymph node metastasis (Lu & Burke, 2000). In early cervical cancer, radical trachelectomy preserves the fertility of the woman, lead ing to a good rate of living

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newborns without impairing the chances of survival (Mathevet et al., 2003; Wright et al., 2007).

2.3.6 Staging and pathology report

Cervical cancer staging is typically based on guidelines produced by the International Federation of Gynecology and Obstetrics staging system. Cancer staging commonly begins from stage 0, the precancerous or non-invasive stage in which the cancer is easily cured, to stage 4, in which the cancer has extended throughout many body parts and is incurable. Cancer staging helps clinicians to design an appropriate treatment plan. Different terms, such as CIS and dysplasia, are used to define cervical precancerous lesions. Figure 2.10 shows two main systems that are used for reporting cervical diagnosis and Pap smear results. CIN system was suggested by Richart in 1973 (Richart, 1973). The CIN system has three levels, namely, mild (CIN 1), moderate (CIN 2), and severe dysplasia and CIS (CIN 3).

Bethesda System was established in 1988 and replaced the CIN system after revision in 1991 and 2001 (Burd, 2003; Solomon et al., 2002). In the Bethesda system, squamous cell abnormalities are classified into four classes: atypical squamous cells (ASC), low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL), and squamous cell carcinoma. Figure 2.10 provides the classification of the CIN and Bethesda systems.

2.3.7 Cervical cancer treatment

Treatment of cervical cancer depends on the stage of the cancer, the size and shape of the tumor, the age and general health of the women and her decision to have children in the future (Dueñas-González et al., 2005).

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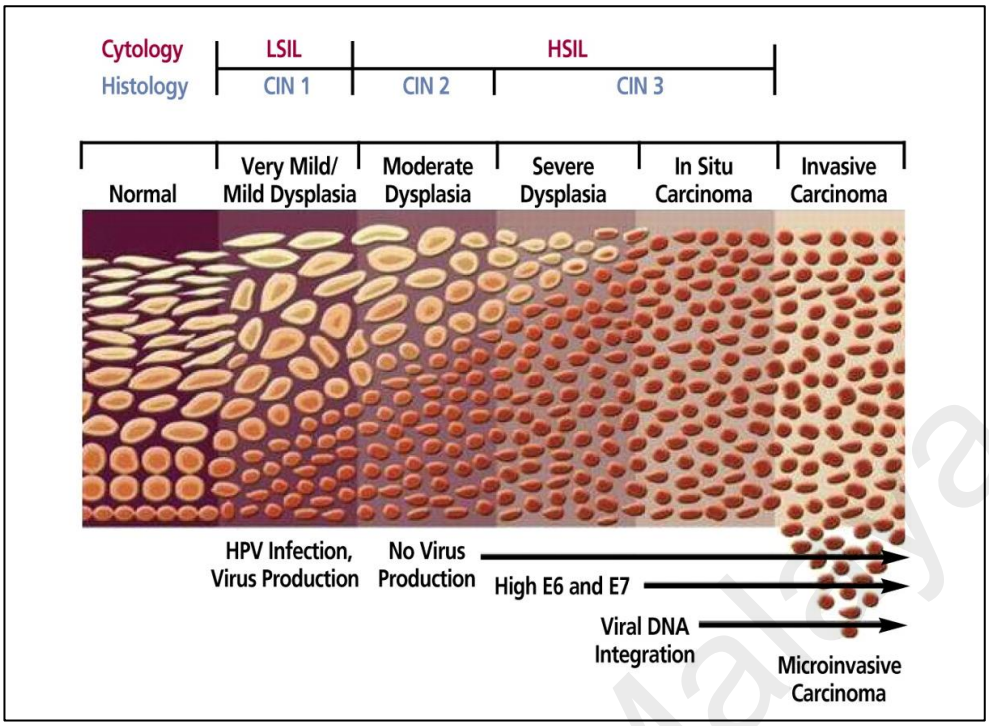

Figure 2.10: Classification of cervical precancerous lesions according to the cervical intraepithelial neoplasia and Bethesda systems (Ortoski & Kell, 2011)

As mentioned before, early cervical cancer can be treated by destroying and removing the precancerous or cancerous tissue without removing the uterus or damaging the cervix so that woman can still have children in the future (Kesic, 2006). This is achieved by hysterectomy, loop electrosurgical excision procedure (LEEP), cryotherapy and laser therapy. For more advanced cervical cancer, treatment may include radical hysterectomy, pelvic exenteration and radiation (Kesic, 2006).

Current treatments of cervical cancer are surgery, radiotherapy and chemotherapy. Some of these chemotherapy drugs include carboplatin (Mabuchi et al., 2009), 5-florouracil (Rose et al., 1999), ifosfamide (Downs et al., 2011), paclitaxel (Higgins et al., 2007), cisplatin (Petrelli et al., 2014), and cyclophosphamide (De Murua et al., 1987).

Paclitaxel is one of the microtubule inhibitors, microtubules are important cellular targets for anticancer therapy because of their key role in mitosis. Microtubule inhibitors induce apoptosis via blocking of cell cycle progression, activation of pro-apoptotic effectors Bax,

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Bad, and Apaf-1, Inactivation of the anti-apoptotic regulators Bcl-2 and BclxL, accumulation of Cytochrome C, Activation of caspase-2 and caspase-9 and increasing ROS levels (Jérôme et al., 2006; Perez, 2009). Paclitaxel has been shown to have strong activity as a single agent or as part of a combined treatment with cisplatin on both advanced (Moore et al., 2004; Papadimitriou et al., 1999) and early stage cervical cancer (Kim et al., 2006).

A combination of paclitaxel and cisplatin is effective in the treatment of recurrent and persistent cervical cancer (Moore et al., 2004). Keeping in mind that an effective and low-toxic regimen is needed in the treatment of cervical cancer, a considerable study has shown that combination of paclitaxel and carboplatin is superior to paclitaxel and cisplatin combination in the treatment of recurrent cervical cancer.This is due to the favorable toxicity profile of carboplatin over cisplatin (Singh et al., 2013), since cisplatin exhibits serious side effects like bone-marrow depression, neutropenia, thrombocytopenia and anemia due to hematological toxicity along with nephrotoxicity and neurotoxicity.

However, acquired chemo-resistance of cervical cancer cells to chemotherapeutic drugs and its severe side effects are the major reasons why chemotherapy-based treatment may fail. Therefore, exploring a novel treatment to be used alone or in combination with other chemotherapeutic drugs to reduce their resistant and side effects appears to be urgently needed. Natural products with anticancer activity

2.4 Natural products with anticancer activity

Plants have been used in cancer therapy for thousands of years. Traditional medicine plays a critical role in treatment of many chronic life-threatening conditions and diseases, including cancer (Mohan et al., 2011).

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Recent cancer treatments include chemotherapy, radiotherapy, and surgery. Chemotherapy is used in treatment of various cancer types, including breast, colorectal, cervical, lung, pancreatic, and ovarian cancers (Siegel et al., 2012). Nevertheless, the efficiency of chemotherapy is restricted by its serious side effects, relapse and developed resistance (Sitzia & Huggins, 1998). Consequently, herbal therapies are used as supportive agents to satisfy the need for a potent and safe cancer treatment. Investigation of cancer treatments from plants started in the middle of the last century with the discovery of vinca alkaloids, the development of its derivatives vinblastine and vincristine and the isolation of the cytotoxic podophyllotoxins (Cragg & Newman, 2005; Gordaliza, 2007). Vinblastine is a vinca alkaloid isolated from *Catharanthus roseus* which inhibits the assembly of microtubules by binding to tubulin. It is indicated for non-Hodgkin's lymphoma, Hodgkin's disease, breast, testicular, lung and neck and head cancers (Bennouna et al., 2005).

Epipodophyllotoxin extracted from root of *Podophyllum peltatu*m, is a non-intercalating dual inhibitor of both topoisomerases I and II and has antitumor activity against Ll210 leukemia (Stähelin & von Wartburg, 1991). Docetaxel and paclitaxel are current chemotherapeutic drugs extracted from clippings of *Taxus brevifolia* which are used effectively in cancers of the breast, lung, ovary and cervix (Cragg & Newman, 2005; De Furia, 1997; Rose et al., 1999). Paclitaxel stabilizes microtubules and interferes with the normal breakdown of microtubules during cell division.

There are several potential anticancer agents derived from natural products in the clinical improvement phase, based on selective activity against cancer-related molecular targets, including flavopiridol and combretastin A4 phosphate (Gordaliza, 2007). Chalcones are prominent secondary metabolites and precursors of flavonoids and isoflavonoids in plants, they are reported to exhibit various pharmacological activities including anticancer (Cabrera et al., 2007; Nowakowska, 2007; Sharma et al., 2010).

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Camptothecins are a quinolone based cytotoxic alkaloids isolated from the bark and stem of *Camptotheca acuminate* and show significant anticancer activity through inhibition of topoisomerase I (Mishra & Tiwari, 2011). Topotecan and irinotecan are the only camptothecins approved for clinical use as anticancer drugs during the last two decades (Garcia-Carbonero & Supko, 2002). Topotecan a synthetic analog of camptothecin is approved as a second-line treatment for metastatic ovarian cancer, lung cancer and recurrent cervical carcinoma in combination with cisplatin, as well as irinotecan is indicated as first-line therapy against advanced colorectal cancer when combined with fluoropyrimidines, particularly if 5-fluorouracil–based chemotherapy failed (Grossman et al., 2008).

Xanthones are one of the potential classes of natural compounds which possess a chemopreventive and therapeutic effect and can effectively inhibit tumor initiation and progression. These compounds showed induction of apoptosis and cell cycle arrest in human colon cancer DLD-1 cells and enhance the inhibitory action of 4-hydroxytamoxifen growth in estrogen receptor-positive breast cancer cell lines (Paiva et al., 2012). The biological activities of xanthones are associated with their tricyclic scaffold but vary according to the type and/or position of the varied substituents (Wong et al., 2013).

2.5 α-Mangostin

α-Mangostin (AM) is a yellow powder with a xanthone core structure (Figure 2.11) and is one of the major secondary metabolite of xanthones. This compound exhibits a wide spectrum of biological activities (Chairungsrilerd et al., 1996; Devi Sampath & Vijayaraghavan, 2007; Ibrahim et al., 2015; Sidahmed et al., 2013). As an antitumor agent, AM has been reported to induce apoptosis in different types of cancer cells *in vitro* and *in vivo* (Ibrahim et al., 2016; Ibrahim et al., 2015) .

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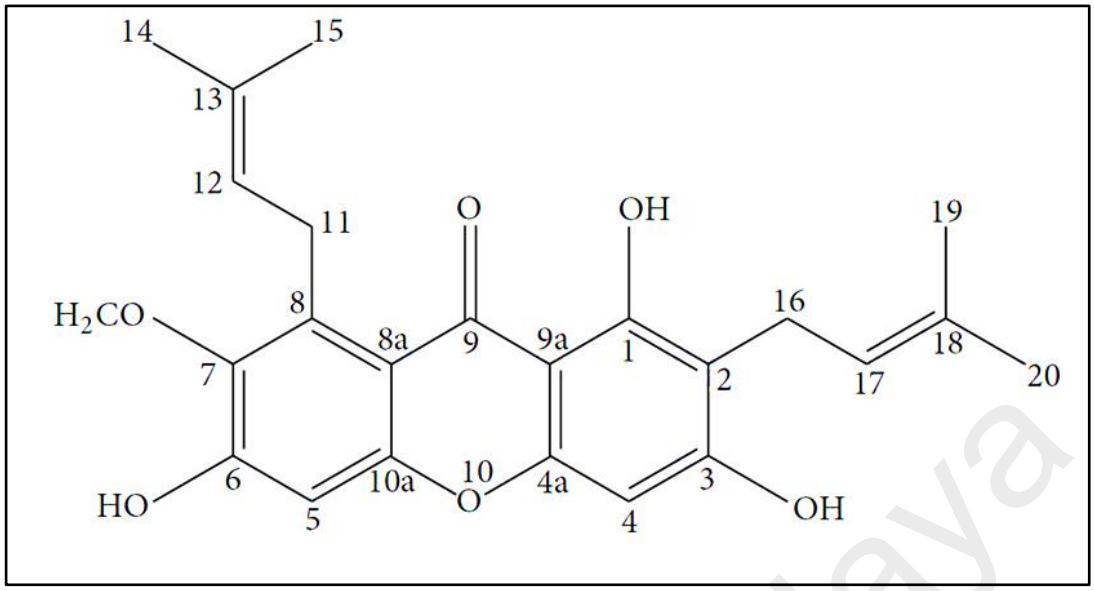

Figure 2.11: Chemical structure of α-Mangostin (AM) isolated from *C. arborescens* (Sidahmed et al., 2013)

In this study, AM was isolated from *Cratoxylum arborescens* stem bark (Sidahmed et al., 2013). *Cratoxylum arborescens* (Blume) is a well-known Asian herbal medicine that belongs to the Guttiferae family. This plant is widely distributed in South Burma, Malaysia, Thailand, Myanmar, Philippines, Sri Lanka, and India. The bark, roots, and leaves of this plant are used in folk medicine to treat fever, cough, diarrhea, itchiness, ulcer, and abdominal complaints.

The main phytochemical compounds found in *C. arborescens* are oxygenated and prenylated xanthones, such as AM, which have remarkable pharmacological activities (Ibrahim et al., 2015).

2.5.1 Anticancer effects of α-mangostin

The anticancer effects of AM have been reported in a number of studies. Matsumoto et al. (2003) explored the inhibitory effects of AM and other xanthones isolated from the pericarps of mangosteen, *Garcinia mangostana* Linn on leukemia cell lines HL60, K562, NB4, and U937. AM shows the best results and inhibits the cell growth of these cell lines

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and the study has provided some insights to AM effect in developing apoptosis in leukemia cell lines. However, this study is still far from being sufficient and further parameters need to be tested to clarify the apoptotic effect of AM.

Matsumoto et al. (2005) investigated the antiproliferative effects of AM, β-mangostin, γ-mangostin, and methoxy-β-mangostin on human colon cancer DLD-1 cells. These xanthones, except methoxy-β-mangostin, strongly inhibited DLD-1 cell growth. The antitumor effect of AM was evaluated by Hoechst 33342 nuclear staining and nucleosomal DNA gel electrophoresis. In addition, AM induces G1 cell cycle arrest, which is associated with apoptosis.

Suksamrarn et al. (2006) evaluated the anticancer effect of AM and other compounds on breast cancer (BC-1) cells. AM was found to be the most potent followed by garcinone E and γ-. In addition, AM exhibits a potent cytotoxic effect on oral epidermoid carcinoma (KB) cells as compared with that of the standard drug ellipticine. This research evaluated the cytotoxic effect of different compounds isolated from the young fruit (7-week maturity stage) of *Garcinia mangostana* on different human cancer cells, however this study did not provide any estimation about how the previous isolated compounds exert their cytotoxic effect.

Hung et al. (2009) examined the antimetastatic effects of α-mangostin on human prostate carcinoma cell line PC-3. This study provides some insights to account for the role of AM in inhibiting migration and invasion of carcinoma cells. AM reduced PC-3 cell metastasis by reducing the expression of the following enzymes: matrix metalloproteinase-2, matrix metalloproteinase-9, and urokinase-plasminogen activator in a concentration-dependent manner. The expression of these enzymes were reduced by suppressing the JNK1/2 signaling pathway and inhibiting NF-κB and AP-1 binding activities.

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AM has also been reported to induce apoptosis in human breast cancer MCF-7 cells by regulating of NF-κB, Bax/Bcl-2 and heat shock protein 70 (HSP 70) with the contribution of caspases. Ingestion of AM significantly reduced tumor size in an animal model of breast cancer (Ibrahim et al., 2016). AM has shown significant inhibitory effects on human mammary cancer cells MDA-MB-231, leading to cell cycle arrest and programmed cell death through both the extrinsic and intrinsic apoptosis pathways with involvement of the NF-κB and HSP70 signaling pathways (Ibrahim et al., 2014).

2.5.2 Other pharmacological effects of α-mangostin

2.5.2.1 Anti-inflammatory and analgesic properties

AM exhibited anti-inflammatory activity, whereby it has been shown to significantly inhibit lipopolysaccharide-stimulated NO and PGE2 production and iNOS expression in mouse leukemic monocyte macrophage cell line (RAW 264.7 cells) (L.-G. Chen et al., 2008). Chairungsrilerd et al. (1996) reported that AM has the ability to induce histamine-prompted contractions of isolated rabbit thoracic aorta. Therefore, AM can be considered a histaminergic receptor-blocking agent. Cui et al. (2010) indicated that AM, as an analgesic, retains potent peripheral and central analgesic and antinociceptive effects on mice.

2.5.2.2 Anti-oxidant properties

Mahabusarakam et al. (2000) demonstrated the ability of AM to terminate the reduction in α-tocopherol consumption induced by LDL oxidation; furthermore, structural modifications of AM adjust its anti-oxidant activity. Buelna–Chontal et al. (2011) reported that AM induces a protective effect in post-ischemic heart by preventing oxidative stress secondary to reperfusion injury.

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2.5.2.3 Anti-obesity properties

Quan et al. (2012) suggested AM as a promising agent for preventing and treating obesity. the study showed that AM has *in vitro* cytotoxicity against 3T3-L1 cells as well as inhibiting fatty acid synthase (FAS, EC 2.3.1.85), AM induced the apoptosis of 3T3-L1 preadipocytes by inhibiting FAS, increasing cell membrane permeability, mitochondrial membrane potential (Dwm) loss and nuclear chromatin condensation. This was associated with AM ability to suppress intracellular lipid accumulation in differentiating adipocytes and stimulated lipolysis in mature adipocytes.

2.5.2.4 Anti-microbial properties

Kaomongkolgit et al. (2009) considered AM as a potential agent for the treatment of oral candidiasis because of its low toxicity and with a more effective antifungal activity than clotrimazole and nystatin. The study showed that AM exerts rapid antifungal activity against *Candida albicans* within 20 min. While clotrimazole and nystatin showed slow antifungal action withen 30 min. It was revealed that AM at 4,000 µg/mL was not toxic to human gingival fibroblast for 480 min. These findings make AM a good candidate for further development as antifungal agent.

In the study by Sakagami et al. (2005), AM exhibited antibacterial activity against vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA). The study showed synergistic effect of AM and gentamicin against VRE, and AM and vancomycin hydrochloride against MRSA. Furthermore, the study showed partial synergistic effect between AM and commercially available antibiotics such as ampicillin and minocycline.

Chen et al. (1996) showed an antiviral activity of AM against HIV-1 protease. The study demonstrated the effective inhibition of HIV-1 protease by the ethanolic extract. AM

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exhibited an IC_{50} value of 5.12 ± 0.41 µM comparing with Pepstatin A as a positive control with an IC_{50} value of 76 ± 5.5 nM.

Keiser et al. (2012) demonstrated low activity of AM against *Ancylostoma ceylanicum* adults *in vitro*, and satisfactory effects against trematodes: *Schistosoma mansoni*, *Echinostoma caproni*, and *Fasciola hepatica in vitro*.

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CHAPTER 3: METHODOLOGY

3.1 Chemicals and reagents

RPMI 1640 media, fetal bovine serum (FBS), trypsin-EDTA, Phosphate buffered saline (PBS), dimethyl sulfoxide (DMSO), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT), ribonuclease (RNase), acridine orange (AO) and propidium iodide (PI) were acquired from Sigma Chemical Co., St. Louis, MO, USA. AM was provided by my supervisor, Dr. Najihah Mohd Hashim from the Department of Pharmacy, Faculty of Medicine, University of Malaya, Malaysia. The compound was isolated from *Cratoxylum arborescens*. The chemical structure and purity of AM were confirmed using nuclear magnetic resonance spectroscopy (NMR), high performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC/MS) (Appendix A).

3.2 Cell lines

Human cervical adenocarcinoma cell (HeLa) and human epidermoid carcinoma cell (Ca Ski), and human normal ovarian cell line (SV40) were obtained from the American Type Culture Collection (ATCC). Cells were maintained in culture media which is prepared by adding 50 mL (10%) FBS and 5 mL (1%) of 100 U penicillin / 0.1 mg/ mL streptomycin to 500 mL of RPMI media. The cells were then incubated at 37 °C with 5% CO_2 saturation (Thermo Scientific Forma Steri Cycle CO_2 Incubator, TC 115). The cells were detached using trypsin-EDTA solution. Cells were used in the linear phase of growth with a passage number of 20 or less.

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3.3 Sample preparation

Stock solution (10 mg/mL) of the compound AM was dissolved in DMSO (100%) and then 100 µg/mL in 1% DMSO solution was prepared by taking 10 µL from the stock solution and topped up to 1 mL with the prepared media (RPMI 1640 medium complemented with 10% FBS and 1% penicillin / streptomycin).

3.4 Viability assay

The cytotoxic effect of AM on cervical cancer cells (HeLa and Ca Ski) and normal ovarian cells (SV40) were evaluated by MTT assay as described by Mosmann, (1983) and Vega-Avila & Pugsley, (2011). The cells were seeded at 1×10^4 cells per well in a 96-well plate and incubated with 5% CO_2 saturation at 37 °C overnight. The medium was then replaced, and the cells were treated with increasing doses of AM by serial dilution starting with 100 µg/mL till 0.78 µg/mL. Upon completion of the treatment period of 24, 48, and 72h, 20 µL of MTT (0.5 mg/mL) solution was added in the dark because of the light sensitivity properties of the solution. The cells were then incubated for 2–4h with 5% CO_2 at 37 °C. The solution was aspirated, and 100 mL of DMSO was added per well to dissolve the formazan crystals formed.

The MTT assay results were obtained using an ELISA microplate reader (Tecan Group Ltd., Mannedorf, Switzerland) at 570 nm. The MTT assay results depend on the formation of purple formazan crystals from the reduction of tetrazolium salt by mitochondrial dehydrogenase enzymes in metabolically active cells, which indicates mitochondrial activity. Since for most cell populations the mitochondrial activity is correlated to the number of viable cells.

