P53 AND E7 EXPRESSION STATUS IN HUMAN CERVICAL CARCINOMA CELL (HELA) TREATED WITH *QUERCUS INFECTORIA* AQUEOUS EXTRACT

By

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LIST OF SYMBOL

%	percentage
°C	degree Celcious
µg/ml	microgram per mililiter
µl	microliter
cm	centimeter
cm^3	centimeter cube
g	gram
h	hour
mg	milligram
mg/ml	milligram per mililiter
ml	milliliter
rpm	rotation per minute

LIST OF ABBREVIATION

BSC	Biosafety cabinet
CO_2	Carbon Dioxide
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EDTA	ethylenediamineteraacetic acid
FBS	Fetal Bovine Serum
HCl	Hydrochloride acid
IC_{50}	Inhibition concentration of 50% cell population
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
Pen-Strep	Penicillin-Streptomycin
PBS	Phosphate Buffer Saline
QI	Quercus Infectoria
WHO	World Health Organization

ABSTRAK

Pencarian agen anti kanser novel telah berubah daripada agen sitotoksik kepada agen sasaran biologi (yang merupakan anti proliferatif) untuk mengelak kesan toksik terapi anti kanser dan mencipta agen yang lebih selektif dan efektif. Perencatan pertumbuhan sel kanser merupakan penanda aras untuk status penurunan pertumbuhan sel dalam rawatan kanser. Dalam hal ini, penggunaan herba adalah disyorkan dalam rawatan kanser. Ekstrak *Quercus infectoria* (manjakani) telah dilaporkan berpotensi sebagai agen anti proliferatif terhadap sel kanser servik, HeLa. Walau bagaimanapun, mekanisma anti proliferatif masih belum ditemui. Justeru itu, kajian semasa dijalankan menggunakan ekstrak akues *Q.infectoria* untuk menyelidik sebahagian daripada mekanisma anti proliferatif yang dilaporkan oleh kajian lepas. Status ungkapan sampel penindas tumor protein p53 dan protein virus human papilloma E7 yang dirawat telah dianalisa dalam kajian ini. Kajian awal mendapati bahawa aktiviti anti proliferatif ekstrak ialah 17.92± 0.42 μg/ml. Kepekatan ini digunakan untuk menentukan perubahan morfologi dan penyerpihan nukleus sebagai penanda aras apoptosis oleh Hoescht noda. Ungkapan protein p53 dan penindasan HPV E7 dinilai oleh kaedah pemendapan Western dengan masa inkubasi yang berbeza iaitu 24, 48 dan 72 jam. Sel yang dirawat menunjukkan perubahan morfologi dan penyerpihan nukleus. Protein p53 telah terungkap dan protein HPV E7 telah tertindas. Keputusan ini mencadangkan bahawa ekstrak akueus *Q.infectoria* yang bertindak untuk menekan proliferatif sel HeLa oleh apoptosis dengan ungkapan protein p53 dan penekanan HPV E7.

ABSRACT

The search for novel anticancer agents has moved from cytotoxic agents to biologically targeted agents (which are primarily antiproliferative) to avoid the traditional toxic effects of anticancer therapy and to develop more selective and effective agents. Inhibition of cancer cells growth is a hallmark for declining the cell proliferation status in the treatment of cancer. In this regard, herbals are more preferable for alternative agent to treat cancer. A plant gall, *Quercus infectoria* (QI) (manjakani) extract had been reported to have antiproliferative activity towards cervical cancer cell, Hela. However, its antiproliferative mechanism is remaining undiscovered. Therefore, the current study has utilized aqueous extract of *Q. infectoria* to investigate partial of antiproliferative mechanism demonstrated by previous study. The expression status of tumor suppressor protein p53 and human papilloma virus protein E7 treated samples were analyzed in this study. Preliminary study revealed the antiproliferative activity of the extract as 17.92± 0.42 µg/ml. This concentration was used to determine the morphological changes and nuclear fragmentation as hallmark of apoptosis by Hoechst stain. The protein expression of p53 and suppression of HPV-E7 both evaluated by Western Blotting method with respective different incubation time 24, 48 and 72 hours. Treated cells demonstrated the morphological changes and fragmented nuclear. The p53 proteins were expressed and HPV E7 was slightly suppressed. These results suggested that, QI aqueous extracts acted to suppress the Hela cells proliferation by induction of apoptosis with expression of p53 and suppression of HPV E7

CHAPTER 1

INTRODUCTION

1.1 Introduction to research project

In recent years, the search for novel anticancer agents has moved from cytotoxic agent to biologically targeted agents (which are primarily antiproliferative) to avoid the traditional toxic effect of anticancer therapy and to develop more selective and effective agents (Michael Wolf *et al.*, 2004). This present study highlighted the expression of the HPV-E7 and p53 protein as a marker for proliferating cells as well as inhibition of cancer cells growth as a hallmark for declining of cell proliferation status in the treatment of cancer. These proteins also reported as biomarker for human cervical cancer (Lee K., *et al.*, 2011).

On the other hand, the apoptosis event will be investigated to understand the nuclear fragmentation of HeLa cell treated with *Q.infectoria* aqueous respective to the time. This is tp understand the structural and morphological changes that resembles the effect of apoptosis mechanism in the body. Thus, it is of great interest to continue the research investigating of the manjakani extract that exhibited antiproliferative activity towards HeLa carcinoma cells. Apart from this, a mechanism of antiproliferative effect of isolated compound from manjakani extract will be also understood.

According to the World Health Organization in 2012, cervical cancer is known as 4th most common cancer in the world after breast, colorectal and lung cancers. About 528 000 cases had been reported recently and it accounts about 12% of all female cancer. Out of this, 26 600 death cases have been reported from cervical cancer which comprises about 7.5% of all female cancer deaths. This cervical cancer is most commonly found in Africa countries and less developing regions due to lack of access to effective screening and economic status. However, lowestt cases were reported in Austria and New Zealand. This cancer frequently occurs among females of age between 30- 34 which indicate that they are in sexually active in their late teens. In Malaysia , about 2145 new cases has been reported in 2012 and its account as 2nd most female cancer in Malaysia. This case was reported to occur in old women of 15 to 44 years old. Besides, 621 cervical cancer death have been reported which bring up to 5th most causes of female death.

Cervical cancer is cancer that form in the tissue of the cervix and it is a slow growing cancer that is asymptomatic. Nowadays it can be diagnosed with regular Pap Smear test and most likely caused by the Human Papilomavirus as the central cause (Clifford, 2003). There are several factors which lead to this cancer which is due to the lower socioeconomic status, high number of sexual partners, smoking, use of oral contraceptive, and history of Sexual Transmitted Disease(STD) (Bosch, 2002).

Symptom of the cervical cancer can be bleeding that occurs between regular menstrual period, after sexual intercourse or pelvic exam and still going after menopause. Besides , menstrual period can be last longer and heavier than before and increase in vaginal discharge. Moreover, the patient may experience pelvic pain and pain during sex. Cervical cancer can be screening and diagnose by using the Pap smear test, cervical examination

and biopsy tissue sample. Hela is a cancerous cell that contain human papilloma virus (HPV 18) and its morphology is a epithelial cell and has adherent culture properties.

1.2 Objectives of the research project

GENERAL OBJECTIVES :

To evaluate the expression of proliferation and apoptotic protein towards HeLa cells treated with *Quercus infectoria* aqueous extract

SPECIFIC OBJECTIVES:

1. To determine the changes of nuclear morphology in the treated HeLa cells with *Q.infectoria* aqueous extract.
2. To determine the expression of E7 and p53 protein in HeLa cell treated with *Q.infectoria* aqueous extract.
3. To evaluate the mechanism of antiproliferative activity of *Q.infectoria* aqueous towards HeLa cells