This experiment was conducted to determine the half-maximal inhibitory concentration (IC_{50}) values (the concentration of substance required to inhibit/kill 50% of the cell

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population) of AM against HeLa, Ca Ski and SV40 cells. All experiments were performed in triplicates. Paclitaxel was used as a positive control because of its potent cytotoxic effect on cervical cancer cells. The relative cell viability (%) was expressed as a percentage relative to the untreated control cells. Viability percentages of cells were determined by equation 1 (Mosmann, 1983; Vega-Avila & Pugsley, 2011)

Equation 1:

Cell Viability (%) = $(A_{sample} - A_b / A_c - A_b) \times 100$

Where, A_{sample} = absorbance reading of sample

A_b = absorbance reading of blank

A c = absorbance reading of control

3.5 Proliferation activity using clonogenic assay

The antiproliferative effect of AM on cervical cancer cells (HeLa and Ca Ski) was evaluated by clonogenic assay as described by Zhang et al. (2012). This assay based on the ability of a single cell to grow into a colony, the colony has to consist of at least 50 cells. The assay tests the ability of every cell in the population to undergo unlimited division. Only a fraction of seeded cells retains the capacity to produce colonies after treatment. A serial dilution was conducted to identify the optimal number of cells to harvest, starting with (1000 cells/plate) till (100 cells/plate). HeLa (400 cells/plate) and Ca Ski (200 cells/plate) cells were plated in 25 mL flasks and incubated with 5% CO_2 saturation at 37 °C overnight. Untreated HeLa and Ca Ski cells were used as control. The incubated cells were then treated with three doses of AM (IC_{50}, $IC_{50} \pm 5$ µg/mL) at 24, 48 and 72h incubation period to choose the optimal AM concentration and the proper time which cause obvious and gradual decrease in colonies number and size. Hence, HeLa cells were treated with series of concentrations 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20 µg/mL for 24, 48 and 72h, while Ca Ski cells were treated with concentrations starting from 12.5,

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15, 17.5, 20, 22.5, 25, 27.5 to 30 μg/mL for 24, 48 and 72h and later three doses were chosen to complete the assay (IC_{50}, $IC_{50} \pm 5$ μg/mL) for 24h. HeLa cells were treated with (5, 10, and 15 μg/mL), Ca Ski cells were treated with (15, 20, and 25 μg/mL) of AM for 24h based on the IC_{50} values obtained from the antiproliferative assay. The cells were then washed by 5 mL PBS, and fresh medium was added. After 14 days of incubation, surviving colonies were first fixed with 4% paraformaldehyde for 15 minutes and then stained with 0.5% w/v crystal violet (Coomassie Blue Stain) and incubate at room temperature for 2h, then the excess crystal violet were washed with dH_2O and flasks were left to dry. After staining, colonies containing more than 50 cells were counted. Plating efficiency (PE) was evaluated, and the fraction of surviving cells at a given treatment was calculated.

Plating Efficiency (PE) = (No. of colonies counted/No. of cells plated) × 100

Survival Fraction = (PE of treated sample/PE of control) × 100

3.6 Assessment of apoptotic morphological changes in cells using propidium iodide and acridine orange double staining (AO/PI)

The morphological changes in apoptotic HeLa and Ca Ski cells were evaluated using AO/PI double staining and visualized by fluorescence microscopy (Leica with Q-Floro Software, Leica Microsystems) as described by Nordin et al. (2015) and Syam et al. (2014). Cells were seeded at a concentration of 5×10^5 cells/mL in a 25 mL culture flask, incubated at 5% CO_2 saturation, 37 °C overnight, and then treated with the IC_{50} concentrations of AM at 24, 48 and 72h. HeLa cells were treated with 10 μg/mL, Ca Ski cells were treated with 20 μg/mL of AM. The flasks were incubated for 24, 48, and 72h.

After the treatment, the cells were centrifuged at 1000 rpm for 10 min (Eppendorf 5417C Centrifuge), and the supernatant was discarded. The cells were then washed twice with

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PBS (2-8 °C). Briefly, 10 µL each of two fluorescent dyes, namely, acridine orange (AO) and propidium iodide (PI), were mixed at equal volumes with 20 µL of cell sample in a dark environment. The cells were then resuspended and 20 µL of freshly stained sample were then applied onto glass slides and covered using cover slips. The slides were visualized within 30 min before the fluorescence intensity weakened.

3.7 Assessment of apoptotic morphological changes in cells using Hoechst 33258

The morphological analysis of HeLa and Ca Ski cell apoptosis was confirmed by nuclear staining with Hoechst 33258 dye as described by Li et al. (2007) and Niu et al. (2002). The cells were seeded at a concentration of 2×10^5 in a 25 mL culture flask and incubated with 5% CO_2 saturation at 37 °C overnight to allow attachment. The cells were then treated with IC_{50} concentration of AM for 24h, HeLa cells were then treated with 10 µg/mL of AM, Ca Ski cells were treated with 20 µg/mL of AM and incubated with 5% CO_2 saturation at 37 °C for 24, 48, and 72h. After removal of the culture medium, the cells were fixed with ethanol 70% (v/v) for 10 min. The cells were then washed twice with PBS and stained with Hoechst 33258 dye (1 µg/mL) for 15 min in an incubator with 5% CO_2 at 37 °C. The samples were evaluated by fluorescence microscopy. Apoptotic cells were identified according to the characteristic nuclear morphological changes in the cells, including reduction in volume and chromatin condensation.

3.8 Cell cycle analysis

Cell cycle kinetics was evaluated by flow cytometry analysis using DNA labeling with propidium iodide (PI) by using a previously described method with minor modifications (Nicoletti et al., 1991). HeLa and Ca Ski cells were seeded at a concentration of 2×10^5 cells/mL in a 25 mL culture flask, incubated overnight with 5% CO_2 saturation at 37 °C. HeLa cells were then treated with 10 µg/mL of AM, Ca Ski cells were treated with 20 µg/mL of AM and incubated with 5% CO_2 saturation at 37 °C for 24, 48, and 72h. After

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the treatment, the cells were centrifuged at 1000 rpm for 10 min. The supernatant was removed, and the pellet was washed with warm PBS (37 °C) twice to discard the remaining media. To preserve the cell structure, the cells were fixed by mixing with 700 µL of 90% pre-cooled ethanol overnight at 4 °C. The cells were then centrifuged at 250x*g* for 10 min, and ethanol was completely discarded. After washing with PBS, the cell pellets were resuspended in PBS (700 µL) and RNase (25 µL, 10 mg/mL). The PI dye (50 µL, 1 mg/mL) was added, and the cells were retained for 30 min at 37 °C. DNA contents of the cells were then analyzed by flow cytometry.

3.9 Caspase activity assays

To evaluate caspases levels in HeLa and Ca Ski cells and illustrate their role in apoptosis process, the activities of caspases 3/7, 8, and 9 were determined by colorimetric-based assays (Caspase 3/7 assay, R&D Systems Kit; Caspase 8, R&D Systems Kit; and Caspase 9 assay, R&D Systems Kit) in a concentration-dependent study as described by Salim et al. (2013). HeLa and Ca Ski cells were seeded at 6×10^6 per well and incubated for 24h at 37 °C. The cells were treated with IC_{50} concentrations of AM for 24, 48, and 72h and collected by centrifugation at 250x*g* for 10 min. The supernatant was eliminated, and 25 µL of cold protein lysis buffer was added per 1×10^6 cells.

The cell lysate was incubated in ice for 10 min and centrifuged at 10000x*g* for 1 min. The supernatant was retained, and about 50 µL of the supernatant (protein) was transferred into each well of 96-well plates. Each well was then added with 50 µL of reaction buffer and 5 µL of caspase and incubated with 5% CO_2 saturation at 37 °C for 1–2 h. The reaction was read using a luminescence reader at a wavelength of 405 nm.

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3.10 DCFH-DA cellular Reactive Oxygen Species (ROS) detection assay

Intracellular ROS production levels in HeLa and Ca Ski cells were determined using 2′, 7′-dichlorofluorescin diacetate (DCFH-DA) assay as described by Mohan et al. (2012) and Salim et al. (2013). Approximately 5×10^3 cells per well were seeded in a 96-well black plate and incubated with 5% CO_2 saturation at 37 °C overnight. The cells were then treated with gradual increasing concentrations of AM (IC_{50}, $IC_{50} \pm 5$ µg/mL) for 24h, HeLa cells were treated with (5, 10, and 15 µg/mL), Ca Ski cells were treated with (15, 20, and 25 µg/mL) of AM for 24h based on the IC_{50} values obtained from the antiproliferative assay.

A working solution with a concentration of 20 µM was prepared by diluting 10 mM DCFH-DA methanol stock solution by 500-fold in Hank's balanced salt solution (HBSS) without other additives. After 2h of the treatment, the cells were washed twice with HBSS, 5 mL of HBSS were added and suspended with cells, cells were then centrifuged, collected and incubated in 100 µL of the working solution for 30 min at 37 °C. Degree of fluorescence was then read using the fluorescence microplate reader (TECAN Infinite®200 PRO) at 485 nm excitation and 520 nm emission.

3.11 Multiple cytotoxicity assay

Multiple cytotoxicity assays were conducted using the Cellomics® Multiparameter Cytotoxicity 3 Kit (Thermo Scientific, Pittsburgh, PA, USA) as described by Mohan et al. (2012). Six different independent parameters of apoptosis were evaluated at the same time, namely cell number, nuclear size, morphological changes, cell permeability, mitochondrial membrane potential, and cytochrome c release. The cells were seeded in a 96-well microplate at a concentration of 5×10^3 cells per well. The cells were then treated with AM, HeLa cells were treated with 10 µg/mL, Ca Ski cells were treated with 20 µg/mL of AM, and incubated for 24, 48, and 72h at 37 °C with 5% CO_2 saturation.

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Concisely, several solutions were added in each well containing 50 μL of live cell staining, 100 μL of fixation solution, 100 μL of 1x permeabilization buffer, and 100 μL of 1x blocking buffer for an incubation duration of 30, 20, 10, and 15 minutes, respectively. 50 μL of two antibodies solutions (primary and secondary antibody) were added. The assay was conducted following procedure provided in appendix B. The plate was read, and the results were evaluated on an Array Scan HCS reader. The experiment was done in triplicates.

3.12 Statistical analysis

Each experiment was performed at least three times. Results were expressed as the mean values ± standard deviation (SD). Statistical analysis was performed with Microsoft Excel and one-way analysis of variance (ANOVA) with Tukey Multiple Comparison Test to compare all columns vs. control using Graph Pad Prism software (version 4.0; Graph Pad Software Inc., San Diego, CA). Statistical significance is expressed as $*p < 0.05$ or lower.

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3.13 Flowchart of the experimental design

The main steps of the research methodology are briefly illustrated in the following flowchart.

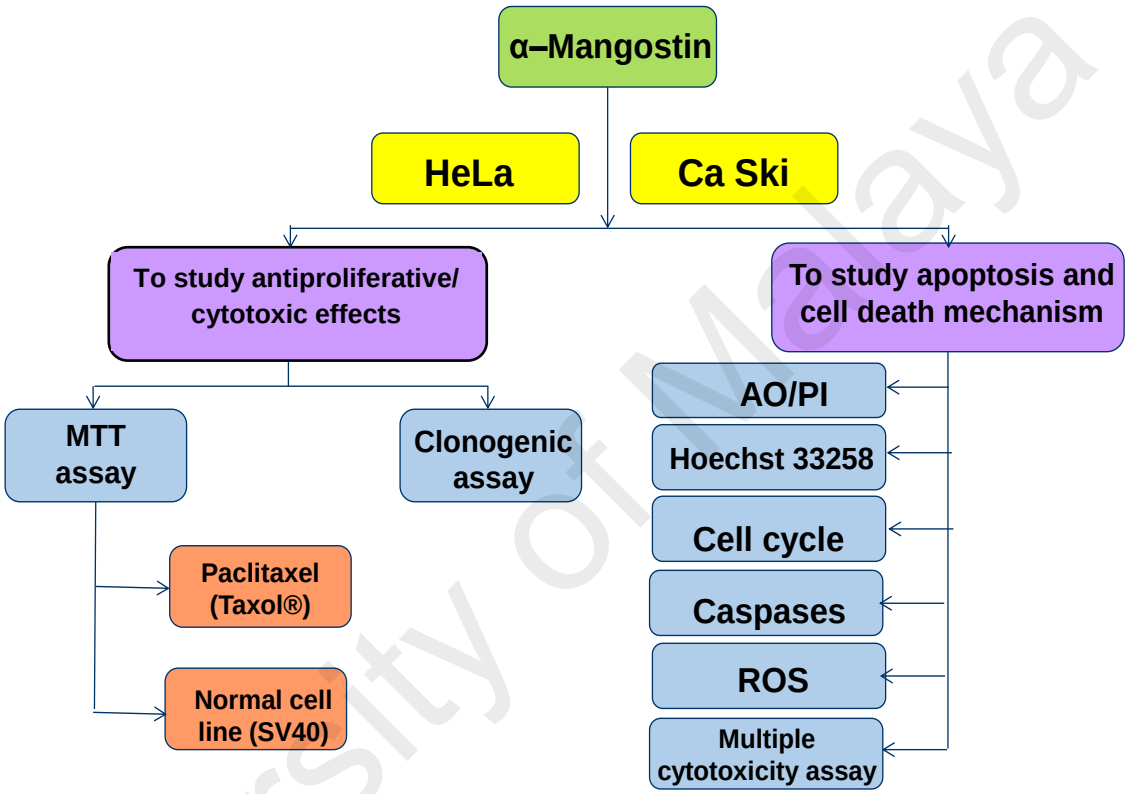

Figure 3.1: Flowchart of the different assays applied on HeLa and Ca Ski cells after treatment with α-mangostin (AM)

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CHAPTER 4: RESULTS

4.1 Cytotoxicity activity of α-mangostin (AM) on HeLa cells

The cytotoxicity potential of AM was determined by using MTT assay. The cell lines used in this study were human cervical adenocarcinoma cell HeLa (HPV-18), human epidermoid carcinoma cell Ca Ski (HPV-16) and normal human epithelial ovarian cell SV40.

The MTT assay results indicated that AM treatment markedly decreased the cell viability of the treated HeLa and Ca Ski cells compared with that of cells unexposed to AM. The IC_{50} values of AM for HeLa and Ca Ski cells at the durations of 24, 48, and 72h are shown in Figure 4.1 and Table 4.2. The IC_{50} values of AM for HeLa and Ca Ski cells were 10.07 ± 0.61 μg/mL and 21.09 ± 0.11 μg/mL, respectively, after 24h (Table 4.1). HeLa cells exhibited higher sensitivity to AM and obtained lower IC_{50} values than Ca Ski cells (Figure 4.1).

The viability percentage of HeLa and Ca Ski cells exposed to AM decreased significantly ($p < 0.05$) from 10.07 ± 0.61 μg/mL and 21.09 ± 0.11 μg/mL after 24h to 5.82 ± 0.89 μg/mL and 15.80 ± 0.59 μg/mL after 48h, respectively, but was not significantly different between 48h and 72h where the IC_{50} values of HeLa and Ca Ski cells were 5.25 ± 0.57 μg/mL and 12.64 ± 0.09 μg/mL after 72h, respectively, (Table 4.2).

AM exerted lower toxicity on SV40 compared to HeLa and Ca Ski cells and showed an IC_{50} value of 38.28 ± 1.61 μg/mL as shown in Table 4.1, AM exhibited a considerable low IC_{50} on HeLa and Ca Ski cells compared with that of cells exposed to paclitaxel.

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The IC_{50} values after 24h of paclitaxel treatment on HeLa and Ca Ski cells were 3.26 ± 0.21 μg/mL and 22.12 ± 1.56 μg/mL, respectively, (Table 4.1).

AM exerted lower toxicity on SV40 compared with that exerted by Paclitaxel (Table 4.1). The IC_{50} values after 24h of paclitaxel treatment on SV40 cells were 28.26 ± 1.23 μg/mL. These findings demonstrated that AM reduced the viability of cervical cancer cell lines in a time and dose dependent manner.

Table 4.1: The IC_{50} values of α-mangostin (AM) and paclitaxel against selected cell lines after 24h of treatment.

Treatment	**IC_{50} (μg/mL)** ± SD		
	HeLa	**Ca Ski**	**SV40**
AM	10.07 ± 0.61	21.09 ± 0.11	38.28 ± 1.61
Paclitaxel	3.26 ± 0.21	22.12 ± 1.56	28.26 ± 1.23

Table 4.2: The IC_{50} values of α-mangostin (AM) against selected cell lines after 24, 48 and 72h of treatment.

Cell Line	**IC_{50} (μg/mL)** ± SD		
	24h	**48h**	**72h**
HeLa	10.07 ± 0.61	5.82 ± 0.89	5.25 ± 0.57
Ca Ski	21.09 ± 0.11	15.80 ± 0.59	12.64 ± 0.09

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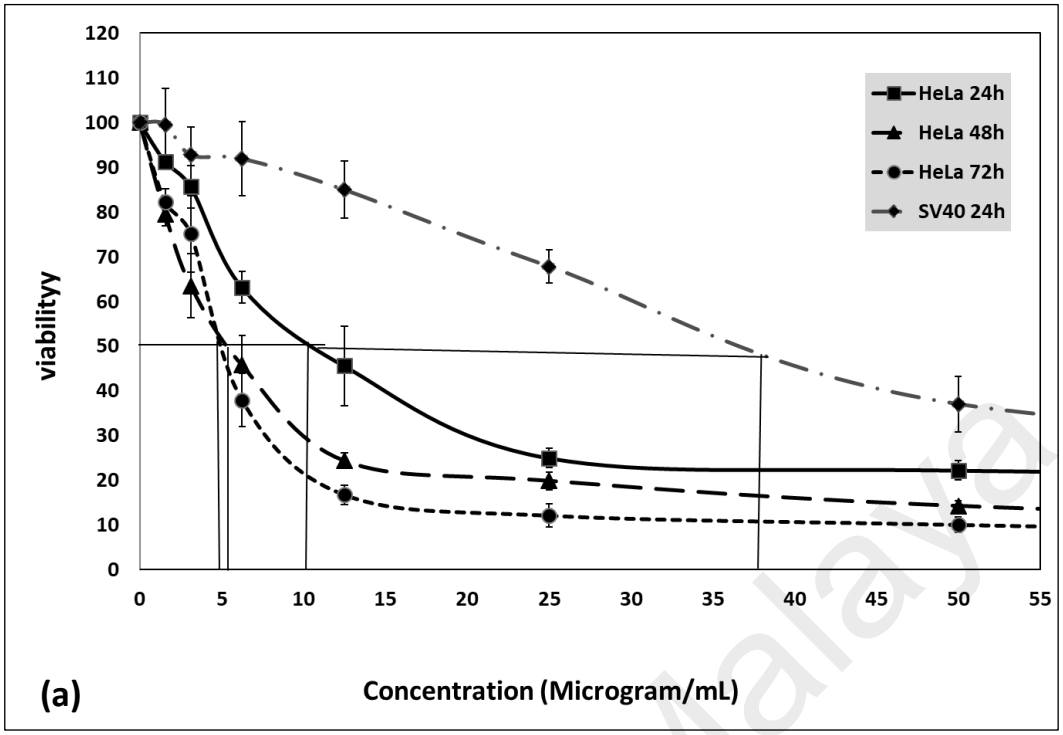

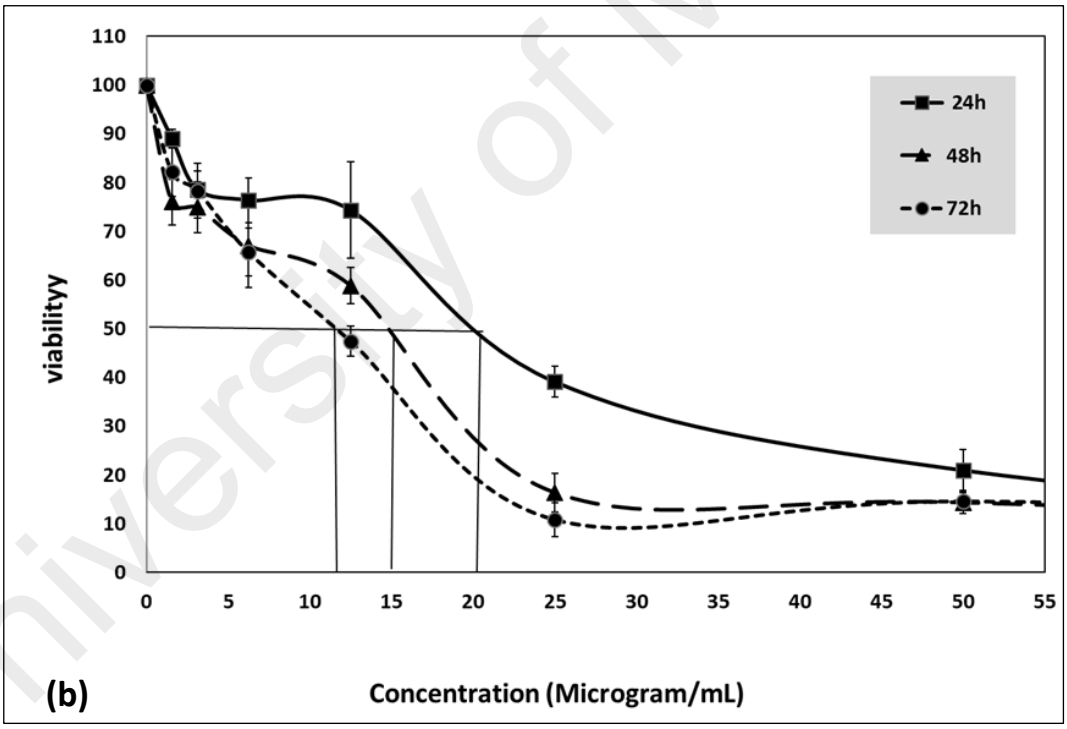

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Figure 4.1: Effects of α-mangostin (AM) on HeLa and Ca Ski cells viability, (a) The viability percentage of HeLa cells, (b) The viability percentage of Ca Ski cells. The cell viability was measured after 24, 48 and 72h of treatment. Each point represents the mean ± S.D. of three separated experiments. The statistical significance is expressed as *p < 0.05.

4.2 Proliferation activity using clonogenic assay

Healthy and active cells can reproduce and form colonies. The same with cancer cells anticancer drugs can lead to loss of reproductive integrity and proliferation of cancer cells. Clonogenic assay is used to study the effect of AM on the ability of HeLa and Ca Ski cells to form colonies. The loss of this ability as a function of dose of AM is described by the dose-survival curve. Plating efficiency is the percentage of cells that can grow into colonies after treatment, whereas survival fraction is the percentage of cells that can reproduce after treatment.

The results indicated that HeLa cells could form a large number of small colonies before treatment (600 colonies) (Figures 4.2), but Ca Ski could only form a smaller number of large colonies (130) (Figures 4.4). The effects of AM on the colony formation efficiency of HeLa and Ca Ski cells were determined. Obviously, AM treatment caused a dose-dependent inhibition of colony formation on both HeLa and Ca Ski cells.

The number of colonies of HeLa cells after treatment with 5, 10, and 15 μg/mL of AM for 24h were 125, 70 and 33, respectively (Figures 4.2). The number of colonies of Ca Ski cells after treatment with 15, 20, and 25 μg/mL of AM for 24h were 35, 25 and 17, respectively (Figures 4.4).The survival fraction of HeLa cells after treatment with 5, 10, and 15 μg/mL of AM for 24h were 20.80 ± 3.61, 11.66 ± 2.35, 5.41 ± 1.09 μg/mL, respectively (Figure 4.3). The survival fraction of Ca Ski cells after treatment with 15, 20, and 25 μg/mL of AM for 24h were 63.2 ± 14.65, 19.80 ± 5.20, 13.30 ± 2.48 μg/mL, respectively (Figure 4.5).

AM inhibitory effect was observed from the lowest concentration and proportionally increased with increasing doses of AM. Compared with Ca Ski cells, HeLa cells seem to be slightly more sensitive to AM (Figures 4.3 and 4.5).