1.3 Experimental design

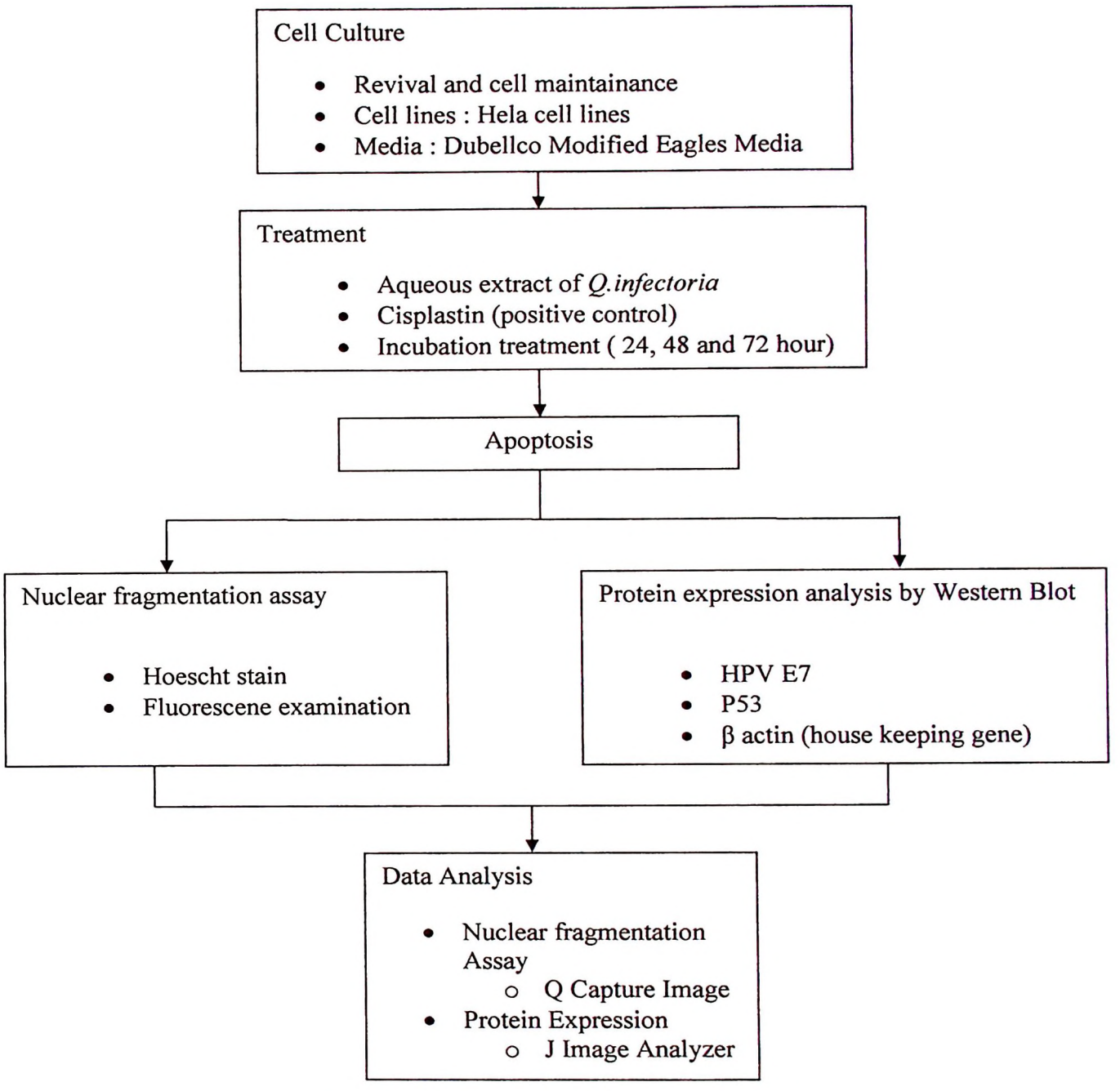

Figure 1.1 the Research Design which explains the IC_{50} of Q.infectoria aqueous extract from preliminiary foundings being utilized to perform determine apoptosis event by nuclear fragmentation assay by mean Hoescht 33342 staining to investigate the morphology changes induced by extract. Further analysis is done by evaluate the protein expression of HPV E7 and p53 protein by western blot analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 Cervical Cancer

Cervical cancer cell is a worldwide disease that infected by the human papillomavirus HPV which are mucosal trophic viruses that infecting basal squamous epithelial cell which are transient infection but in some patient th viral is exhibit in basal squamous epithelial cells of the cervix, underlies of neoplastic progression and emergence of invasive malignancies. The common HPV types were HPV types 16, 18, 31, 33,39,45,52, 58 and 69 which also known as high risk types which give malignant lesion, whereas HPV6 and HPV 11 are considered as low risk type of HPV types which detected in genital warts (condylomata acuminate) (Riley *et al.*, 2003) .