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All the tested concentrations of AM (5, 10, 15, 20, 25 μg/mL) significantly ($p < 0.001$) inhibited cell proliferation and colony formation of HeLa and Ca Ski cells compared to the control. These findings suggested that cervical cancer cells could not actively reproduce and form colonies when treated with different concentrations of AM.

4.3 Assessment of apoptotic morphological changes in cells by using propidium iodide and acridine orange double staining

The effect of AM on the morphology of HeLa and Ca Ski cells was determined with AO/PI double staining method and by using a fluorescence microscope. The toxicity of AM causes a number of morphological changes, indicating its apoptotic effect in a time-dependent manner. After 24h of 10 and 20 μg/mL treatment of AM on HeLa and Ca Ski cells, respectively, both cell lines exhibited early apoptosis, as evidenced by the bright green fluorescence caused by AO binding to the fragmented DNA (Figures 4.6B and 4.8B). By contrast, control cells (untreated) showed green normal nuclear structures (Figure 4.6A and 4.8A).

After AM treatment for 48h, moderate apoptotic features, such as cell blebbing and nuclear chromatin condensation, were recognized (Figures 4.6C and 4.8C). Moreover, cells treated with AM for 72h exhibited features of late apoptosis and showed reddish-orange fluorescence caused by AO intervention within the denatured DNA (Figures 4.6D and 4.8D).

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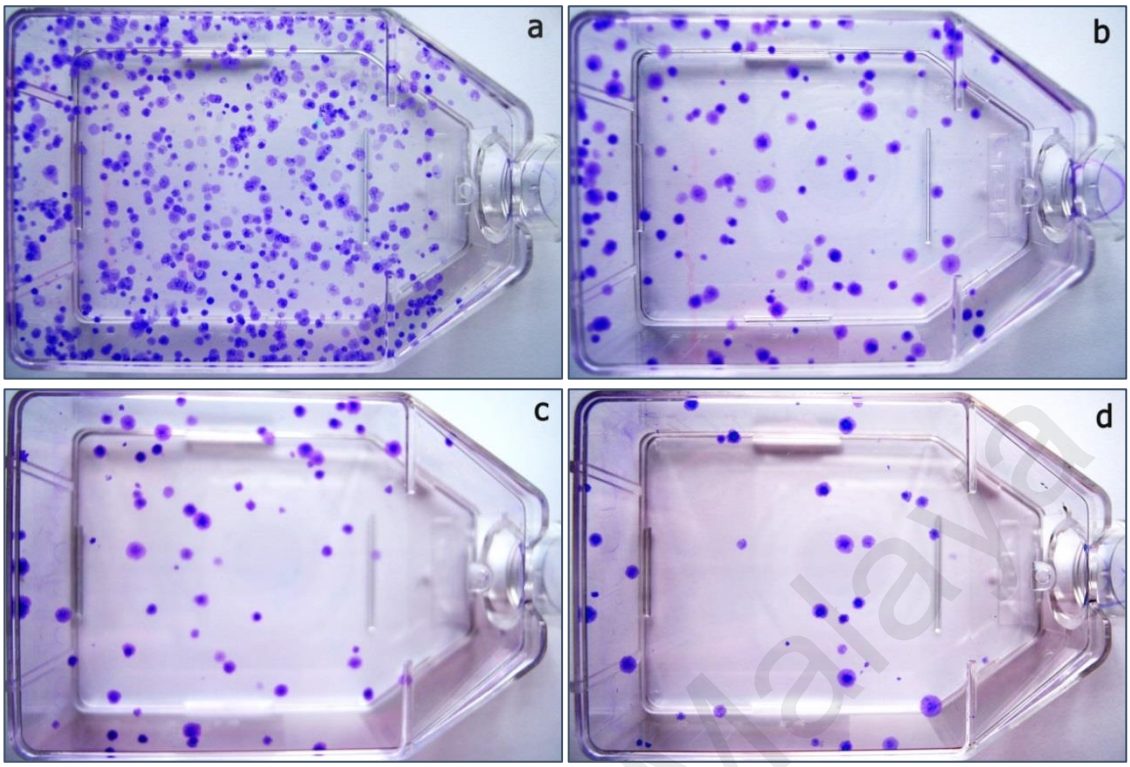

Figure 4.2: Effects of α-mangostin (AM) on HeLa colony formation as measured by clonogenic assay. Cells were treated with gradually increased concentrations of AM for 24 h and then cultured for 14 days, (a) HeLa control, (b, c, d) HeLa treated with (5, 10, 15 µg/mL) of AM, respectively.

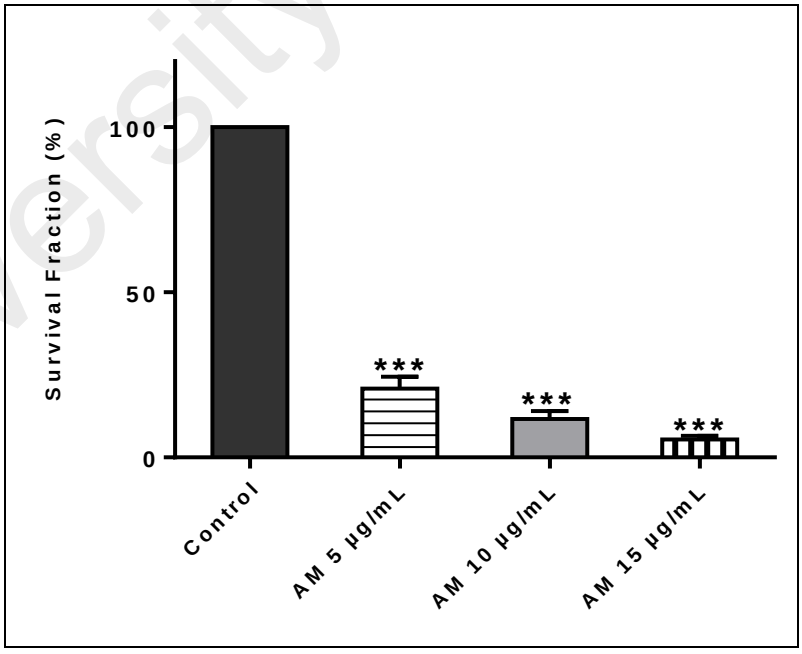

Figure 4.3: Percentage of colony formation of HeLa cells after treatment with increasing concentrations of α-mangostin (AM). Values as mean ± S.D, The statistical significance is expressed as ***$p < 0.001$.

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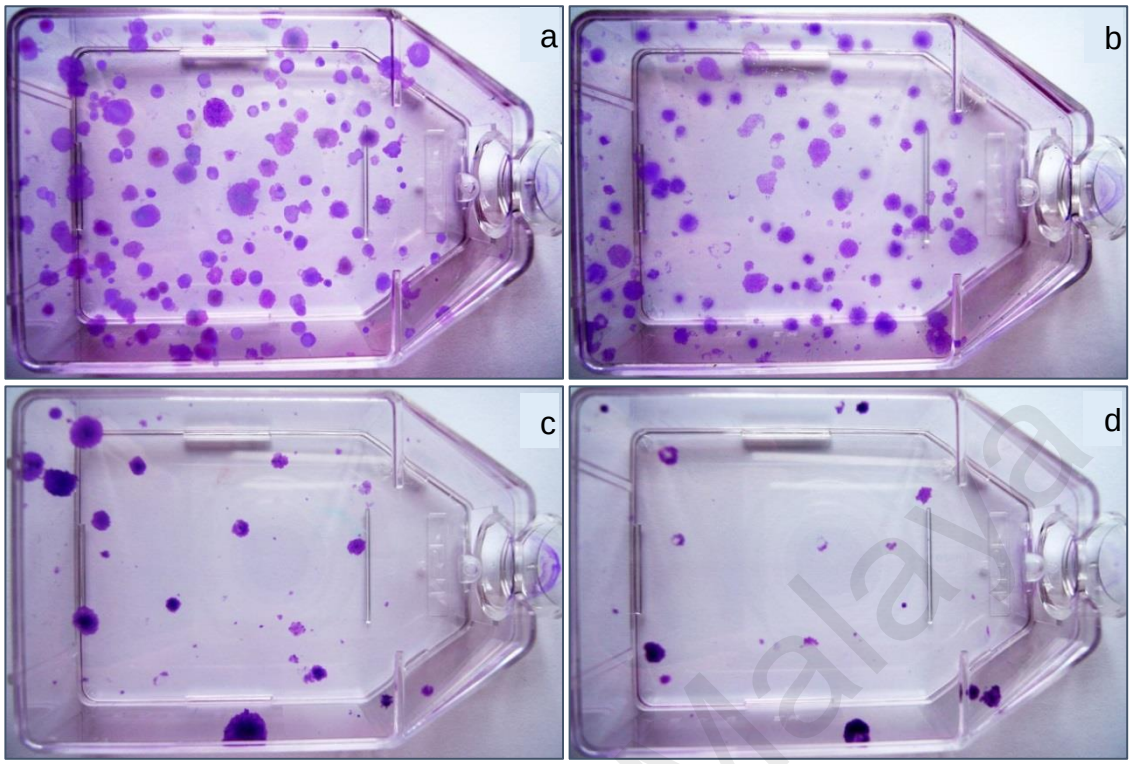

Figure 4.4: Effects of α-mangostin (AM) on Ca Ski colony formation as measured by clonogenic assay. Cells were treated with gradually increased concentrations of AM for 24 h and then cultured for 14 days. (a) Ca Ski control, (b, c, d) Ca Ski treated with (15, 20, 25µg/mL) of AM respectively.

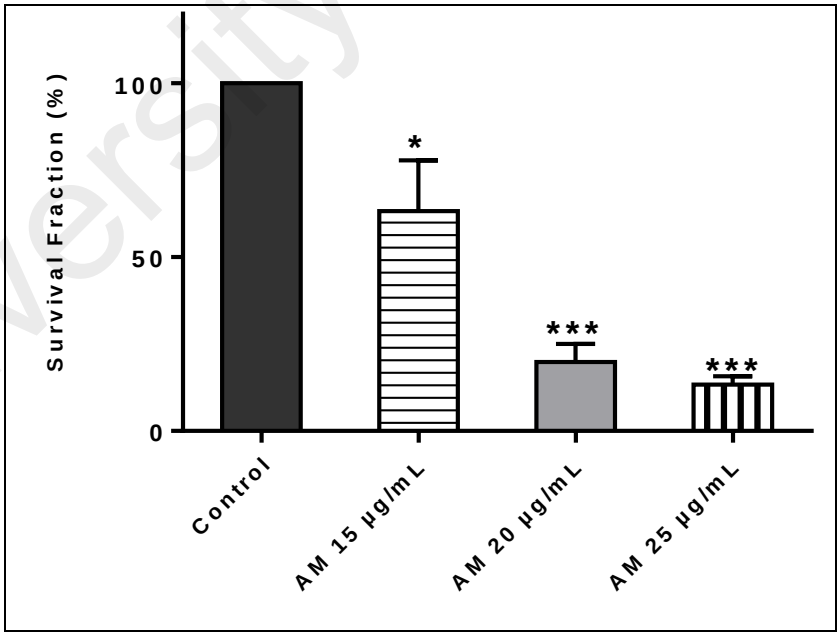

Figure 4.5: Percentage of colony formation of Ca Ski cells after treatment with increasing concentrations of α-mangostin (AM). Values as mean ± S.D, The statistical significance is expressed as *$p < 0.05$; ***$p < 0.001$.

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In addition, percentages of viable cells decreased significantly after treatment with AM. Percentages of viable cells at control, 24, 48 and 72h of AM treatment for HeLa cells were 81%, 48%, 26.33% and 11.67%, respectively. Percentages of viable cells at control, 24, 48 and 72h of AM treatment for Ca Ski cells were 76.67%, 50%, 26% and 11.33%, respectively. Numbers of apoptotic cells increased proportionally with treatment duration (Figures 4.7 and 4.9).

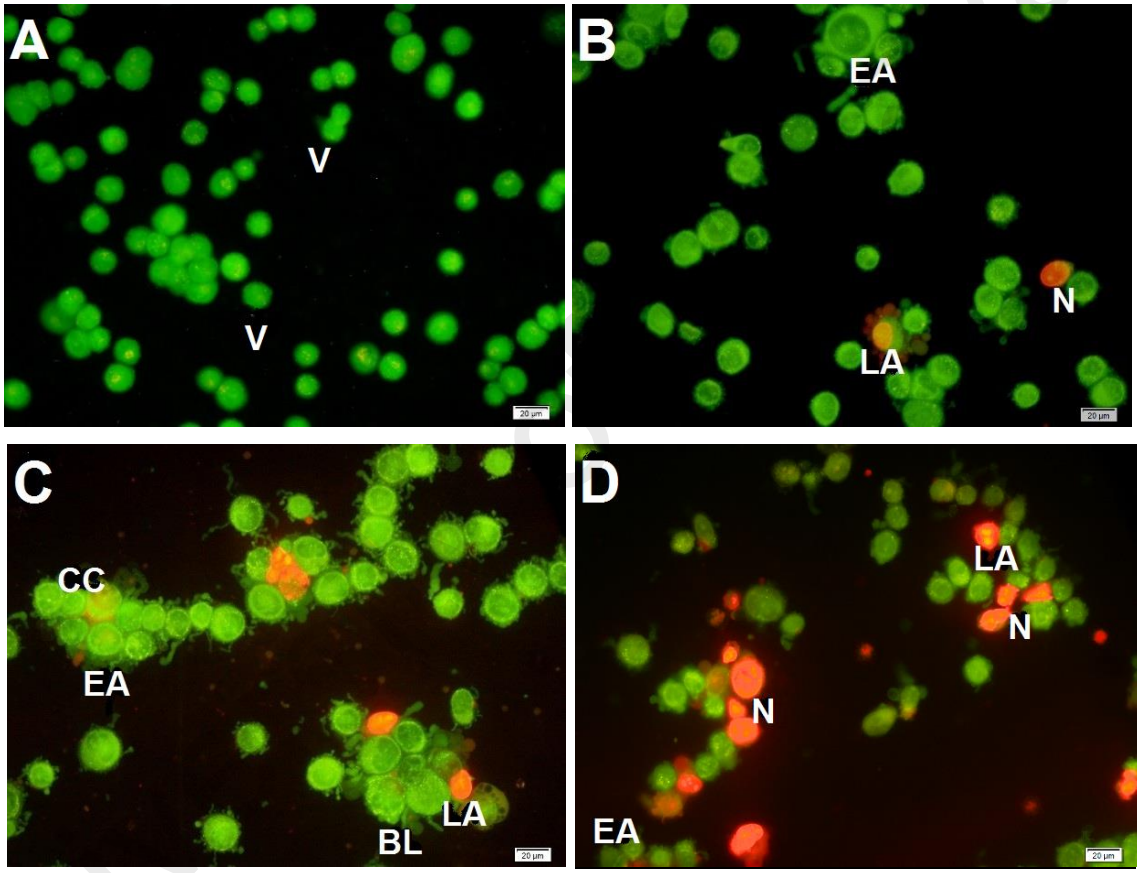

Figure 4.6: Effects of α-mangostin (10 µg/mL AM) on HeLa cells as measured by acridine orange and propidium iodide double-staining after 24h (B), 48h (C) and 72h (D) of treatment. Viable (A), early apoptotic (B), blebbing (BL), chromatin condensation (CC), late apoptotic (C) and secondary necrotic (D) were seen. Note: V: viable, EA: early apoptosis, LA late apoptosis and N: necrosis.The statistical significance is expressed as *p < 0.05; **p < 0.01, Magnification power 20X.

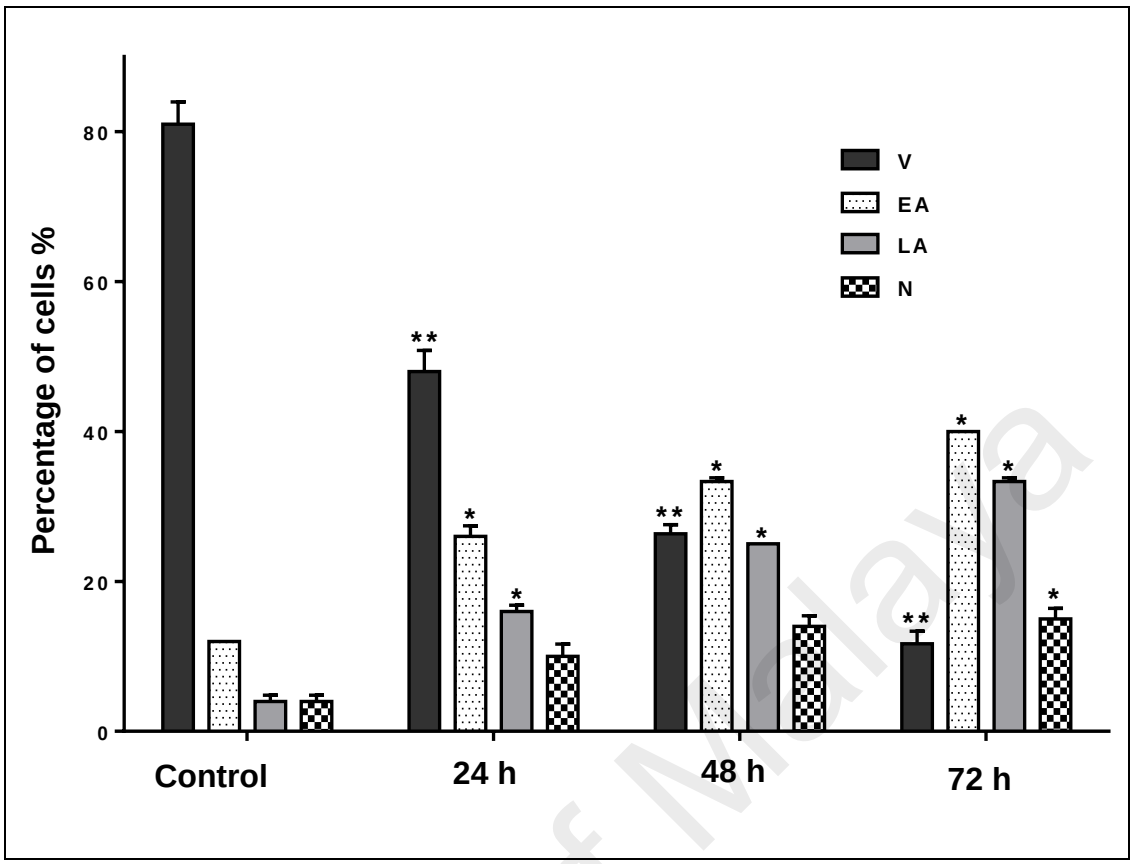

Figure 4.7: Percentages of viable, early apoptotic, late apoptosis and necrotic HeLa cells after α-mangostin (10 μg/mL AM) treatment. The statistical significance is expressed as *$p < 0.05$; **$p < 0.01$.

Percentages of early apoptotic cells at control, 24, 48 and 72h of AM treatment for HeLa cells were 12%, 26%, 33.33% and 40%, respectively, while early apoptotic cell percentages for Ca Ski cells at control, 24, 48 and 72h of AM treatment were 13.67%, 24%, 33% and 39%, respectively.

For HeLa cells, late apoptotic cell proportions were 4%, 16%, 25% and 33.33% at control, 24, 48 and 72h of AM treatment, respectively, whereas for Ca Ski cells, proportions of late apoptotic cells were 5.33%, 16.33%, 24.33% and 33.33% at control, 24, 48 and 72h of AM treatment, respectively. Percentages of necrotic cells at control, 24, 48 and 72h of AM treatment for HeLa cells were 4%, 10%, 14% and 15%, respectively. Percentages of necrotic cells at control, 24, 48 and 72h of AM treatment for Ca Ski cells were 4%, 9.67%,

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15% and 16.33%, respectively. These findings demonstrated that AM exerts an apoptogenic effect on HeLa and Ca Ski cells in a time-dependent manner.

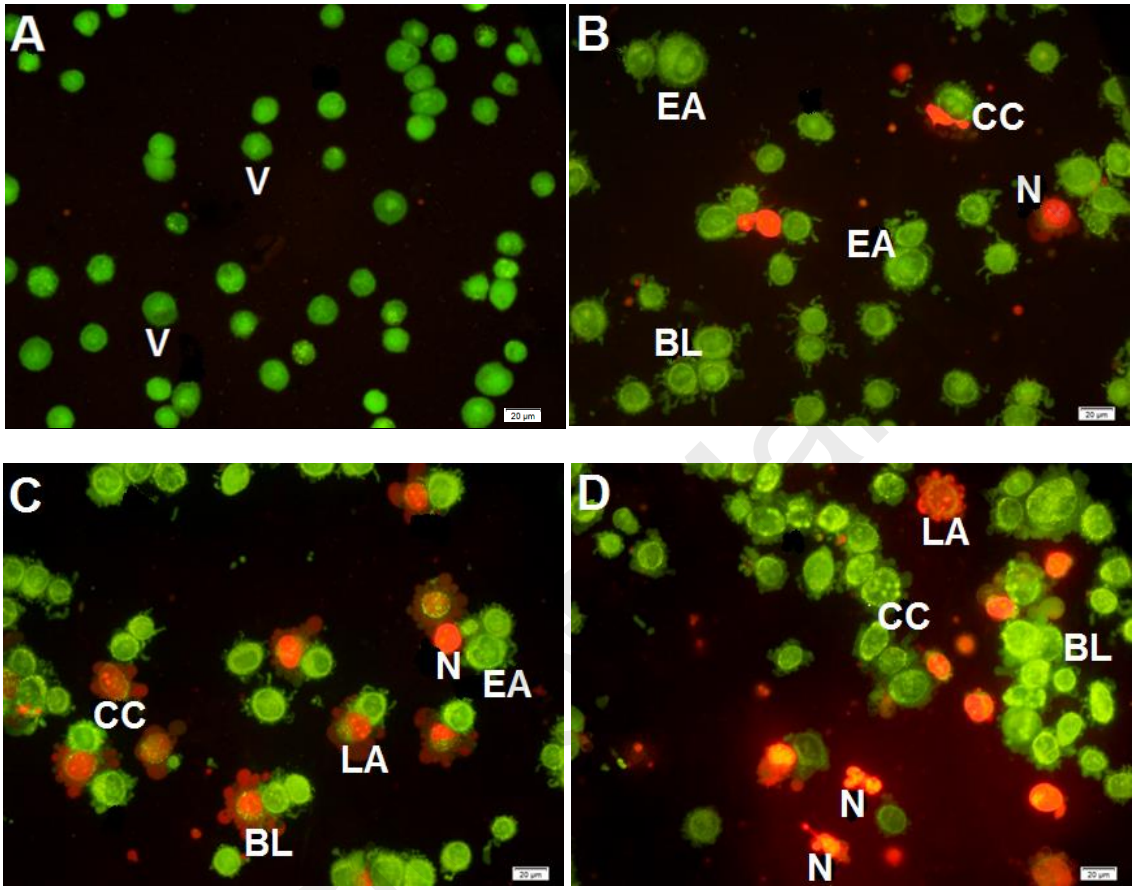

Figure 4.8: Effects of α-mangostin (20 μg/mL AM) on Ca Ski cells as measured by acridine orange and propidium iodide double-staining after 24h (B), 48h (C) and 72h (D) of treatment. Viable (A), early apoptotic (B), blebbing (BL), chromatin condensation (CC), late apoptotic (C) and secondary necrotic (D) were seen. Note: V: viable, EA: early apoptosis, LA late apoptosis and N: necrosis.The statistical significance is expressed as *$p < 0.05$; **$p < 0.01$, Magnification power 20X.

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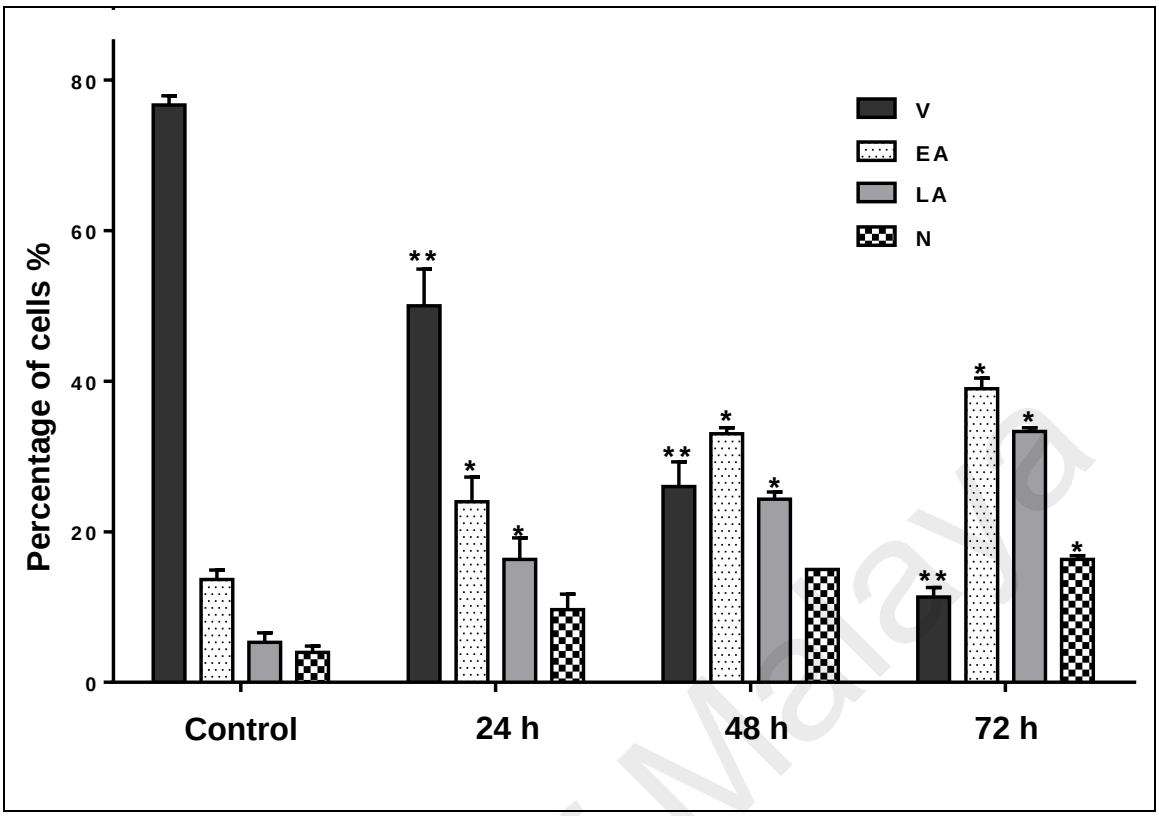

Figure 4.9: Percentages of viable, early apoptotic, late apoptosis and necrotic Ca Ski cells after α-mangostin (20 μg/mL AM) treatment. The statistical significance is expressed as *p < 0.05; **p < 0.01.

4.4 Nuclear changes and apoptotic features by Hoechst 33258 staining

Hoechst 33258 staining results revealed classic apoptotic hallmarks, such as nuclear chromatin condensation, DNA fragmentation, plasma membrane blebbing, and formation of apoptotic bodies.

The morphological changes in HeLa and Ca Ski cells caused by apoptosis were examined using cell-permeable DNA dye Hoechst 33258 through fluorescence microscopy analysis. The normal untreated control cells showed normal intact nuclei with faint staining (Figures 4.10A and 4.11A). HeLa and Ca Ski cells were treated with 10 and 20 μg/mL of AM, respectively. The cells treated for 24h showed fragmented nuclei and nuclear blebbing, which are indicative of apoptosis (Figures 4.10B and 4.11B). Moreover, cells treated with AM for 48h showed numerous apoptotic cells with condensed,

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fragmented nucleus and a much brighter color (Figures 4.10C and 4.11C). Furthermore, cells treated with AM for 72h exhibited marked characteristic features of apoptosis, including marked nuclear condensation, intense brightness, and formation of dense, apoptotic bodies (Figures 4.10D and 4.11D). These results indicated that AM significantly increased the number of apoptotic cells in a time-dependent manner. The morphological changes in cells caused by apoptosis showed that HeLa cells were more sensitive to AM than Ca Ski cells. These findings show that AM can induce the death of human cervical cancer cells by inducing apoptosis.

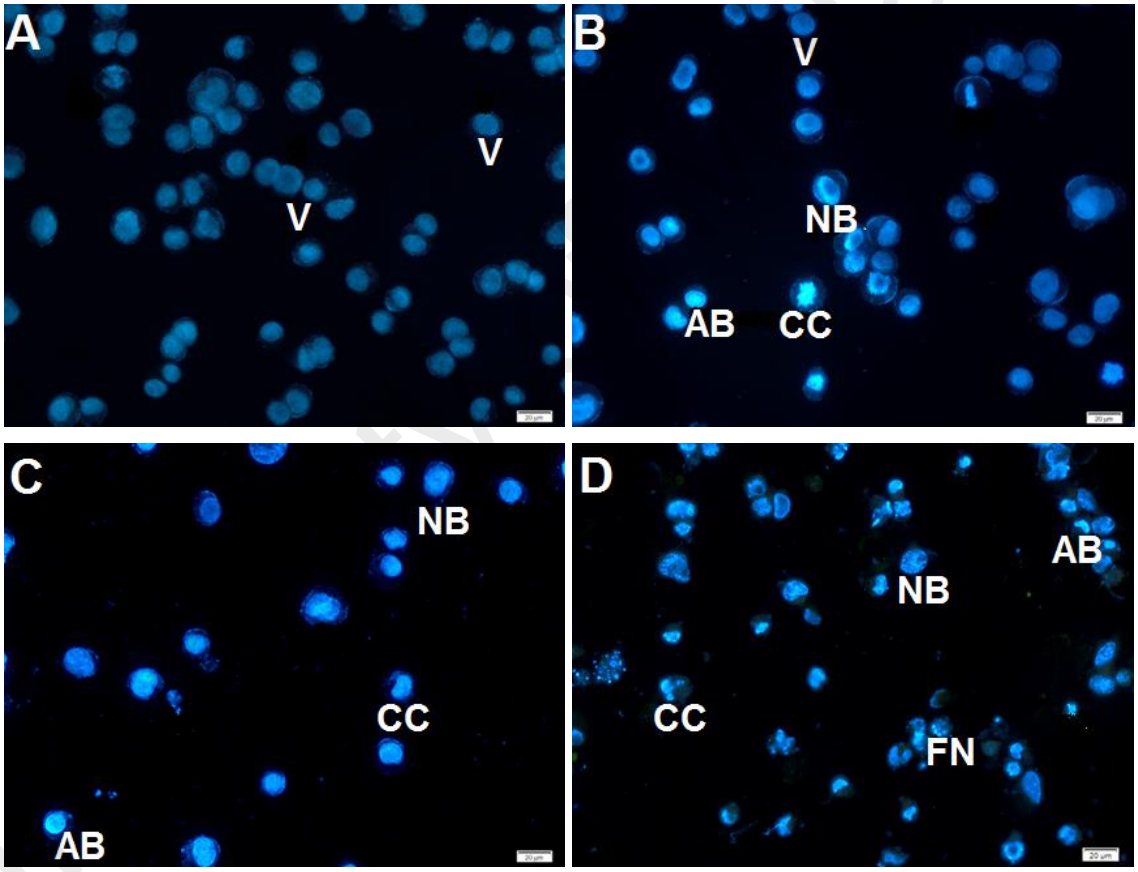

Figure 4.10: Fluorescent micrograph of Hoechst 33258 dye stained HeLa. (A) HeLa control, (B, C, D) HeLa treated with α-mangostin (AM) (10 µg/mL) for 24, 48, and 72h respectively. Note, V= viable, CC= chromatin condensation, and AB= Apoptotic bodies, FN= fragmented nucleous, NB= nuclear blebbing. Magnification power, 20X.

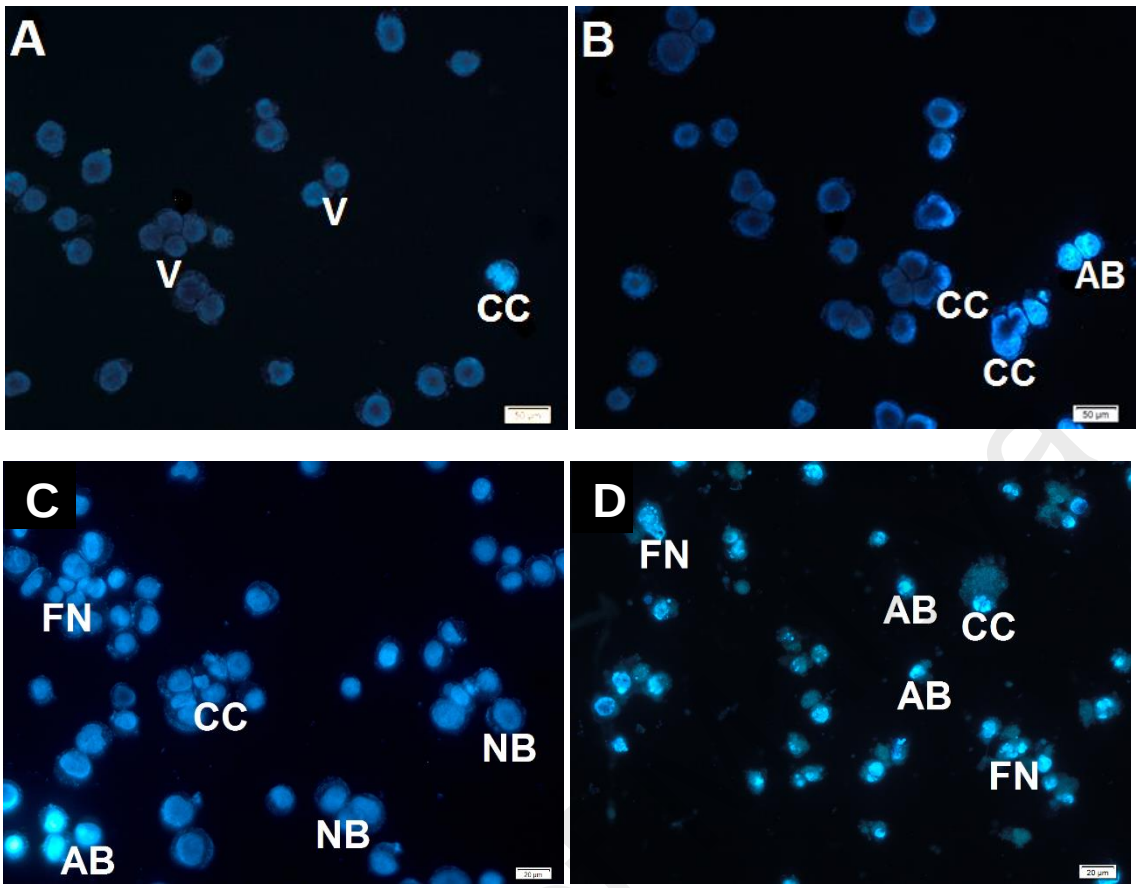

Figure 4.11: Fluorescent micrograph of Hoechst 33258 dye stained Ca Ski. (A) Ca Ski control, (B, C, D) Ca Ski treated with α-mangostin (AM) (20 µg/mL) for 24, 48, and 72h respectively. Note, V= viable, CC= chromatin condensation, and AB= Apoptotic bodies, FN= fragmented nucleous, NB= neclear blebbing. Magnification power, 20X.

4.5 Cell cycle analysis

The response of cancer cells to anticancer drugs is affected by the integrity of cell cycle checkpoints (G1and G2 arrest), which are a potential target of anticancer drugs. The kinetics of the cell cycle was evaluated after 24, 48, and 72h by flow cytometry analysis to determine the influence of 10 µg/mL and 20 µg/mL of AM on the DNA content of HeLa and Ca Ski cells, respectively. Results demonstrated that AM arrested the cell cycle progression in HeLa and Ca Ski cells at G2/M phase (Figure 4.12) and S phase (Figure 4.14), respectively. As shown in figure 4.13, HeLa cells displayed G2/M phase in a time-dependent manner, and account for 18.91%, 25.42%, and 30.19 of cells following

treatment with IC_{50} of AM for 24, 48, and 72h, respectively. Ca Ski cells displayed S phase arrest in a time-dependent manner (Figure 4.15), and account for 31.11%, 39.99%, and 54.55% of cells following treatment with IC_{50} of AM for 24, 48, and 72h, respectively.

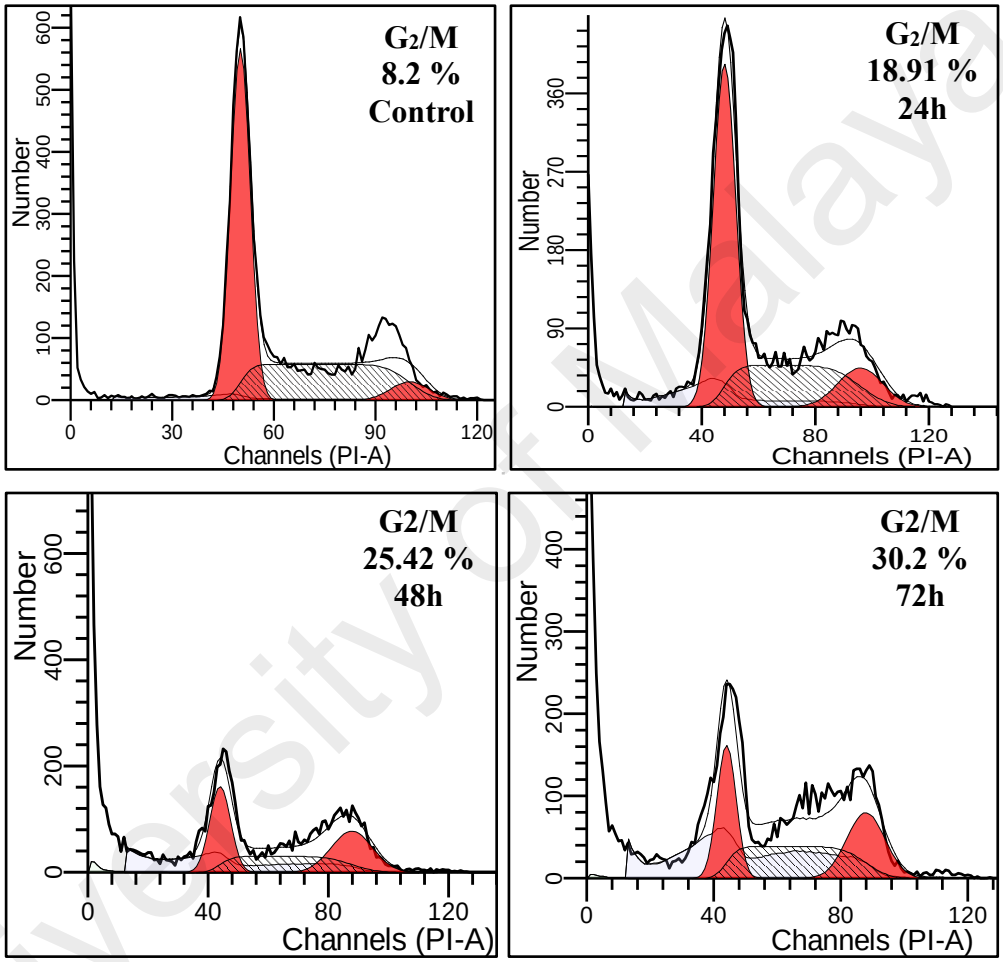

Figure 4.12: Histograms of cell cycle flow cytometry analysis for HeLa cells treated with α-mangostin (AM) (10 μg/mL) for 24, 48, and 72h. Results represent one of three separated experiments.

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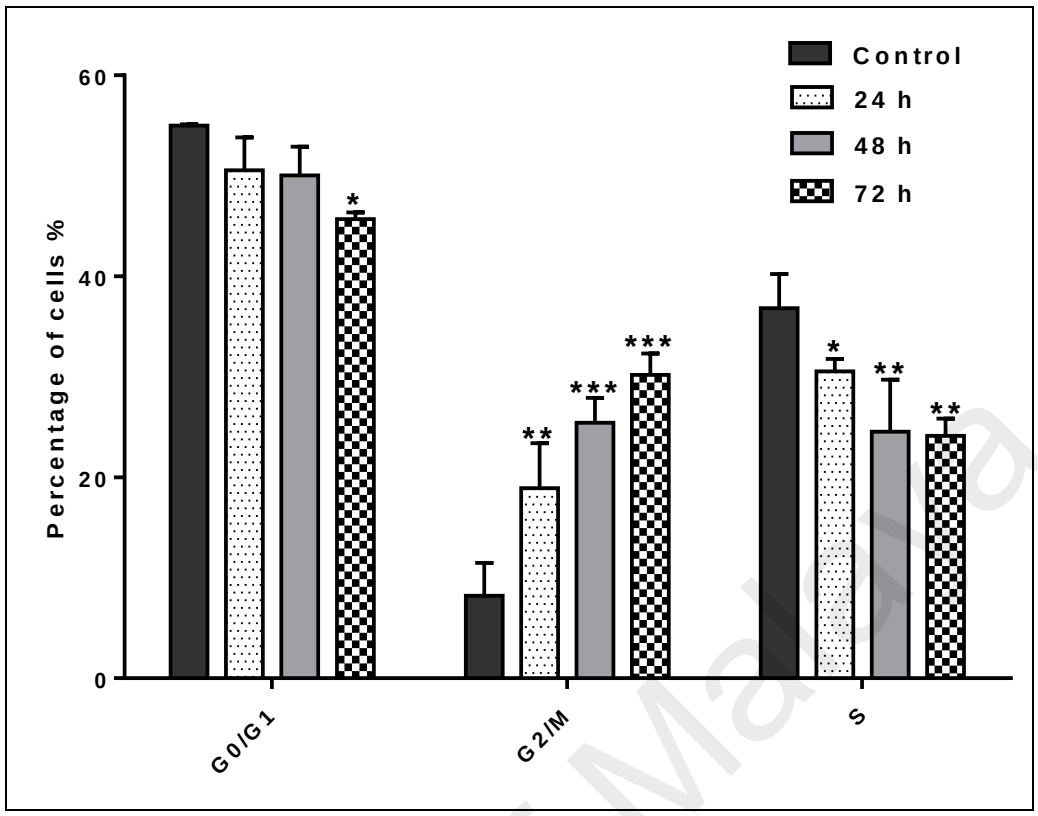

Figure 4.13: Promotion of G2/M phase accumulation in cell cycle progression of HeLa cells treated with α-mangostin (AM) (10 μg/mL), statistical significance are expressed as ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

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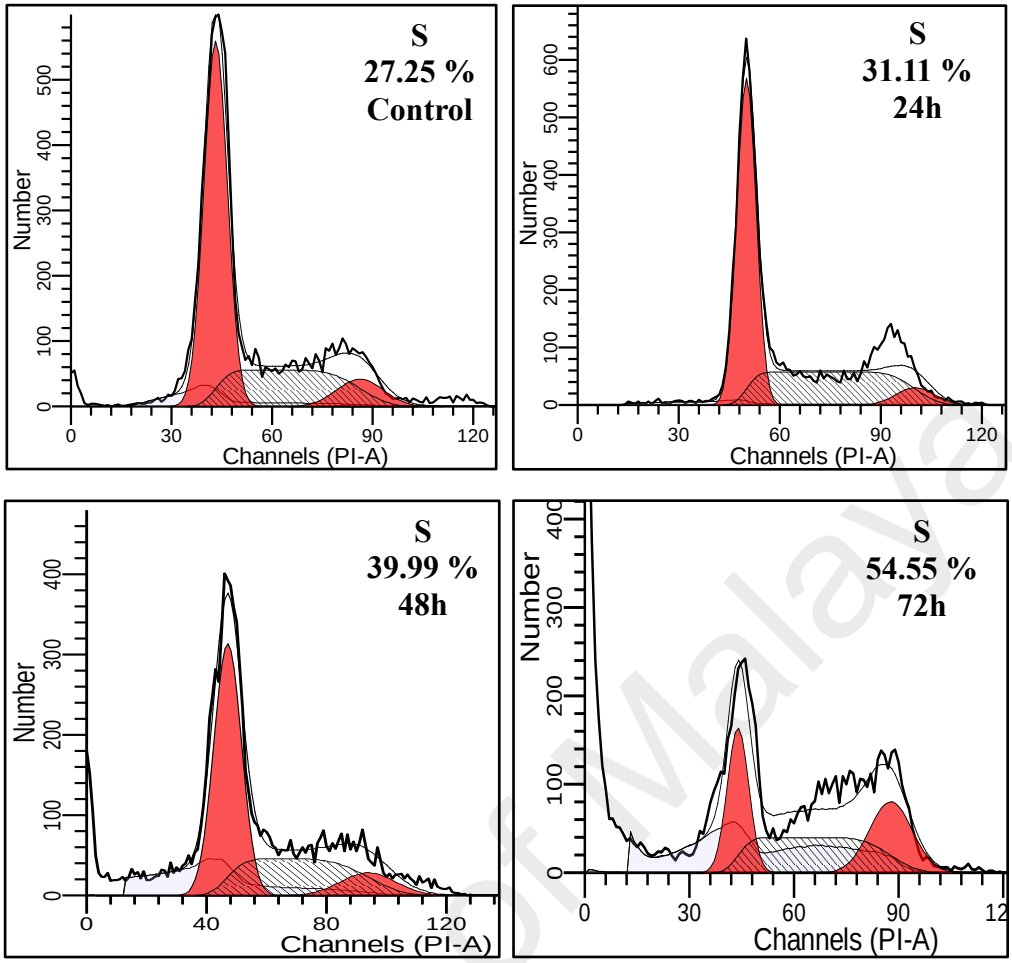

Figure 4.14: Histograms of cell cycle flow cytometry analysis for Ca Ski cells treated with α-mangostin (AM) (20 µg/mL) for 24, 48, and 72h. Results represent one of three separated experiments.

4.6 Caspases 3/7, 9 and 8 assays

The enzyme activities of caspases 3/7, 8, and 9 were measured on HeLa and Ca Ski cells treated with AM. The results indicated that high caspases-3/7 and 9 levels were present in HeLa and Ca Ski cells, after treatment with 10 and 20 µg/mL of AM respectively. HeLa and Ca Ski cells were treated for 24, 48 and 72h. HeLa cell line were highly positive to activate caspase-3/7 and caspase-9 with a significant variance from the control cells (untreated cells).