There is major viral oncogene that modified cellular protein which regulates cell cycle such E6 protein which binds to the p53 tumor suppressor protein to degrade the ubiquitin-mediated and trigger the telomerase activity in the cultured keratinocytes. Besides , E7 proteins binds to the pRb (retinoblastoma) and lead to the destabilization and loss of Rb/E2F complexes that repress the transcription of genes required for cell cycle progression (Zur Hausen, 2000). Squamous cell carcinoma represent the most common type of cervical carcinoma which approximately 80-85% and currently, chemotherapy play main rol in the treatment of cervical cancer such as platinum based chemotherapy

concomitant with radiotherapy for locally advanced cervical cancer (Eifel, 1999; DeFilippis *et al.*, 2003; Green *et al.*, 2005).

The epidemiologic study on the association between human papiloma virus and cervical intraepithelial neoplasia have reported that the risk factor that contributing to this disease is a the positive HPV patient is had more sexual partners, cigarette smokers, ealier age at first sexual intercourse and lower socioeconomic status (Schiffman *et al.*, 1993). The main etiologic factor that lead to the cervical cancer is a sexually transmitted disease (STD) just as syphilis, gonorrhea, chlamydia which brought to the longer latent period between infection amd the production of obvious clinical disease such genital tract squamous neoplasm. Besides that, the HPV may induce clinical symptom such caudiflower-like, veneral warts, flat white lesion in the genital tract which cytologically had commonly thought to be associated with the production of squamous cell cancer (Richart, 1987).

Furthermore, treatment for cervical cancer basically, surgery which has two types such as radical trachelectomy(lymph node removal) and complete hysterectomy (removal of the uterus or cervix). Other than that, cervical cancer patient can be treated by the chemotheraphy and radiation theraphy which basically done by diagnosed the patient which have consume the anti cancer drugs to see the rogression of the drug kill the cancer cell (Schiffman *et al.*, 1993). For chemotherapy there are certain side effect for the patient which is depend mainly on which how drug are administered and dosage given. Chemotherapy commonly kills fast-growing cancer cells, but sometimes the drugs can also harm normal cells that divide rapidly such as when chemotherapy kill healthy blood cell, it will lead the person got infection, bruise or bleed easily and feel very weak and tired. Other

than that it will cause hair loss but if it grow back , it may change in color and texture. Furthermore, it will cause poor appetite, nausea and vomiting, diarrhea, or mouth and lip sores (National Cancer Institute, 2012).