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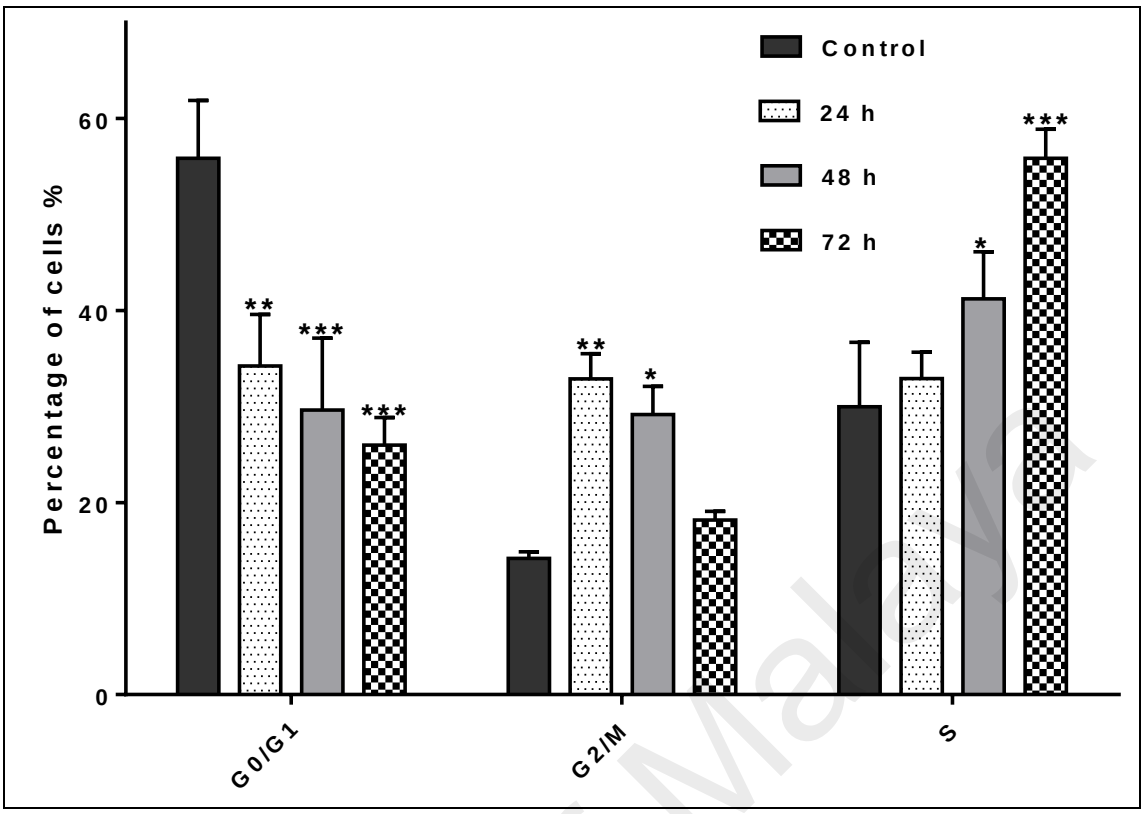

Figure 4.15: Promotion of S phase accumulation in cell cycle progression of Ca Ski cell treated with α-mangostin (AM) (20 μg/mL), statistical significance are expressed as ***p < 0.001, **p < 0.01, *p < 0.05

In the present study showed that there were increasing in luminescence units over time upon treatment with AM for caspases 3/7 and 9 in both cell lines. Caspase-3/7 concentrations were increased from 0.20 ± 0.001 before treatment to 0.63 ± 0.07, 0.97 ± 0.07 and 1.28 ± 0.12 after 24, 48 and 72h of treatment, respectively. Caspase-9 concentrations were increased from 0.19 ± 0.003 before treatment to 0.79 ± 0.03, 1.33 ± 0.05 and 2.02 ± 0.08 after 24, 48 and 72h of treatment, respectively. (Figure 4.16). Treatment of Ca Ski cells with AM activated caspases 3/7 and 9 but in lower amounts than those produced with AM in HeLa cells. Caspase-3/7 concentrations were increased from 0.19 ± 0.001 before treatment to 0.70 ± 0.01, 1.44 ± 0.05 and 2.07 ± 0.02 after 24, 48 and 72h of treatment, respectively. Caspase-9 concentrations were increased from 0.20 ± 0.003 before treatment to 0.32, 0.98 ± 0.01 and 1.69 ± 0.05 after 24, 48 and 72h of

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treatment, respectively (Figure 4.16). By contrast, the level of caspase-8 enzyme did not change in both cell lines before and after AM treatment (Figure 4.16).

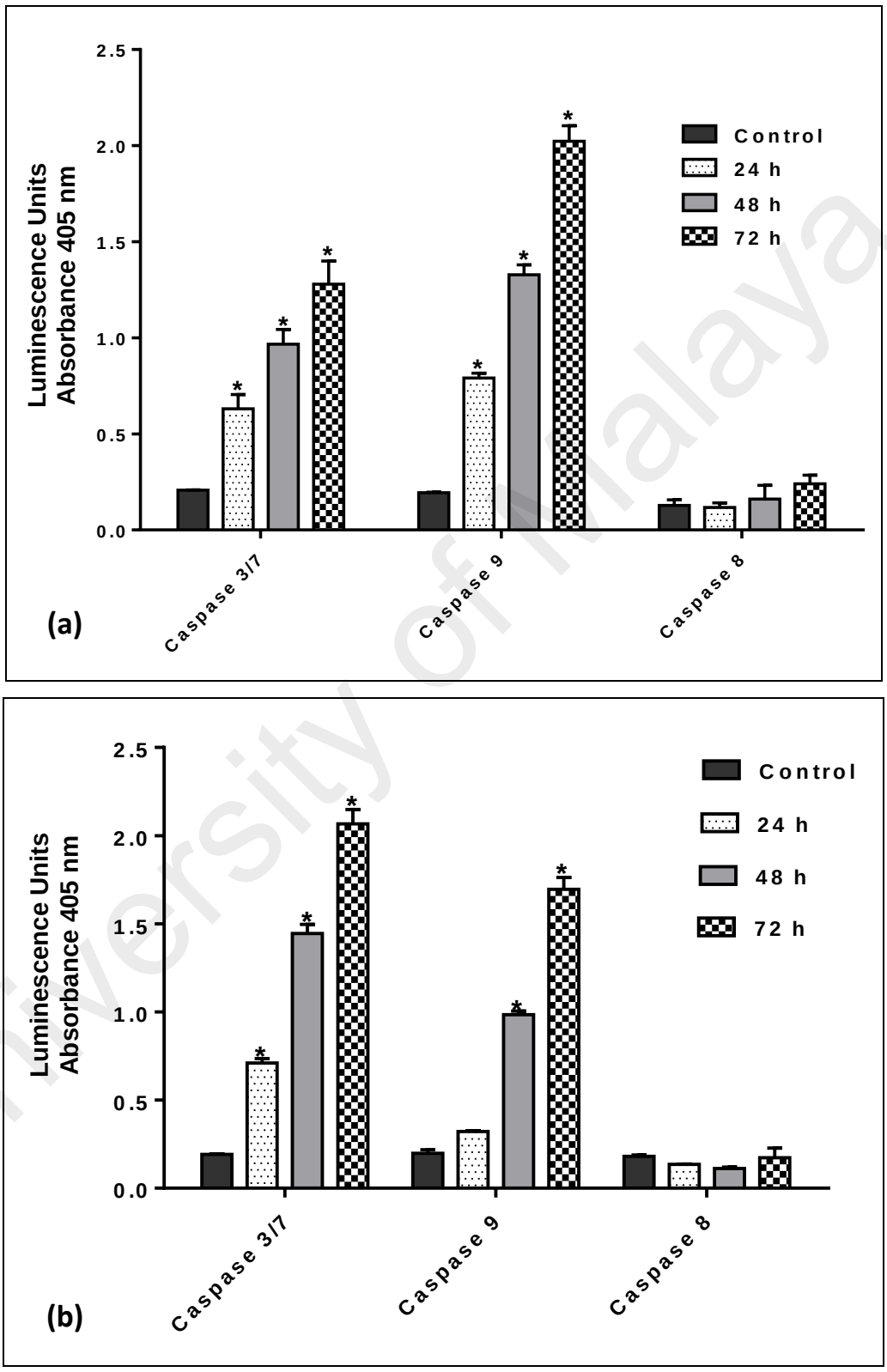

Figure 4.16: Proportional expressions of caspases 3/7, 9 and 8 in (a) HeLa and (b) Ca Ski cells treated with 10 and 20 μg/mL of α-mangostin (AM) at 24, 48 and 72h, respectively. Each treatment group was done in triplicate, *$p < 0.05$.

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4.7 DCFH-DA cellular Reactive Oxygen Species (ROS) detection assay

ROS comprises reactive molecules and free radicals containing oxygen molecules, which serve as inter- and intracellular messengers. ROS plays a critical role in cell signaling as apoptosis gene expression and cell signaling cascade activation.

These molecules are generated as byproducts during mitochondrial electron transport in aerobic respiration. Studies demonstrated that the role of ROS in cancer is not limited to genetic toxicity and mutation leading to cancer but also as signal transduction messengers (Liou & Storz, 2010; Sabharwal & Schumacker, 2014).

ROS can initiate either cell proliferation or death according to the actual endogenous and exogenous conditions in the cancer cells. 2', 7' –dichlorofluorescin diacetate (DCFH-DA) cellular reactive oxygen species detection assay uses DCFH-DA, a cell permeable fluorescent dye that measures ROS activity within the cell. After diffusion into the cell, DCFH-DA is de-acetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into 2', 7' –dichlorofluorescein (DCF), a highly fluorescent compound which can be detected by fluorescence spectroscopy with maximum excitation and emission spectra of 495nm and 529nm respectively.

As shown in Figure 4.17, AM could increase the ROS levels in HeLa and Ca Ski cells in a concentration-dependent manner. HeLa cells were treated with 5, 10 and 15 μg/mL of AM. Ca Ski cells were treated with 15, 20 and 25 μg/mL of AM. After AM treatment ROS levels (which is represented by fluorescence intensity) increased significantly. In HeLa cells, fluorescence intensity increased from 450.66 ± 23.80 before treatment up to 559 ± 36.45, 619.33 ± 17.02 and 937 ± 30.43 after 5, 10 and 15 μg/mL of AM treatment, respectively. In Ca Ski cells, fluorescence intensity increased from 412 ± 29.02 before treatment up to 579.34 ± 34.32, 851 ± 38.27 and 1016 ± 14.35 after 15, 20 and 25 μg/mL of AM treatment. ROS levels were higher in the treated cells than those in the control

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cells, and the difference increases with prolonged treatment duration as represented in the previous results.

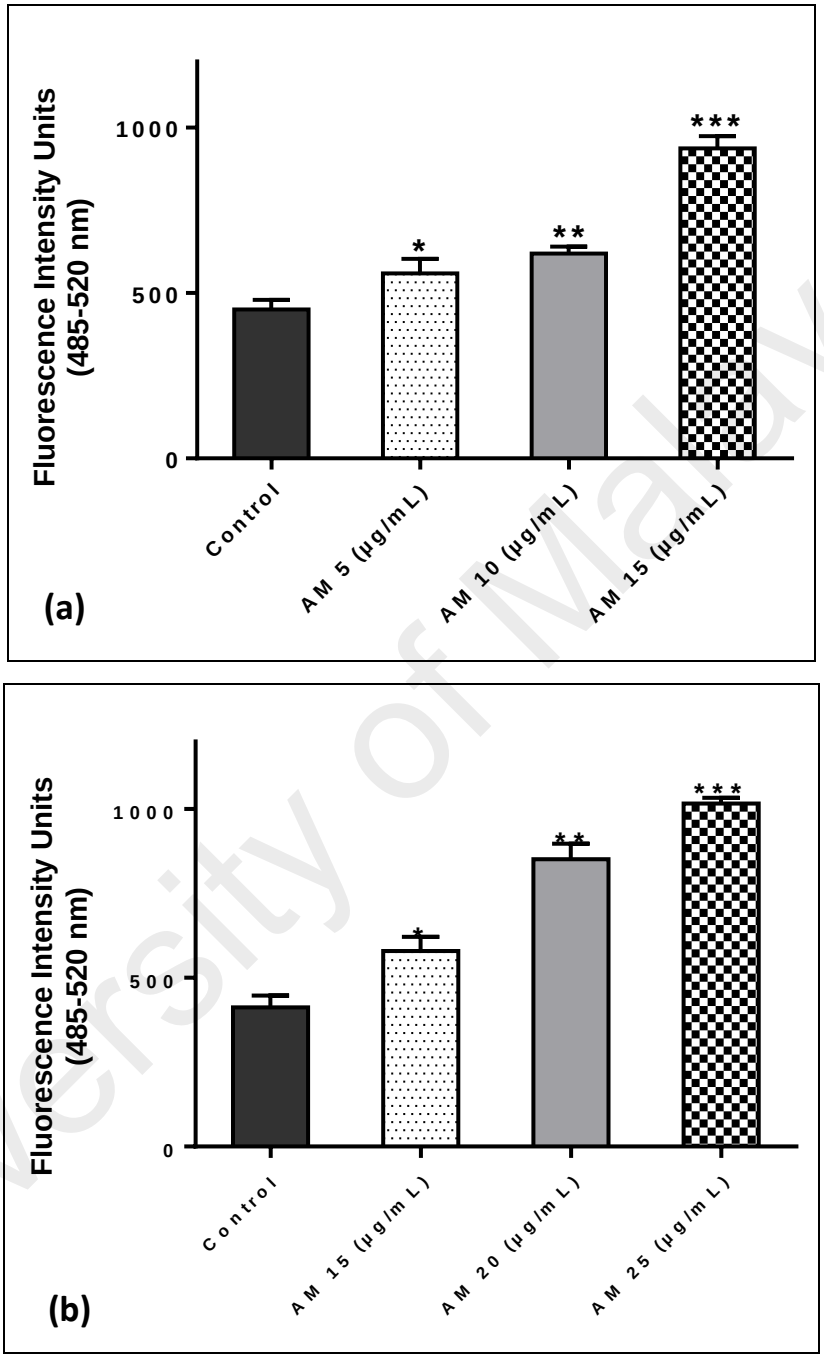

Figure 4.17: Effect of α-mangostin (AM) on ROS production in (a) HeLa and (b) Ca Ski cells. ROS levels increased in HeLa and Ca Ski cells, after treatment with AM in a concentration-dependent manner, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

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4.8 Analysis of multiple cytotoxicity assays

Multiple cytotoxicity assays were carried out to measure various parameters related to cell viability, including cell number, nuclear size, morphological changes, cell permeability (CP), mitochondrial membrane potential (MMP), and cytochrome c (Cyto C). Data were analyzed using the ArrayScan HCS system (Cellomics, PA, USA). Images of 10 μg/mL AM treated-HeLa cells and 20 μg/mL AM treated-Ca Ski cells for 24h are showed in Figure 4.18 and 4.20, respectively. Quantitative information on the changes in the commonly used cellular parameters in both cell lines are provided in Figure 4.19 and 4.21 in a dose dependent manner.

Cell viability: the results showed that HeLa and Ca Ski cell lines which were treated at 24h with 10 and 20 μg/mL of AM, respectively, exhibited cell viability decreasing which were indicated by reduction in cell number after AM treatment (Figure 4.18 and 4.20).

Total nuclear intensity (TNI): TNI was detected using Hoechst dye, TNI increasing was indicated by strong fluorescence intensity of 10 μg/mL AM treated-HeLa cells and 20 μg/mL AM treated-Ca Ski cells compared to untreated cells (Figure 4.18 and 4.20). HeLa cells which were treated with 5, 10 and 15 μg/mL AM and Ca Ski cells which were treated with 15, 20 and 25 μg/mL AM exhibited a significant dose-dependent total nuclear intensity increasing as shown in Figure 4.19 and 4.21.

Mitochondrial membrane potential: MMP reduction was indicated by weakened fluorescence intensity of HeLa and Ca Ski cells treated for 24h with 10 and 20 μg/mL of AM, respectively. The MMP dye stained strongly in the cytoplasm of control cells compared to treated cells as shown in Figure 4.18 and 4.20. HeLa cells which were treated with 5, 10 and 15 μg/mL AM and Ca Ski cells which were treated with 15, 20 and 25 μg/mL AM exhibited a significant dose-dependent MMP reduction as shown in Figure 4.19 and 4.21.

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Cell membrane permeability: permeability dye stained strongly 10 μg/mL AM treated-HeLa cells and 20 μg/mL AM treated-Ca Ski cells compared to untreated cells, a significant increase in plasma membrane permeability was noticed which was indicated by increased green-fluorescence emission after AM treatment, (Figure 4.18 and 4.20). HeLa cells which were treated with 5, 10 and 15 μg/mL AM and Ca Ski cells which were treated with 15, 20 and 25 μg/mL AM exhibited a significant dose-dependent increment in cell membrane permeability as shown in Figure 4.19 and 4.21.

Cytochrome c: Cyto C stained weakly in control cells. In contrast, 10 μg/mL AM treated-HeLa cells and 20 μg/mL AM treated-Ca Ski cells showed strong staining around the nucleus (Figure 4.18 and 4.20). This suggests that AM triggered the translocation of Cytochrome c from mitochondria into the cytosol. HeLa cells which were treated with 5, 10 and 15 μg/mL AM and Ca Ski cells which were treated with 15, 20 and 25 μg/mL AM exhibited a significant dose-dependent increase in Cyto C release into the cystole as shown in Figure 4.19 and 4.21.

The previous results showed that AM can trigger increasing in fluorescence intensities in total nuclear intensity, cell permeability and Cytochrome c in treated HeLa and Ca Ski cells compared with the control cells. In contrast, MMP showed a significant dose-dependent decrease in fluorescence intensity in treated HeLa and Ca Ski cells compared with the control cells (Figures 4.19 and 4.21).

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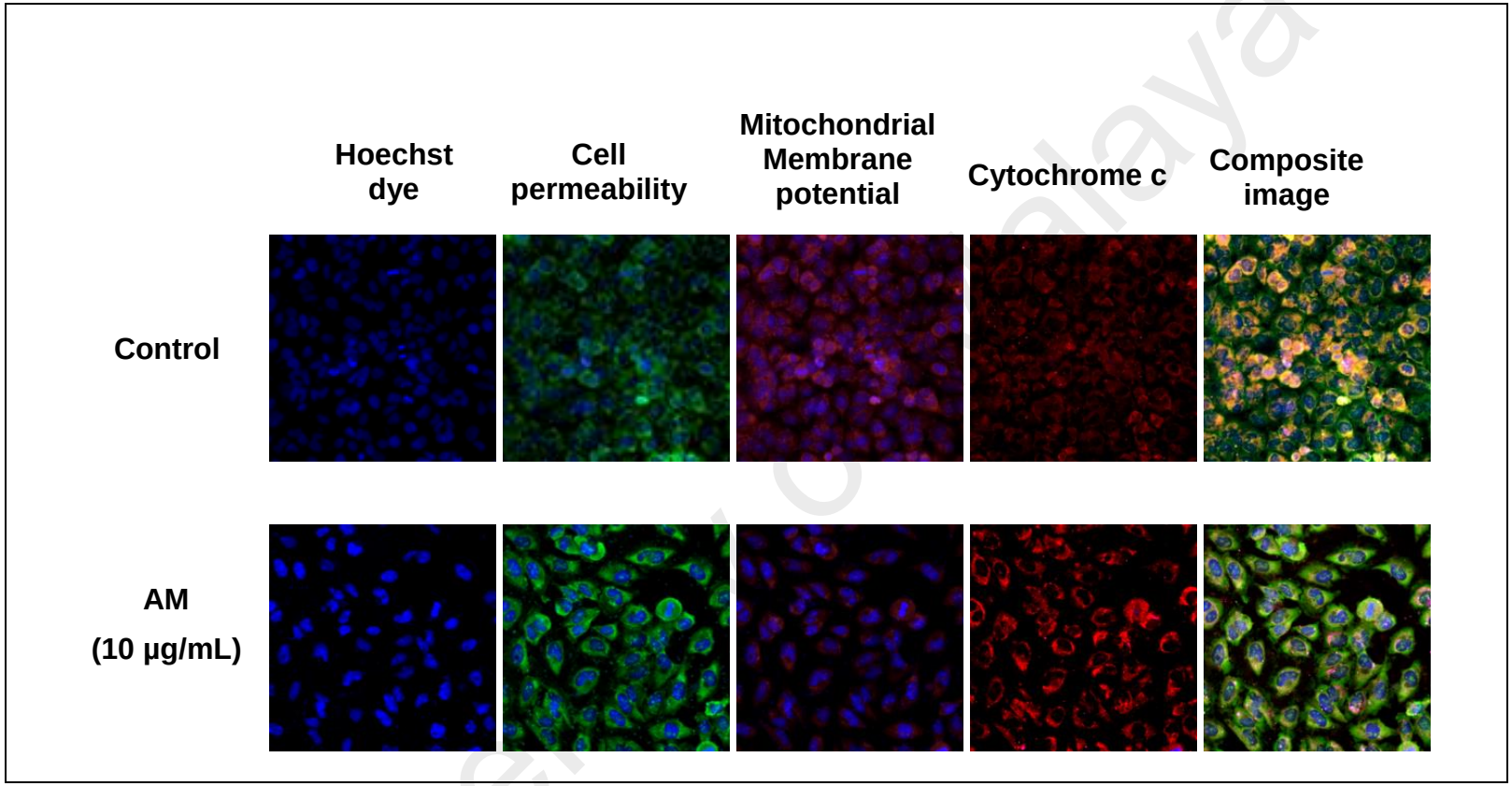

Figure 4.18: Apoptotic parameters of HeLa cells treated with (10 µg/mL) of α-mangostin (AM). Representative images of HeLa cells treated with AM compared to untreated cells, and stained with Hoechst dye, cell permeability dye, mitochondrial membrane potential dye and cytochrome c. The images of each row are acquired from the same field of the same treatment sample (magnification 20 X).

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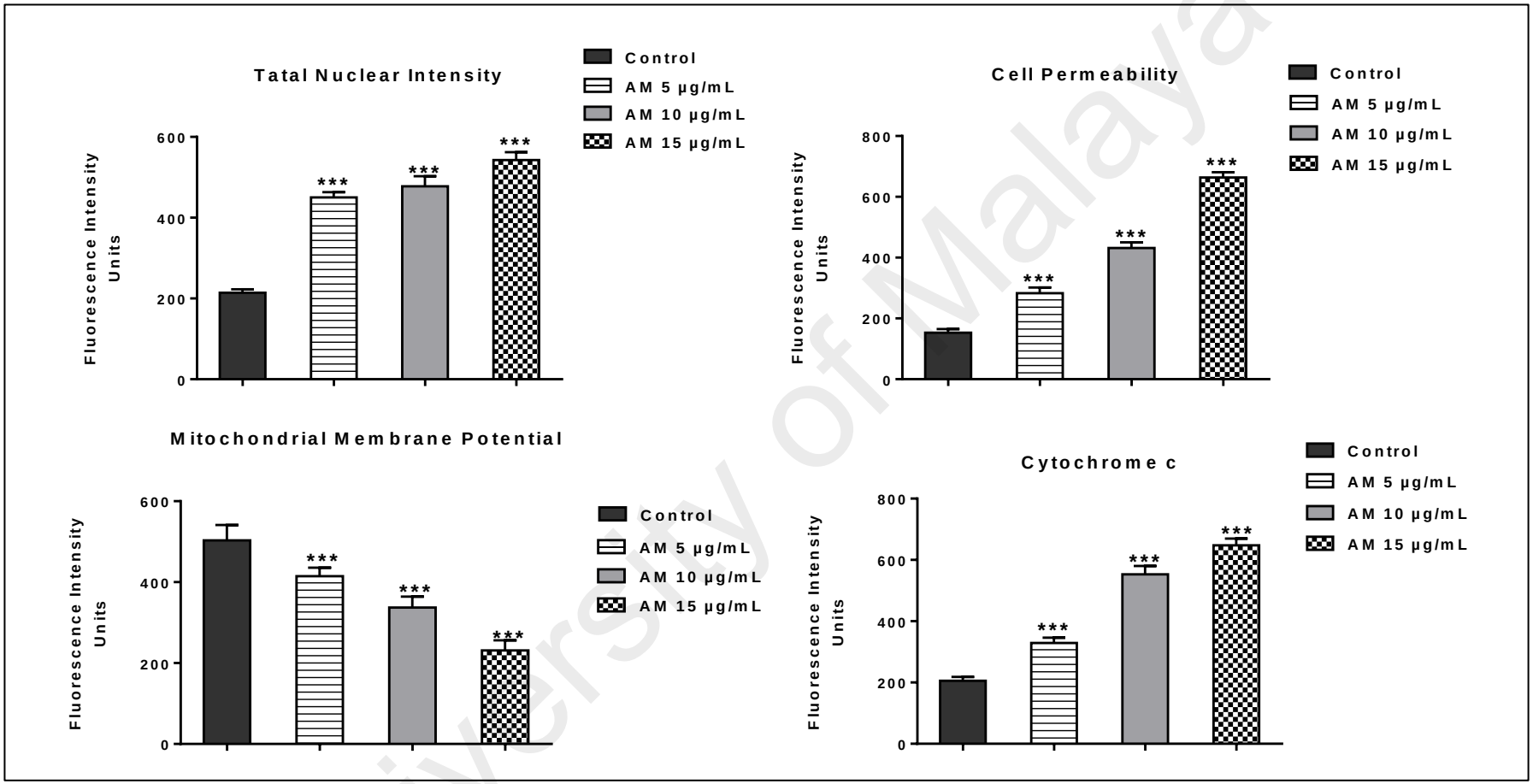

Figure 4.19: Quantitative analysis of α-mangostin AM mediated apoptosis parameters on HeLa cells. Changes in (a) Total nuclear intensity, (b) Cell permeability, (c) Mitochondrial membrane potential and (d) Cytochrome c localization were measured simultaneously in HeLa cells. Statistical significance is expressed as **p < 0.01; ***p < 0.001.

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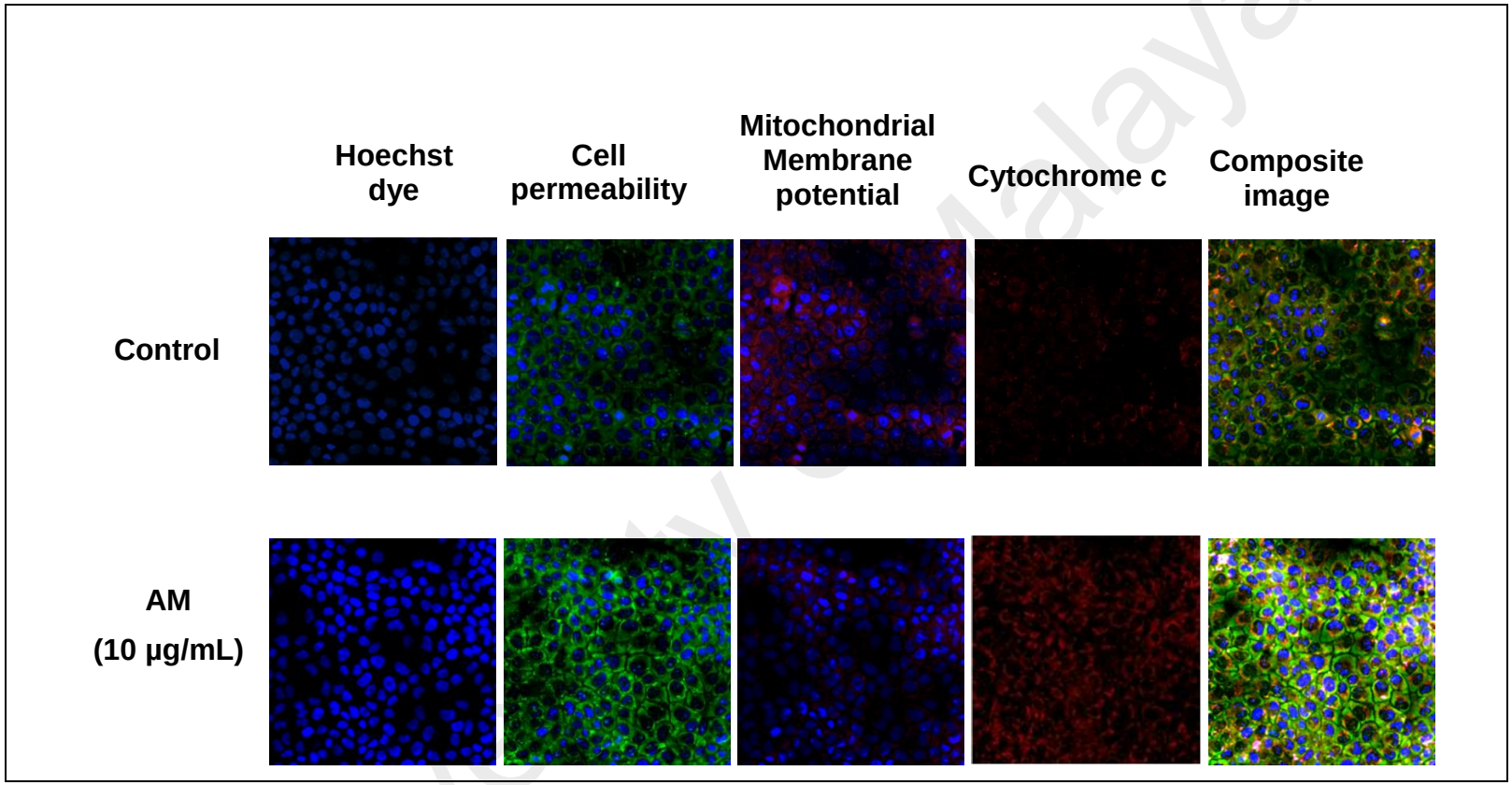

Figure 4.20: Apoptotic parameters of Ca Ski cells treated with 20 µg/mL of α-mangostin (AM). Representative images of Ca Ski cells treated with AM compared to untreated cells, and stained with Hoechst dye, cell permeability dye, mitochondrial membrane potential dye and cytochrome c. The images of each row are acquired from the same field of the same treatment sample (magnification 20 X).

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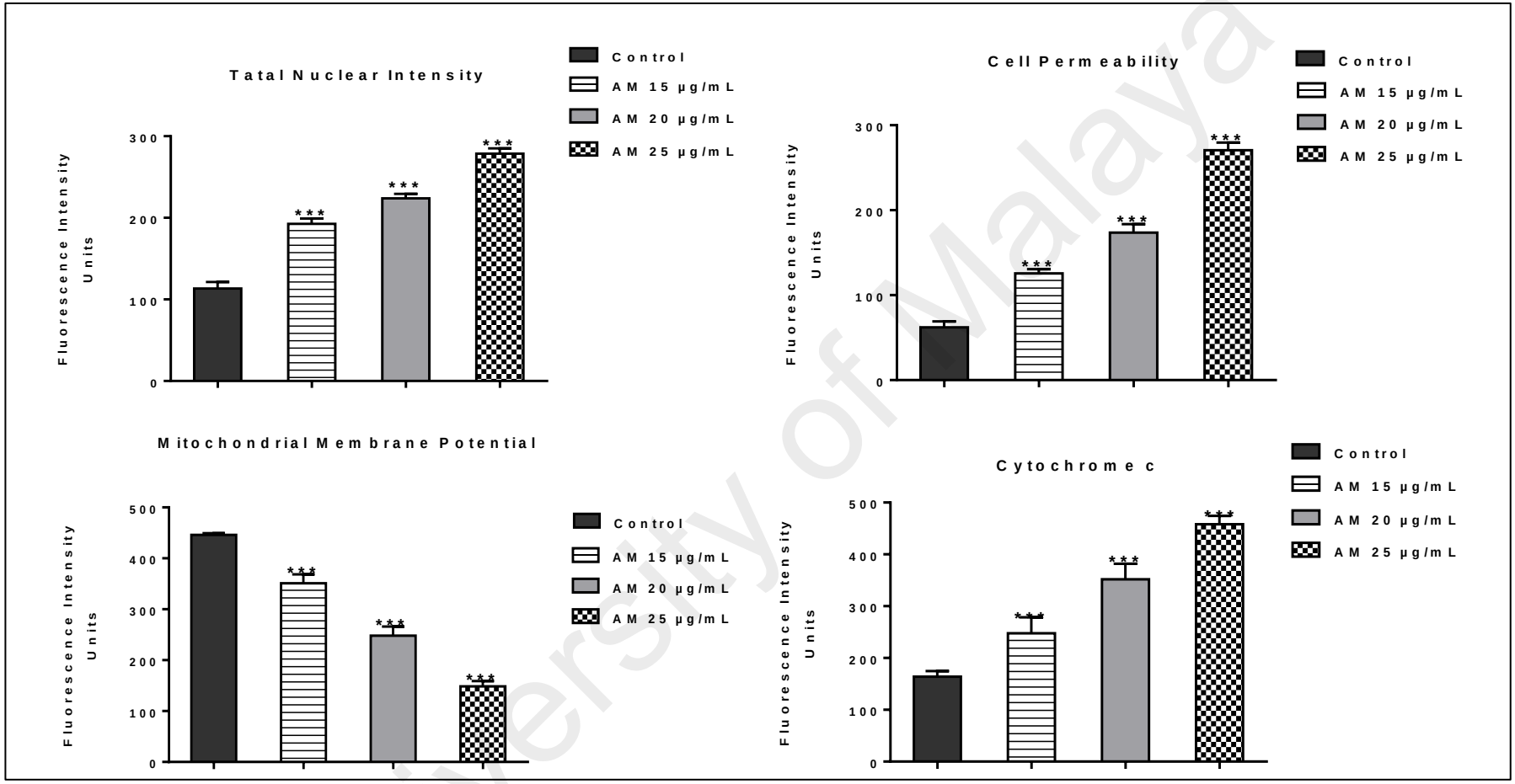

Figure 4.21: Quantitative analysis of α-mangostin AM mediated apoptosis parameters on Ca Ski cells. Changes in (a) Total nuclear intensity, (b) Cell permeability, (c) Mitochondrial membrane potential and (d) Cytochrome c localization were measured simultaneously in Ca Ski cells. Statistical significance is expressed as **p < 0.01; ***p < 0.001.

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CHAPTER 5: DISCUSSION

Plants have been used in cancer therapy for thousands of years. Traditional medicine plays a critical role in treatment of many chronic life-threatening conditions and diseases, including cancer (Mohan et al., 2011). The severity of side-effects and acquired resistance to conventional chemotherapeutic agents particularly at high doses limit its usage and boost researchers to investigate other options of anticancer treatments (Loehrer & Einhorn, 1984; Sitzia & Huggins, 1998).

AM is an oxygenated and prenylated xanthone compound isolated from *C. arborescens*, and has shown strong pharmacological effects *in vitro* and *in vivo* through various mechanisms of action (Ibrahim et al., 2015; Morelli et al., 2015). The present study was attempted to explore the mechanism of action involved in the promising effects of AM on human cervical cancer cell lines, HeLa and Ca Ski. Previous study has shown that AM has a suppressive effect on many cancer cell lines including breast cancer MCF-7 cells. This effect was mediated through the induction of an apoptotic process with regulation of NF-κB and Hsp70 protein modulation (Ibrahim et al., 2016). AM was also reported to induce apoptosis via mitochondrial dysfunction in human leukemia HL60 cells (Matsumoto et al., 2004) . In the present study, the cytotoxicity of AM on HeLa and Ca Ski cells was evaluated by MTT assay and was influenced by mitochondrial NADPH-dependent oxidoreductase, which can reduce the tetrazolium dye to its insoluble purple formazan. Thus, the cytotoxicity of AM on HeLa and Ca Ski cells was measured with a reliable quantitative test (Mosmann, 1983).

The results indicated that AM treatment in HeLa and Ca Ski cells induced cytotoxic effects in a dose- and time-dependent manner. AM treated-HeLa cells had IC_{50} values of

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10.07 ± 0.61, 5.82 ± 0.89 and 5.25 ± 0.57 µg/mL for 24, 48 and 72h, respectively. AM treated-Ca Ski cells had IC_{50} values of 21.09 ± 0.11, 15.80 ± 0.59 and 12.64 ± 0.09 µg/mL for 24, 48 and 72h, respectively. The IC_{50} of AM on SV40 cells was 38.28 ± 1.61 µg/mL after 24h of treatment. According to (Shier, 1991), compounds that exhibit an IC_{50} value of more than 30 µg/mL are considered as not potentially cytotoxic, compounds which exhibit IC_{50} value between 25.0 - 30.0 µg/mL were classified as very weak cytotoxic compounds, compounds which exhibit IC_{50} value between 10.0 - 25.0 µg/mL were classified as weak cytotoxic compounds, compounds which exhibit an IC_{50} value between 5.0 - 10.0 µg/mL were classified as moderately active compounds, while compounds which exhibit an IC_{50} value of less than 5.0 µg/mL are considered very active compounds. Moreover, AM has exerted higher cytotoxic effects on HeLa cells than those on Ca Ski cells and not potentially cytotoxic on SV40 cells with IC_{50} value 38.28 ± 1.61 µg/mL. We conclude that AM could be a potential antitumor agent with a considerable cytotoxic effect on cervical cancer cell lines HeLa and Ca Ski.

To evaluate the effectiveness of AM on the survival and proliferation of cervical cancer cells before and after exposure to AM, clonogenic assay was applied. This assay determines the ability of the cell to display "unlimited" division and only a portion of the treated cells maintained the ability to continue division and form colonies. AM significantly inhibited the colony formation of HeLa cells compared to that of the control (untreated cells). These results indicated that cells could not actively replicate and form colonies after AM treatment. Moreover, inhibition of colony formation increased gradually with increasing AM concentration. AM exhibited the same effect on Ca Ski cells but showed varied influence on colony number and size. HeLa formed more and smaller colonies compared to Ca Ski, but the ability of both cell lines to form colonies decreased significantly. The results showed that AM toxic effect can obviously be realized by loss of reproductive integrity and proliferation in HeLa and Ca Ski cells.

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Apoptosis induced in HeLa and Ca Ski cells was then confirmed with a morphological study by fluorescence microscopy analysis by using AO/PI staining. AO and PI dyes intercalated to DNA fluorochromes; thus, green fluorescence and orange fluorescence were emitted, respectively. Only the AO dye could pass through the membrane of viable and early apoptotic cells. Thus, the viable cells exhibited green nuclei with normal structures, whereas the cells in early apoptosis showed bright-green nuclei with condensed or fragmented chromatins in the nuclei. Both AO and PI dyes could pass through cell membranes in late apoptotic and necrotic cells. Thus, late apoptotic cells exhibited condensed and fragmented orange chromatins; secondary necrotic cells showed orange nuclei with intact structures. These characteristic of morphological changes revealed the apoptogenic effect of AM, whereas the antiproliferative effect was assessed by decreased number of viable cells after the AM treatment. By calculating the number of viable HeLa and Ca Ski cells after the treatment, we found that the number of viable cells significantly decreased, thereby indicating the antiproliferative effect of AM on both cell lines.

Apoptotic morphological features, such as membrane blebbing, chromatin condensation, and formation of apoptotic bodies, appeared clearly under a fluorescence microscope, indicating the apoptotic effect of AM on both cell lines. No marked changes in the number of necrotic cells were observed with increasing AM concentration in HeLa and Ca Ski cells. Therefore, AM induces cell death via the apoptotic pathway and not by necrosis. Common apoptotic hallmarks, such as nuclear chromatin condensation, DNA fragmentation, plasma membrane blebbing, and formation of apoptotic bodies were examined using cell-permeable DNA dye Hoechst 33258 by fluorescence microscopy analysis. The untreated HeLa and Ca Ski cells showed normal, intact nuclei, whereas the AM-treated cells showed fragmented nuclei and nuclear blebbing. The cells showed condensed, fragmented nuclei, and dense, apoptotic bodies were formed with increasing

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AM concentration. The morphological changes in cells caused by apoptosis indicated that HeLa cells were more sensitive to AM than Ca Ski cells. These results confirmed the previous findings on AOPI assay. The characteristic apoptogenic morphological changes revealed by Hoechst 33258 dye on both cell lines After AM treatment confirmed the previous apoptogenic morphological changes showed by AO/PI staining on both cell lines after AM treatment.

Cell cycle control is an important mechanism for protection of cellular division. The link between the cell cycle and apoptosis is based on several instances in which apoptosis is regulated by genes that are involved in cell cycle progression. These genes control and regulate the main episodes in the cell cycle, to ensure that cells divide normally. The loss of this regulation is the hallmark of cancer. For example, Myc directly activates genes involved in proliferation and apoptosis. Mitogens stimulate Myc's proliferation pathway, while anti-apoptotic factors, such as Bcl-2, shut down Myc's apoptotic pathway. Another important protein is *p53,* which induces either cell death or cell cycle arrest depending on a variety of factors such as the extent of DNA damage and the genetic background of the cell. In addition, functional p21 and retinoblastoma protein (pRb) are critical determinants in *p53*'s role (Pucci et al., 2000). Numerous carcinogenic progressions generate defects in cell cycle regulators (Shoja et al., 2015). Therefore, changing the cell cycle regulation in cancer cells is important for chemoprevention and chemotherapy (Saleh et al., 2015; Yang et al., 2015).

The events in cell cycle are monitored during cell cycle checkpoints which serves as a potential point along the cell cycle and ensure proper division of the cell. These checkpoints occur at the G1/S boundary, in S-phase, and during the G2/M-phases. The checkpoints enable cell proliferation only in the presence of stimulatory signals such as growth factors. They are also activated by DNA damage leading to growth arrest to allow the cell to repair the damage and the cell cycle will continue to progress. If the damage

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cannot be repaired, the cell will be eliminated through apoptosis (Pucci et al., 2000). Studies on HeLa's cell cycle after treatment with AM have revealed a higher percentage of cells in the G2/M phase. By contrast, the percentage of cells in S and G0/G1 phases decreased compared with that in the untreated cells. Ca Ski cells exhibited similar antiproliferative effects, which led to cell accumulation in the S phase and decreased cell number in the G2/M and G0/G1 phases. These results indicated that HeLa and Ca Ski cells underwent apoptosis which caused G2/M-phase and S-phase arrest, respectively, in a time-dependent manner.

Caspases belong to a family of proteases that play a critical role in regulating cell death (Chang & Yang, 2000; McIlwain et al., 2013). AM induced the activation of caspases 9 and 7/3 but not caspase 8, suggesting that AM can trigger apoptosis through the mitochondrial pathway. The mitochondrial pathway was confirmed by MMP disruption and increased cell permeability, concurrent with cytochrome c release (Salim et al., 2013) AM significantly stimulated the cells to release cytochrome c from the mitochondria into the cytosol during early apoptosis, thereby controlling apoptosome formation. Apoptosome activates procaspase-9 to caspase-9, which led to activation of caspase-3 in the activation cascade of the mitochondrial intrinsic pathway, resulting in apoptosis.

Apoptosis can be confirmed by increased ROS production (Gundala et al., 2014); as such, the effect of ROS on cancer is not limited to be cancer promoters that facilitate mutagenesis but also as signal transduction messengers; which may induce either proliferation or death of cancer cells according to cellular endogenous and exogenous conditions . ROS levels were assessed after the AM treatment of HeLa and Ca Ski. The result exhibited a considerable correlation. Intracellular ROS level increased by almost twofold in HeLa and Ca Ski cells treated with 15 and 25 µg/mL of AM, respectively. This effect may be attributed to the production of free radicals during cytotoxicity.

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Changes in MMP of HeLa and Ca Ski cells treated with 10 and 20 g/mL of AM, respectively, for 24h showed significantly reduced fluorescence intensity, which reflected MMP collapse. High content screening (HCS analysis) showed characteristic morphological features of apoptotic cell death, such as nuclear condensation, loss of membrane symmetry, release of cytochrome c, and reduction in MMP (Vitale et al., 2016). Disruption in MMP, an early episode in apoptosis, triggered cytochrome c release from the mitochondria to the cytosol and led to caspase-9 stimulation. Stimulation of caspase-9 resulted in the activation of the downstream caspase-3/7, which is accountable for the characteristic apoptosis-associated morphological alterations, such as chromatin condensation, membrane blebbing, and loss of the general cell form. This assay revealed that treatment with AM increased the activities of caspases-3/-7 and 9. This phenomenon led to MMP collapse and cytochrome c release.

These results suggested that AM can trigger apoptosis via the mitochondrial pathway, as confirmed by MMP disruption and increased cell permeability. Similarly, AM has significantly induced apoptosis of HeLa and Ca Ski cells via the intrinsic or mitochondrial pathway as illustrated in Figure 5.1. To the best of my knowledge, the antiproliferative effect of AM has not been tested yet on cervical cancer cell lines (HeLa and Ca Ski). No known intensive study has been done so far to explain the antitumor effect of AM on cervical cancer cell lines, thereby lending this research findings have contribute to a great significance and uniqueness of the study.

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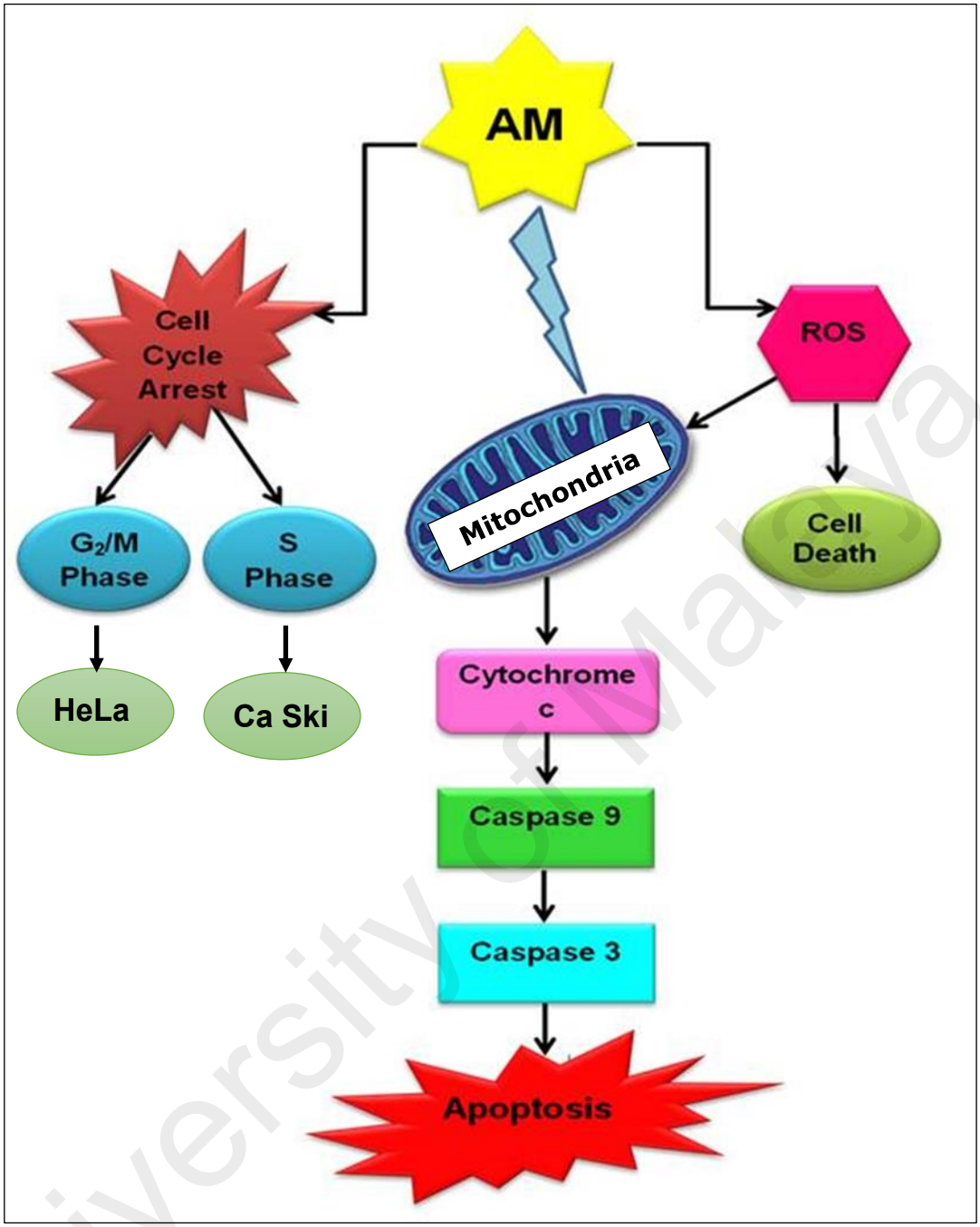

Figure 5.1: Flow chart depicting the mechanisms involved in α-mangostin (AM)-induced apoptosis in human cervical cancer cells HeLa and Ca Ski

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CHAPTER 6: CONCLUSION

This study illustrates the apoptotic effect and the mechanism of apoptosis triggered by AM in the human cervical cancer cell lines namely HeLa and Ca Ski. The results of the MTT assay demonstrated the cytotoxic effects of AM on HeLa and Ca Ski cells. The antitumor effects of AM were then confirmed by AO/PI double staining, Hoechst 33258 staining, and clonogenic assay. AM induces the apoptosis of HeLa and Ca Ski cells through the intrinsic pathway and promotion of intracellular ROS production. In addition, AM can induce cell cycle arrest in HeLa and Ca Ski cells in the G2/M and S phases, respectively.

AM can also adjust MMP to trigger cytochrome c release from the mitochondria to the cytosol. As cytochrome c enters the cytosol, caspase-9 and -3 are activated, leading to apoptosis. AM does not alter the level of caspase-8, indicating that apoptosis occurs via the intrinsic pathway only and without the involvement of the extrinsic pathway.

Our findings may elucidate the apoptotic mechanism through which AM exerts its tumour effects on cervical cancer cells (HeLa and Ca Ski). This study provides a basis for the development of new chemotherapeutic approaches for cancer therapy especially of cervical cancer. Further studies need to be conducted to validate the safety and efficacy of a potential compound to provide more scientific evidences or correct information to the public and researchers.

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6.1 Suggestion for future studies

This thesis has presented a general view of AM effects on different types of cancer. Then it concentrated on cervical cancer to study in details. We studied the antitumour and apoptogenic effect of AM on cervical cancer cell lines HeLa and Ca Ski, many studies still need to be done in the future

1- Study AM cytotoxic effect on further cervical cancer cell lines from different cancer stages such as C4 (I, II), SiHa, MS751 and SW756 cell lines.

2- *In vivo* study of AM on cervical cancer.

3- Detect HPV status in different cervical cancer cell lines, as many cell lines are not detectable yet.

4- Identify genetic changes in p53 status in HeLa and Ca Ski cell lines if it is still normal or mutated, and to investigate more about the role of p53 in cervical cancer initiation and treatment and study the effect of AM on it.

5- Identify genetic changes in pRb status in HeLa and Ca Ski cell lines if it is still normal or mutated, and to investigate more about the role of pRb in cervical cancer initiation and treatment and study the effect of AM on it.

6- Most Recent studies focusing on molecular targets of AM and have yielded promising results, but the details of the anticancer mechanisms involved need to be clarified further and preclinical and clinical studies must be done to determine the potential of AM.

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REFERENCES

Abdul-Karim, F. W., Fu, Y. S., Reagan, J. W., & Wentz, W. B. (1982). Morphometric study of intraepithelial neoplasia of the uterine cervix. *Obstetrics and Gynecology Journal, 60* (2), 210-214.

Akinlotan, M., Bolin, J. N., Helduser, J., Ojinnaka, C., Lichorad, A., & McClellan, D. (2017). Cervical cancer screening barriers and risk factor knowledge among uninsured women. *Journal of Community Health*, 42 (4), 770-778.

Alejo, M., Alemany, L., Clavero, O., Quiros, B., Vighi, S., Seoud, M., …Loveras, B. (2018). Contribution of human papillomavirus in neuroendocrine tumors from a series of 10,575 invasive cervical cancer cases. *Papillomavirus Research,* 5, 134-142.

Aljunid, S., Zafar, A., Saperi, S., & Amrizal, M. (2010). Burden of disease associated with cervical cancer in Malaysia and potential costs and consequences of HPV vaccination. *Asian Pacific Journal of Cancer Prevention, 11* (6), 1551-1559.

Althuis, M. D., Fergenbaum, J. H., Garcia-Closas, M., Brinton, L. A., Madigan, M. P., & Sherman, M. E. (2004). Etiology of hormone receptor–defined breast cancer: A systematic review of the literature. *Cancer Epidemiology Biomarkers & Prevention, 13* (10), 1558-1568.

Arcand, S. L., Maugard, C. M., Ghadirian, P., Robidoux, A., Perret, C., Zhang, P., …Tonin, P. N. (2008). Germline TP53 mutations in BRCA1 and BRCA2 mutation-negative French Canadian breast cancer families. *Breast Cancer Research and Treatment, 108* (3), 399-408.

Badial, R. M., Dias, M. C., Stuqui, B., Melli, P., Quintana, S. M., Bonfim, C. M. D., …Rahal, P. (2018). Detection and genotyping of human papillomavirus (HPV) in HIV-infected women and its relationship with HPV/HIV co-infection. *Medicine (Baltimore), 97* (14), e9545.

Baldauf, J. J., Dreyfus, M., Monlun, E., Ritter, J., & Obert, G. (1995). Increased prevalence of herpes viruses in high grade CIN and carcinoma of the cervix: The potential for synergistic effect. *Journal of the European Academy of Dermatology and Venereology, 4* (1), 104-109.

University of Malaya

Bennouna, J., Campone, M., Delord, J. P., & Pinel, M.-C. (2005). Vinflunine: A novel antitubulin agent in solid malignancies. *Expert Opinion on Investigational Drugs, 14* (10), 1259-1267.

Bodaghi, S., Wood, L. V., Roby, G., Ryder, C., Steinberg, S. M., & Zheng, Z. (2005). Could human papillomaviruses be spread through blood? *Journal of Clinical Microbiology, 43* (11), 5428-5434.

Bolick, D. R., & Hellman, D. J. (1998). Laboratory implementation and efficacy assessment of the ThinPrep cervical cancer screening system. *The Journal of Clinical Cytology and Cytopathology, 42* (1), 209-213.

Bosch, F. X., Burchell, A. N., Schiffman, M., Giuliano, A. R., De Sanjose, S., Bruni, L., …Muñoz, N. (2008). Epidemiology and natural history of human papillomavirus infections and type-specific implications in cervical neoplasia. *Vaccine, 26* (10), 1-16.

Bosch, F. X., Tsu, V., Vorsters, A., Van Damme, P., & Kane, M. A. (2012). Reframing cervical cancer prevention. Expanding the field towards prevention of human papillomavirus infections and related diseases. *Vaccine, 30 Suppl 5*, F1-11.

Bray, F. C. M., Mery, L., Piñeros, M., Znaor, A., Zanetti, R., & Ferlay, J. (2017). Cancer incidence in five continents international agency for research on cancer (*IARC)*, XI.

Brown, S. B., & Hankinson, S. E. (2015). Endogenous estrogens and the risk of breast, endometrial, and ovarian cancers. *Steroids, 99* (A), 8-10.

Bruni, L., Barionueyo-Rosas, L., Albero, G., Aldea, M., Serano, Valencia, S., ...Castellsague, X. (2015). ICO information centre on HPV and cancer (HPV Information Centre). *Humanpapillomavirus and related diseases in the world (20 Mar 2015 ed.)*. Barcelona, Spain: ICO

Burd, E. M. (2003). Human papillomavirus and cervical cancer. *Clinical Microbiology Reviews, 16* (1), 1-17.

Cabrera, M., Simoens, M., Falchi, G., Lavaggi, M. L., Piro, O. E., Castellano, E. E., …González, M. (2007). Synthetic chalcones, flavanones, and flavones as

University of Malaya

antitumoral agents: Biological evaluation and structure–activity relationships. *Bioorganic & Medicinal Chemistry, 15* (10), 3356-3367.

Castellsagué, X., Díaz, M., de Sanjosé, S., Muñoz, N., Herrero, R., Franceschi, S., …Bosch, F. X. (2006). Worldwide human papillomavirus etiology of cervical adenocarcinoma and its cofactors: Implications for screening and prevention. *Journal of the National Cancer Institute, 98* (5), 303-315.

CDC. (2014). Genital HPV Infection - Fact Sheet. USA: *Centers for Disease Control and Prevention.*

Chairungsrilerd, N., Furukawa, K.-I., Ohta, T., Nozoe, S., & Ohizumi, Y. (1996). Pharmacological properties of α-mangostin, a novel histamine H1 receptor antagonist. *European Journal of Pharmacology, 314* (3), 351-356.

Chang, H. Y., & Yang, X. (2000). Proteases for cell suicide: Functions and regulation of caspases. *Microbiology and Molecular Biology Reviews, 64* (4), 821-846.

Chatzistamatiou, K., Moysiadis, T., Vryzas, D., Chatzaki, E., Kaufmann, A. M., Koch, I., …Agorastos, T. (2018). Cigarette smoking promotes infection of cervical cells by high-risk human papillomaviruses, but not subsequent E7 oncoprotein expression. *International Journal of Molecular Sciences, 19* (2), 422.

Chelimo, C., Wouldes, T. A., Cameron, L. D., & Elwood, J. M. (2013). Risk factors for and prevention of human papillomaviruses (HPV), genital warts and cervical cancer. *Journal of Infection, 66* (3), 207-217.

Chen, J. J., Long, Z. J., Xu, D. F., Xiao, R. Z., Liu, L. L., Xu, Z. F., …Liu, Q. (2014). Inhibition of autophagy augments the anticancer activity of alpha-mangostin in chronic myeloid leukemia cells. *Leuk Lymphoma, 55* (3), 628-638.

Chen, L.-G., Yang, L.-L., & Wang, C.-C. (2008). Anti-inflammatory activity of mangostins from *Garcinia mangostana*. *Food and Chemical Toxicology, 46* (2), 688-693.

Cocuzza, C. E., Martinelli, M., Sina, F., Piana, A., Sotgiu, G., Dell'Anna, T., ...Musumeci, R. (2017). Human papillomavirus DNA detection in plasma and cervical samples of women with a recent history of low grade or precancerous cervical dysplasia. *PLoS ONE, 12* (11), e0188592.

University of Malaya

Cragg, G. M., & Newman, D. J. (2005). Plants as a source of anti-cancer agents. *Journal of Ethnopharmacology, 100* (1-2), 72-79.

Daniel, D. G., Basiletti, J., Schelover, E., Díaz Vásquez, N., Mario Alonso, J., Marcelo Marín, H., …Alejandra Picconi, M. (2011). *Chlamydia trachomatis* as a probable cofactor in human papillomavirus infection in aboriginal women from Northeastern Argentina. *The Brazilian Journal of Infectious Diseases, 15* (6), 567-572.

De Furia, M. D. (1997). Paclitaxel (Taxol®): A new natural product with major anticancer activity. *International Journal of Phytotherapy and Phytopharmacology, 4* (3), 273-282.

De Murua, E. O., George, M., Pejovic, M. H., Dewailly, J., & Wolff, J. P. (1987). Combination cyclophosphamide, adriamycin, and cis-platinum in recurrent and metastatic cervical carcinoma. *Gynecologic Oncology, 26* (2), 225-227.

De Sanjose, S., Quint, W. G., Alemany, L., Geraets, D. T., Klaustermeier, J. E., Lloveras, B., …Bosch, F. X. (2010). Human papillomavirus genotype attribution in invasive cervical cancer: A retrospective cross-sectional worldwide study. *The Lancet Oncology,* 11 (11), 1048-1056.

Della, T. G., Pilotti, S., de Palo, G., & Rilke, F. (1978). Viral particles in cervical condylomatous lesions. *Tumori, 64* (5), 549-553.

Devi Sampath, P., & Vijayaraghavan, K. (2007). Cardioprotective effect of α-mangostin, a xanthone derivative from mangosteen on tissue defense system against isoproterenol-induced myocardial infarction in rats. *Journal of Biochemical and Molecular Toxicology, 21* (6), 336-339.

Downs, J. L. S., Chura, J. C., Argenta, P. A., Judson, P. L., Ghebre, R., Geller, M. A., & Carson, L. F. (2011). Ifosfamide, paclitaxel, and carboplatin, a novel triplet regimen for advanced, recurrent, or persistent carcinoma of the cervix: A phase II trial. *Gynecologic Oncology, 120* (2), 265-269.

Dueñas-González, A., Cetina-Pérez, L., Oñate-Ocaña, L. F., Rivera, L., Lopez-Graniel, C., González-Enciso, A., & Mohar, A. (2005). Multimodal treatment of locally advanced cervical cancer. *Archives of Medical Research, 36* (2), 129-135.

University of Malaya

Dürst, M., Gissmann, L., Ikenberg, H., & zur Hausen, H. (1983). A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. *Proceedings of the National Academy of Sciences, 80* (12), 3812-3815.

Elmore, S. (2007). Apoptosis: A review of programmed cell death. *Toxicologic Pathology, 35* (4), 495-516.

Emery, J., Lucassen, A., & Murphy, M. (2001). Common hereditary cancers and implications for primary care. *The Lancet, 358* (9275), 56-63.

Faridi, R., Zahra, A., Khan, K., & Idrees, M. (2011). Oncogenic potential of human papillomavirus (HPV) and its relation with cervical cancer. *Virology, 8*, 269.

Ferlay, J., S. I., Ervik, M., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., …Bray, F. (2013). Cancer incidence and mortality worldwide. *GLOBOCAN 2012*.

Ferlay, J., Soerjomataram, I., Ervik, M., Dikshit, R., Eser, S., Mathers, C., …Bray, F. (2015). GLOBOCAN 2012, Cancer incidence and mortality worldwide: *IARC*. Retrieved 8 Feb. 2015.

Foresta, C., Bertoldo, A., Garolla, A., Pizzol, D., Mason, S., Lenzi, A., & De Toni, L. (2013). Human papillomavirus proteins are found in peripheral blood and semen Cd20+ and Cd56+ cells during Hpv-16 semen infection. *BMC Infectious Diseases, 13*, 593-593.

Garcia-Carbonero, R., & Supko, J. G. (2002). Current perspectives on the clinical experience, pharmacology, and continued development of the camptothecins. *Clinical Cancer Research, 8* (3), 641-661.

Gariglio, P., Gutiérrez, J., Cortés, E., & Vázquez, J. (2009). The Role of retinoid deficiency and estrogens as cofactors in cervical cancer. *Archives of Medical Research, 40* (6), 449-465.

Goldstein, M. A., Goodman, A., del Carmen, M. G., & Wilbur, D. C. (2009). Case records of the Massachusetts General Hospital. Case 10-2009. A 23-year-old woman with an abnormal papanicolaou smear. *New England Journal of Medicine, 360* (13), 1337-1344.

University of Malaya

Gordaliza, M. (2007). Natural products as leads to anticancer drugs. *Clinical and Translational Oncology, 9* (12), 767-776.

Grodstein, F., Martinez, M. E., Platz, E. A., Giovannucci, E., Colditz, G. A., Kautzky, M., …Stampfer, M. J. (1998). Postmenopausal hormone use and risk for colorectal cancer and adenoma. *Annals of Internal Medicine, 128* (9), 705-712.

Grossman, S. A., Carson, K. A., Phuphanich, S., Batchelor, T., Peereboom, D., Nabors, L. B., …Consortium, f. (2008). Phase I and pharmacokinetic study of karenitecin in patients with recurrent malignant gliomas. *Journal of Neuro-Oncology, 10* (4), 608-616.

Gundala, S. R., Yang, C., Mukkavilli, R., Paranjpe, R., Brahmbhatt, M., Pannu, V., …Aneja, R. (2014). Hydroxychavicol, a betel leaf component, inhibits prostate cancer through ROS-driven DNA damage and apoptosis. *Toxicology and Applied Pharmacology, 280* (1), 86-96.

Hall, M. T., Simms, K. T., Lew, J.-B., Smith, M. A., Saville, M., & Canfell, K. (2018). Projected future impact of HPV vaccination and primary HPV screening on cervical cancer rates from 2017–2035: Example from Australia. *PLoS ONE, 13* (2), e0185332.

Häring, J., Schüler, S., Lattrich, C., Ortmann, O., & Treeck, O. (2012). Role of estrogen receptor β in gynecological cancer. *Gynecologic Oncology, 127* (3), 673-676.

Hasima, N., Aun, L. I. L., Azmi, M. N., Aziz, A. N., Thirthagiri, E., Ibrahim, H., & Awang, K. (2010). 1′S-1′-Acetoxyeugenol acetate: A new chemotherapeutic natural compound against MCF-7 human breast cancer cells. *International Journal of Phytotherapy and Phytopharmacology, 17* (12), 935-939.

Hernandez, B. Y., Wilkens, L. R., Zhu, X., Thompson, P., McDuffie, K., Shvetsov, Y. B., …Goodman, M. T. (2008). Transmission of human papillomavirus in heterosexual couples. *Emerging Infectious Diseases, 14* (6), 888-894.

Higgins, R., Bussey, M., Naumann, W., Hall, J., Tait, D., & Haake, M. (2007). Concurrent carboplatin and paclitaxel with pelvic radiation therapy in the primary treatment of cervical cancer. *American Journal of Obstetrics and Gynecology, 197* (2), 205.e201-205.e207.

University of Malaya

Huo, D., Anderson, D., Palmer, J. R., & Herbst, A. L. (2017). Incidence rates and risks of diethylstilbestrol-related clear-cell adenocarcinoma of the vagina and cervix: Update after 40-year follow-up. *Gynecologic Oncology, 146* (3), 566-571.

Ibrahim, M. Y., Hashim, N. M., Mariod, A. A., Mohan, S., Abdulla, M. A., Abdelwahab, S. I., & Arbab, I. A. (2016). α-Mangostin from *Garcinia mangostana Linn*: An updated review of its pharmacological properties. *Arabian Journal of Chemistry, 9* (3), 317-329.

Ibrahim , M. Y., Hashim, N. M., Mohan, S., Abdulla, M. A., Abdelwahab, S. I., Arbab, I. A., …Ishag, O. E. (2015). α-Mangostin from *Cratoxylum arborescens*: An *in vitro* and *in vivo* toxicological evaluation. *Arabian Journal of Chemistry, 8* (1), 129-137.

Ibrahim , M. Y., Hashim, N. M., Mohan, S., Abdulla, M. A., Abdelwahab, S. I., Kamalidehghan, B., …Ali, H. M. (2014). Involvement of NF-κB and HSP70 signaling pathways in the apoptosis of MDA-MB-231 cells induced by a prenylated xanthone compound, α-mangostin, from *Cratoxylum arborescens*. *Drug Design, Development and Therapy, 8*, 2193-2211.

Islaslab-Wiki, (2015). Cervical cancer: *What Is Cervical Cancer?* Retrieved from https://islaslab.wikispaces.com/Cervical/Cancer.

Itharat, A., Houghton, P. J., Eno-Amooquaye, E., Burke, P. J., Sampson, J. H., & Raman, A. (2004). *In vitro* cytotoxic activity of Thai medicinal plants used traditionally to treat cancer. *Journal of ethnopharmacology, 90* (1), 33-38.

Jérôme, A., Frédéric, B., Carole, N., Christiane, C., Alexis, L., Loïc, G., Bernard, W., & François, G. (2006). Accumulation of hydrogen peroxide is an early and crucial step for paclitaxel-induced cancer cell death both *in vitro and in vivo. International Journal of Cancer*, 119 (1), 41-48.

Jo's-trust. (2016). Cervical cancer. Abnormal cervical cells and treatment: Cells of the cervix and uterus. Retrieved from https://www.jostrust.org.uk/about-cervical-cancer/the-cervix.

Kane, M. A. (2012). Preventing cancer with vaccines: Progress in the global control of cancer. *Cancer Prevention Research (Philadelphia), 5* (1), 24-29.

University of Malaya

Kaomongkolgit, R., Chaisomboon, N., & Pavasant, P. (2011). Apoptotic effect of alpha-mangostin on head and neck squamous carcinoma cells. *Archives of Oral Biology, 56* (5), 483-490.

Kaomongkolgit, R., Jamdee, K., & Chaisomboon, N. (2009). Antifungal activity of alpha-mangostin against *Candida albicans*. *Journal of Oral Science*, 51 (3), 401-406.

Kelley, J. R., & Duggan, J. M. (2003). Gastric cancer epidemiology and risk factors. *Journal of Clinical Epidemiology, 56* (1), 1-9.

Keiser, J., Vargas, M., & Winter, R. (2012). Anthelminthic properties of mangostin and mangostin diacetate. *Parasitology International*, 61 (2), 369-371.

Kesic, V. (2006). Management of cervical cancer. *European Journal of Surgical Oncology, 32* (8), 832-837.

Kim, K., Chie, E. K., Wu, H.-G., Ha, S. W., Kim, J. S., Kim, I. A., & Lee, H.-P. (2006). Efficacy of paclitaxel and carboplatin as a regimen for postoperative concurrent chemoradiotherapy of high risk uterine cervix cancer. *Gynecologic Oncology, 101* (3), 398-402.

Kushi, L. H., Doyle, C., Cullough, M., Rock, C. L., Demark-Wahnefried, W., Bandera, E. V., ...Gansler, T. (2012). American cancer society guidelines on nutrition and physical activity for cancer prevention. *CA Cancer J Clin*, 62 (1), 30-67.

Liou, G.-Y., & Storz, P. (2010). Reactive oxygen species in cancer. *Free Radical Research, 44* (5), 10.3109/10715761003667554.

Loehrer, P. J., & Einhorn, L. H. (1984). Drugs five years later. Cisplatin. *Annals of Internal Medicine, 100* (5), 704-713.

Lu, K., & Burke, T. (2000). Early cervical cancer. *Current Treatment Options in Oncology, 1* (2), 147-155.

University of Malaya

Lynch, H. T., Snyder, C., & Casey, M. J. (2013). Hereditary ovarian and breast cancer: what have we learned? *Annals of Oncology, 24* (8), 83-95.

Mabuchi, S., Morishige, K., Fujita, M., Tsutsui, T., Sakata, M., Enomoto, T., & Kimura, T. (2009). The activity of carboplatin and paclitaxel for recurrent cervical cancer after definitive radiotherapy. *Gynecologic Oncology, 113* (2), 200-204.

Mahabir, S. (2013). Association between diet during preadolescence and adolescence and risk for breast cancer during adulthood. *Journal of Adolescent Health, 52* (5, Supplement), 30-35.

Mahabusarakam, W., Proudfoot, J., Taylor, W., & Croft, K. (2000). Inhibition of lipoprotein oxidation by prenylated xanthones derived from mangostin. *Free Radical Research,* 33 (5), 643-59.

Majno, G., & Joris, I. (1995). Apoptosis, oncosis, and necrosis. An overview of cell death. *The American Journal of Pathology, 146* (1), 3-15.

Mathevet, P., Laszlo de Kaszon, E., & Dargent, D. (2003). Fertility preservation in early cervical cancer. *Gynecologie, Obstetrique and Fertilite, 31* (9), 706-712.

Matsumoto, K., Akao, Y., Ohguchi, K., Ito, T., Tanaka, T., Iinuma, M., & Nozawa, Y. (2005). Xanthones induce cell-cycle arrest and apoptosis in human colon cancer DLD-1 cells. *Bioorganic and Medicinal Chemistry, 13* (21), 6064-6069.

Matsumoto, K., Akao, Y., Yi, H., Ohguchi, K., Ito, T., Tanaka, T., …Nozawa, Y. (2003). Preferential target is mitochondria in α-mangostin-induced apoptosis in human leukemia HL60 cells. *Bioorganic and Medicinal Chemistry, 12* (22), 5799-5806.

McGraw, S. L., & Ferrante, J. M. (2014). Update on prevention and screening of cervical cancer. *World Journal of Clinical Oncology, 5* (4), 744-752.

McIlwain, D. R., Berger, T., & Mak, T. W. (2013). Caspase functions in cell death and disease. *Cold Spring Harbor Perspectives in Biology, 5* (4), 1-28.

Meisels, A., & Fortin, R. (1977). Condylomatous Lesions Of The Cervix And Vagina. I. Cytologic Patterns. *Obstetrical and Gynecological, 32* (5), 322-324.

University of Malaya

Mendes, D., Mesher, D., Pista, A., Baguelin, M., & Jit, M. (2018). Understanding differences in cervical cancer incidence in Western Europe: Comparing Portugal and England. *Eur J Public Health, 28* (2), 343-347.

Michels, K. B., & Ekbom, A. (2004). CAloric restriction and incidence of breast cancer. *The Journal of the American Medical Association, 291* (10), 1226-1230.

Miller, J. W., Hanson, V., Johnson, G. D., Royalty, J. E., & Richardson, L. C. (2014). From cancer screening to treatment: service delivery and referral in the national breast and cervical cancer early detection program. *Cancer, 120* (0 16), 2549-2556.

Min, K.-J., Lee, J.-K., So, K. A., & Kim, M. K. (2018). Association between passive smoking and the risk of cervical intraepithelial neoplasia 1 in korean women. *Journal of Epidemiology, 28* (1), 48-53.

Mishra, B. B1., & Tiwari, V. K. (2011). Natural products: An evolving role in future drug discovery. *European Journal of Medicinal Chemistry,* 46 (10), 4769-807.

Mohan , S., Bustamam, A., Ibrahim, S., Al-Zubairi, A. S., Aspollah, M., Abdullah, R., & Elhassan, M. M. (2011). *In Vitro* ultramorphological assessment of apoptosis on CEMss induced by linoleic acid-rich fraction from *typhonium flagelliforme* tuber. *Evidence-Based Complementary and Alternative Medicine, 2011*, 1-12.

Monsuez, J.-J., Charniot, J.-C., Vignat, N., & Artigou, J.-Y. (2010). Cardiac side-effects of cancer chemotherapy. *International Journal of Cardiology, 144* (1), 3-15.

Moore, D. H., Blessing, J. A., McQuellon, R. P., Thaler, H. T., Cella, D., Benda, J., …Rocereto, T. F. (2004). Phase III Study of cisplatin with or without paclitaxel in stage IVB, recurrent, or persistent squamous cell carcinoma of the cervix: A gynecologic oncology group study. *Journal of Clinical Oncology, 22* (15), 3113-3119.

Morelli, C. F., Biagiotti, M., Pappalardo, V. M., Rabuffetti, M., & Speranza, G. (2015). Chemistry of alpha-mangostin. Studies on the semisynthesis of minor xanthones from *Garcinia mangostana*. *Natural Product Research, 29* (8), 750-755.

University of Malaya

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods, 65* (1–2), 55-63.

Nakagawa, Y., Iinuma, M., Naoe, T., Nozawa, Y., & Akao, Y. (2007). Characterized mechanism of α-mangostin-induced cell death: Caspase-independent apoptosis with release of endonuclease-G from mitochondria and increased miR-143 expression in human colorectal cancer DLD-1 cells. *Bioorganic and Medicinal Chemistry, 15* (16), 5620-5628.

Nanda, K., Bastian, L. A., Hasselblad, V., & Simel, D. L. (1999). Hormone replacement therapy and the risk of colorectal cancer: A meta-analysis. *Obstetrics and Gynecology, 93* (5, Part 2), 880-888.

Ng, J., & Shuryak, I. (2015). Minimizing second cancer risk following radiotherapy: Current perspectives. *Cancer Management and Research, 7*, 1-11.

Nicoletti, I., Migliorati, G., Pagliacci, M. C., Grignani, F., & Riccardi, C. (1991). A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *Journal of Immunological Methods, 139* (2), 271-279.

Nour, N. M. (2009). Cervical cancer: A preventable death. *Reviews in Obstetrics and Gynecology, 2* (4), 240-244.

Nowakowska, Z. (2007). A review of anti-infective and anti-inflammatory chalcones. *European Journal of Medicinal Chemistry, 42* (2), 125-137.

Olaitan, A. (2014). Diagnosing cervical cancer: What does screening involve? Colposcopy. Retrieved from https://www.totalhealth.co.uk/clinical-experts/dr-adeola-olaitan/diagnosing-cervical-cancer.

Ortoski, R. A., & Kell, C. S. (2011). Anal cancer and screening guidelines for human papillomavirus in men. *The Journal of the American Osteopathic Association, 111* (3), 35-43.

University of Malaya

Paiva, A., Sousa, M., Camões, A., Nascimento, M., & Pinto, M. (2012). Prenylated xanthones: Antiproliferative effects and enhancement of the growth inhibitory action of 4-hydroxytamoxifen in estrogen receptor-positive breast cancer cell line. *Medicinal Chemistry Research, 21* (5), 552-558.

Pan, L., Chai, H.-B., & Kinghorn, A. D. (2012). Discovery of new anticancer agents from higher plants. *Frontiers in Bioscience (Scholar Edition), 4*, 142-156.

Papadimitriou, C. A., Sarris, K., Moulopoulos, L. A., Fountzilas, G., Anagnostopoulos, A., Voulgaris, Z., …Dimopoulos, M. A. (1999). Phase II trial of paclitaxel and cisplatin in metastatic and recurrent carcinoma of the uterine cervix. *Journal of Clinical Oncology, 17* (3), 761.

Patel, A., & Jackson, B. (2018). Low-dose radiation use in diagnostic imaging and cancer therapy settings. *Radiologia Medica, 123* (8), 618-619.

Pedraza-Chaverri, J., Cardenas-Rodriguez, N., Orozco-Ibarra, M., & Perez-Rojas, J. M. (2008). Medicinal properties of mangosteen (*Garcinia mangostana*). *Food and Chemical Toxicology, 46* (10), 3227-3239.

Perez, E. A. (2009). Microtubule inhibitors: Differentiating tubulin-inhibiting agents based on mechanisms of action, clinical activity, and resistance. *Mol Cancer Ther, 8* (8), 2086-2095.

Petitjean, A., Achatz, M. I. W., Borresen-Dale, A. L., Hainaut, P., & Olivier, M. (2007). TP53 mutations in human cancers: Functional selection and impact on cancer prognosis and outcomes. *Oncogene, 26* (15), 2157-2165.

Petrelli, F., De Stefani, A., Raspagliesi, F., Lorusso, D., & Barni, S. (2014). Radiotherapy with concurrent cisplatin-based doublet or weekly cisplatin for cervical cancer: A systematic review and meta-analysis. *Gynecologic Oncology, 134* (1), 166-171.

Pleasance, E. D., Stephens, P. J., O'Meara, S., McBride, D. J., Meynert, A., Jones, D., …Campbell, P. J. (2009). A small-cell lung cancer genome with complex signatures of tobacco exposure. *Nature, 463*, 184.

Plummer, M., de Martel, C., Vignat, J., Ferlay, J., Bray, F., & Franceschi, S. (2016). Global burden of cancers attributable to infections in 2012: a synthetic analysis. *Lancet Glob Health, 4* (9), e609-616.

University of Malaya

Pucci, B., Kasten, M., & Giordano, A. (2000). Cell cycle and apoptosis. *Neoplasia, 2* (4), 291-299.

Quan, W., Hur, K. Y., Lim, Y., Oh, S. H., Lee, J. C., Kim, H. C., …Lee, M. K. (2012). Autophagy deficiency in beta cells leads to compromised unfolded protein response and progression from obesity to diabetes in mice. *Diabetologia,* 55 (2), 392–403.

Reid, R. (1983). Genital warts and cervical cancer: II. Is human papillomavirus infection the trigger to cervical carcinogenesis? *Gynecologic Oncology, 15* (2), 239-252.

Richart, R. M. (1973). Cervical intraepithelial neoplasia. *Pathology annual, 8*, 301-328.

Riman, T., Dickman, P. W., Nilsson, S., Correia, N., Nordlinder, H., Magnusson, C. M., …Persson, I. R. (2002). Hormone replacement therapy and the risk of invasive epithelial ovarian cancer in swedish women. *Journal of the National Cancer Institute, 94* (7), 497-504.

Rose , P. G., Blessing, J. A., Gershenson, D. M., & McGehee, R. (1999). Paclitaxel and cisplatin as first-line therapy in recurrent or advanced squamous cell carcinoma of the cervix: A gynecologic oncology group study. *Journal of Clinical Oncology, 17* (9), 2676.

Rose, P. G., Bundy, B. N., Watkins, E. B., Thigpen, J. T., Deppe, G., Maiman, M. A., …Insalaco, S. (1999). Concurrent cisplatin-based radiotherapy and chemotherapy for locally advanced cervical cancer. *New England Journal of Medicine, 340* (15), 1144-1153.

Roura, E., Travier, N., Waterboer, T., de Sanjosé, S., Bosch, F. X., Pawlita, M., …Castellsagué, X. (2016). The Influence of hormonal factors on the risk of developing cervical cancer and pre-cancer: Results from the EPIC cohort. *PLOS ONE, 11* (1), e0147029.

Sabharwal, S. S., & Schumacker, P. T. (2014). Mitochondrial ROS in cancer: Initiators, amplifiers or an Achilles' heel? *Nature Review Cancer, 14* (11), 709-721.

University of Malaya

Sakagami, Y., Iinuma, M., Piyasena, K. G., & Dharmaratne, H. R. (2005). Antibacterial activity of alpha-mangostin against vancomycin resistant *Enterococci* (VRE) and synergism with antibiotics. *Phytomedicine*, 12 (3), 203-208.

Saleh, A. M., El-Abadelah, M. M., Aziz, M. A., Taha, M. O., Nasr, A., & Rizvi, S. A. A. (2015). Antiproliferative activity of the isoindigo 5′-Br in HL-60 cells is mediated by apoptosis, dysregulation of mitochondrial functions and arresting cell cycle at G0/G1 phase. *Cancer Letters, 361* (2), 251-261.

Salim , L., Mohan, S., Othman, R., Abdelwahab, S., Kamalidehghan, B., Sheikh, B., & Ibrahim, M. (2013). Thymoquinone induces mitochondria-mediated apoptosis in acute lymphoblastic leukaemia *in vitro*. *Molecules, 18* (9), 11219-11240.

Samaras, V., Rafailidis, P. I., Mourtzoukou, E. G., Peppas, G., & Falagas, M. E. (2010). *Chronic bacterial and parasitic infections and cancer: A review. Journal of Infection in Developing Countries, 4 (5)*, 267-81.

Saslow, D., Solomon, D., Lawson, H. W., Killackey, M., Kulasingam, S., Cain, J., …Myers, E. R. (2012). American cancer society, american society for colposcopy and cervical pathology, and american society for clinical pathology screening guidelines for the prevention and early detection of cervical cancer. *CA: A Cancer Journal for Clinicians, 62* (3), 147-172.

Savard, J., Ivers, H., Savard, M.-H., & Morin, C. M. (2015). Cancer treatments and their side effects are associated with aggravation of insomnia: Results of a longitudinal study. *Cancer, 121* (10), 1703-1711.

Shafei, M. N., Zainon, N., Zulkifli, N. F., & Ibrahim, M. I. (2014). Knowledge and perception on human papilloma virus infection and vaccination among medical students of a university in Malaysia. *Procedia - Social and Behavioral Sciences, 116* (0), 2707-2710.

Shang, Y. (2007). Hormones and cancer. *Cell Research, 17* (4), 277-279.

Sharma, A., Chakravarti, B., Gupt, M. P., Siddiqui, J. A., Konwar, R., & Tripathi, R. P. (2010). Synthesis and anti-breast cancer activity of biphenyl based chalcones. *Bioorganic & Medicinal Chemistry, 18* (13), 4711-4720.

University of Malaya

Shier, W. T. (1991). Mammalian cell culture on $5 a day: A laboratory manual of low cost methods. *Los Banos, University of the Philippines, 64* (8), 9-16.

Shoja, M. H., Reddy, N. D., Nayak, P. G., Srinivasan, K. K., & Rao, C. M. (2015). *Glycosmis pentaphylla* (Retz.) DC arrests cell cycle and induces apoptosis via caspase-3/7 activation in breast cancer cells. *Journal of Ethnopharmacology, 168* (0), 50-60.

Sidahmed , H. M. A., Abdelwahab, S. I., Mohan, S., Abdulla, M. A., Mohamed Elhassan Taha, M., Hashim, N. M., …Yahayu, M. (2013). α-Mangostin from *Cratoxylum arborescens* (Vahl) Blume demonstrates anti-ulcerogenic property: A mechanistic study. *Evidence-Based Complementary and Alternative Medicine, 2013*, 1-10.

Siegel, R., DeSantis, C., Virgo, K., Stein, K., Mariotto, A., Smith, T., …Ward, E. (2012). Cancer treatment and survivorship statistics. *CA : A Cancer Journal for Clinicians, 62* (4), 220-241.

Sinal, S. H., & Woods, C. R. (2005). Human papillomavirus infections of the genital and respiratory tracts in young children. *Seminars in Pediatric Infectious Diseases, 16* (4), 306-316.

Singh, R. B., Chander, S., Mohanti, B. K., Pathy, S., Kumar, S., Bhatla, N., …Kumar, L. (2013). Neoadjuvant chemotherapy with weekly paclitaxel and carboplatin followed by chemoradiation in locally advanced cervical carcinoma: A pilot study. *Gynecologic Oncology, 129* (1), 124-128.

Sitzia, J., & Huggins, L. (1998). Side effects of cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) chemotherapy for breast cancer. *Cancer Practice, 6* (1), 13-21.

Sogukpınar, N., Saydam, B. K., Can, H. O., Hadımlı, A., Bozkurt, O. D., Yücel, U., Kocak, Y. C., Akmese, Z. B., Demir, D., & Ceber, E. (2013). Assessment of cervical cancer risk in women between 15 and 49 years of age: case of Izmir. *Asian Pacific Journal of Cancer Prevention, 14* (3), 2119-2125.

Solomon, D., Davey, D., Kurman, R., Moriarty, A., O'Connor, D., Prey, M., …Young, N. (2002). The 2001 Bethesda System: Terminology for reporting results of cervical cytology. *The Journal of the American Medical Association, 287* (16), 2114-2119.

University of Malaya

Stähelin, H. F., & von Wartburg, A. (1991). The Chemical and biological route from *Podophyllotoxin* glucoside to etoposide: Ninth cain memorial award lecture. *Cancer Research, 51* (1), 5-15.

Stanley, M., Winder, D., Sterling, J., & Goon, P. (2012). HPV infection, anal intra-epithelial neoplasia (AIN) and anal cancer: Current issues. *BMC Cancer, 12* (1), 398.

Suksamrarn S., Komutiban O., Ratananukul P., Chimnoi N., Lartpornmatulee N., Suksamrarn A. (2006). Cytotoxic prenylated xanthones from the young fruit of *Garcinia mangostana*. *Chemecal and Pharmaceutical Bulletin.* (Tokyo), 54 (3), 301–305.

Tornesello, M. L., Buonaguro, L., & Buonaguro, F. M. (2013). Mutations of the TP53 gene in adenocarcinoma and squamous cell carcinoma of the cervix: A systematic review. *Gynecologic Oncology, 128* (3), 442-448.

Van Kriekinge, G., Castellsague, X., Cibula, D., & Demarteau, N. (2014). Estimation of the potential overall impact of human papillomavirus vaccination on cervical cancer cases and deaths. *Vaccine, 32* (6), 733-739.

Vega-Avila, E., & Pugsley, M. K. (2011). An overview of colorimetric assay methods used to assess survival or proliferation of mammalian cells. *Proceedings of the Western Pharmacology Society, 54*, 10-14.

Vitale, I., Manic, G., Kroemer, G., & Galluzzi, L. (2016). Mitochondria in cell death regulation. In R. A. Bradshaw & P. D. Stahl (Eds.), *Encyclopedia of Cell Biology* (pp. 341-353). Waltham: Academic Press.

Weinstock, H., Berman, S., & Cates, W., Jr. (2004). Sexually transmitted diseases among American youth: Incidence and prevalence estimates, 2000. *Perspect Sex Reprod Health, 36* (1), 6-10.

Winer, R. L., Hughes, J. P., Feng, Q., Xi, L. F., Cherne, S., O'Reilly, S., Kiviat, N. B., & Koutsky, L. A. (2010). Detection of genital HPV types in fingertip samples from newly sexually active female university students. *Cancer Epidemiology, Biomarkers & Prevention, 19* (7), 1682-1685.

University of Malaya

Winer, R. L., Lee, S.-K., Hughes, J. P., Adam, D. E., Kiviat, N. B., & Koutsky, L. A. (2003). Genital human papillomavirus infection: Incidence and risk factors in a cohort of female university students. *American Journal of Epidemiology, 157* (3), 218-226.

Wong , L. P., Wong, Y. L., Low, W. Y., Khoo, E. M., & Shuib, R. (2009). Knowledge and awareness of cervical cancer and screening among Malaysian women who have never had a Pap smear: A qualitative study. *Singapore Medical Journal, 50* (1), 49-53.

Wong, Y.-L., Chinna, K., Mariapun, J., & Shuib, R. (2013). Correlates between risk perceptions of cervical cancer and screening practice. *Preventive Medicine, 57*, 24-26.

Wright, T. C., Jr., Massad, L. S., Dunton, C. J., Spitzer, M., Wilkinson, E. J., & Solomon, D. (2007). 2006 consensus guidelines for the management of women with cervical intraepithelial neoplasia or adenocarcinoma *in situ*. *Journal of Lower Genital Tract Disease, 11* (4), 223-239.

Wyre, H., & Thrasher, J. B. (2016). Chapter 21 - Effects of smoking, alcohol, and exercise on prostate cancer. In J. H. M. J. Godec (Ed.), *Prostate Cancer (2nd Edition)* (pp. 183-189). San Diego: Academic Press.

Yang, L., Zhou, Y., Li, Y., Zhou, J., Wu, Y., Cui, Y., …Hong, Y. (2015). Mutations of p53 and KRAS activate NF-κB to promote chemoresistance and tumorigenesis via dysregulation of cell cycle and suppression of apoptosis in lung cancer cells. *Cancer Letters, 357* (2), 520-526.

Yin, S.-Y., Wei, W.-C., Jian, F.-Y., & Yang, N.-S. (2013). Therapeutic Applications of herbal medicines for cancer patients. *Evidence-based Complementary and Alternative Medicine : eCAM, 2013*, 302426.

Yoo, J.-H., Kang, K., Jho, E. H., Chin, Y.-W., Kim, J., & Nho, C. W. (2011). α- and γ-Mangostin inhibit the proliferation of colon cancer cells via β-catenin gene regulation in Wnt/cGMP signalling. *Food Chemistry, 129* (4), 1559-1566.

Zaridah, S. (2014). A review of cervical cancer research in Malaysia. *Medical Journal of Malaysia, 69* (A), 33-41.

University of Malaya

Zhang, X., Dai, B., Zhang, B., & Wang, Z. (2012). Vitamin A and risk of cervical cancer: A meta-analysis. *Gynecologic Oncology, 124* (2), 366-373.

Zhang, X., Samadi, A. K., Roby, K. F., Timmermann, B., & Cohen, M. S. (2012). Inhibition of cell growth and induction of apoptosis in ovarian carcinoma cell lines CaOV3 and SKOV3 by natural withanolide Withaferin A. *Gynecologic Oncology, 124* (3), 606-612.

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List of Publications and Papers Presented

Peer Reviewed Journal Papers

1. *In vitro* Assessment of the anti-cancer effect induced by α-mangostin from Cratoxylum arborescens on HeLa cells. Has been published for publication in *PeerJ*.

Conference Proceedings

1. International Conference on Chemical, Agriculture and Medical Science (CAMS-2013). 28-29th Dec, 2013. *In Vitro* assessment of anti-proliferative effect induced by α-Mangostin on Cervical Cancer. Kuala Lumpur, Malaysia.

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