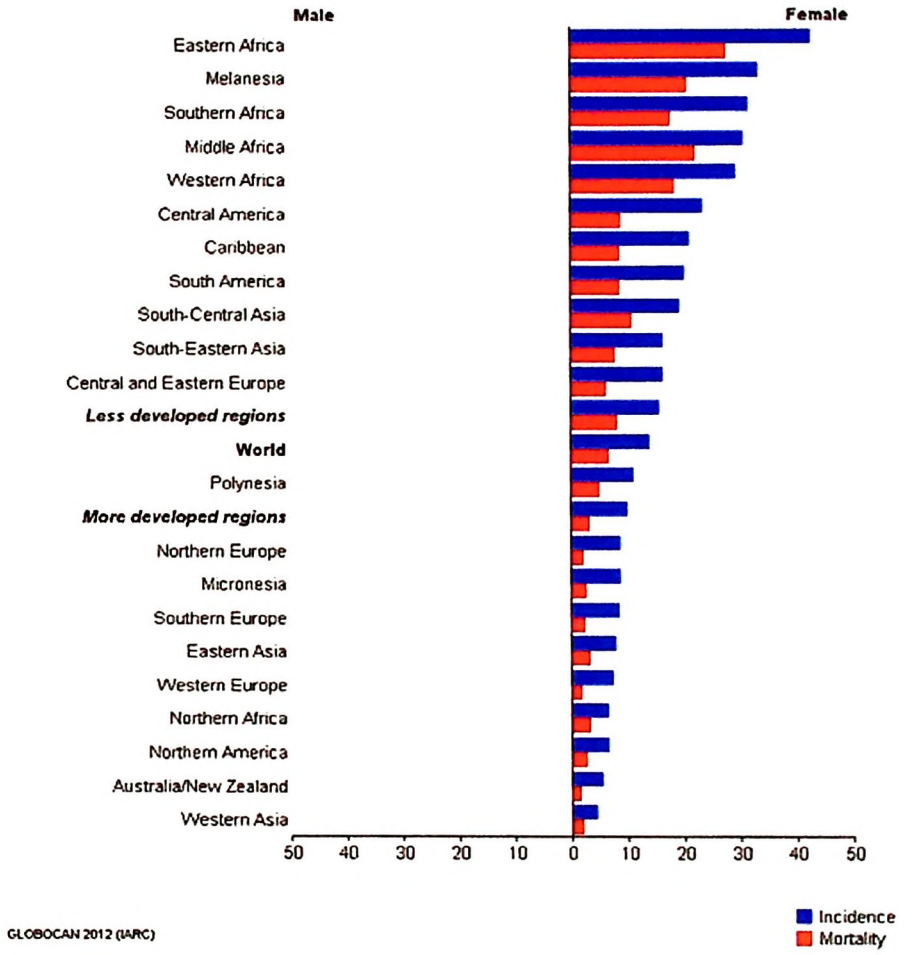

Figure 2.1 Estimated age-standardised rates of Incidence and Mortality cases (World) per 10000 of cervical cancer (GlOBOCON, 2012)

2.2 Cervical Cancer in Malaysia

The incidence of cervical cancer in Malaysia that has been updated on 14 July 2014 reported that about 2145 new cervical cancer cases are diagnosed yearly in Malaysia. Besides that cervical cancer has been ranked as the 2nd cases of female cancer in Malaysia and is the 2nd most common female cancer in women aged 15 to 44 years in Malaysia.

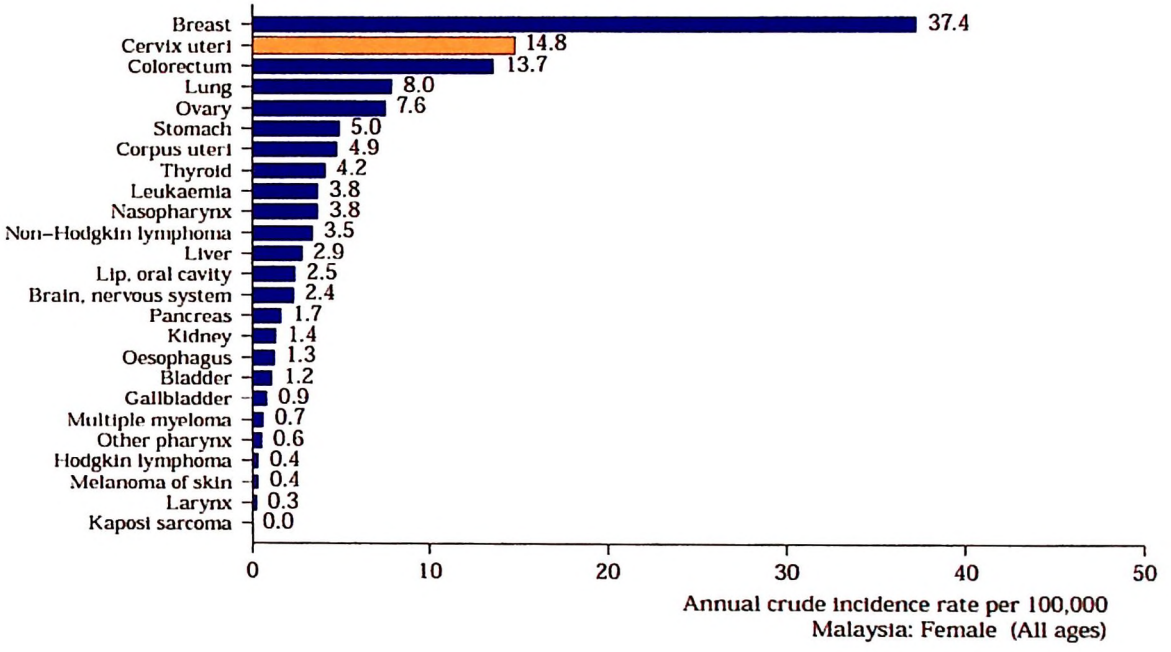

Figure 2.2 Incidence of cervical cancer compared to other cancers in women of all ages in Malaysia (estimation for 2012) (HPV Centre, 2015)

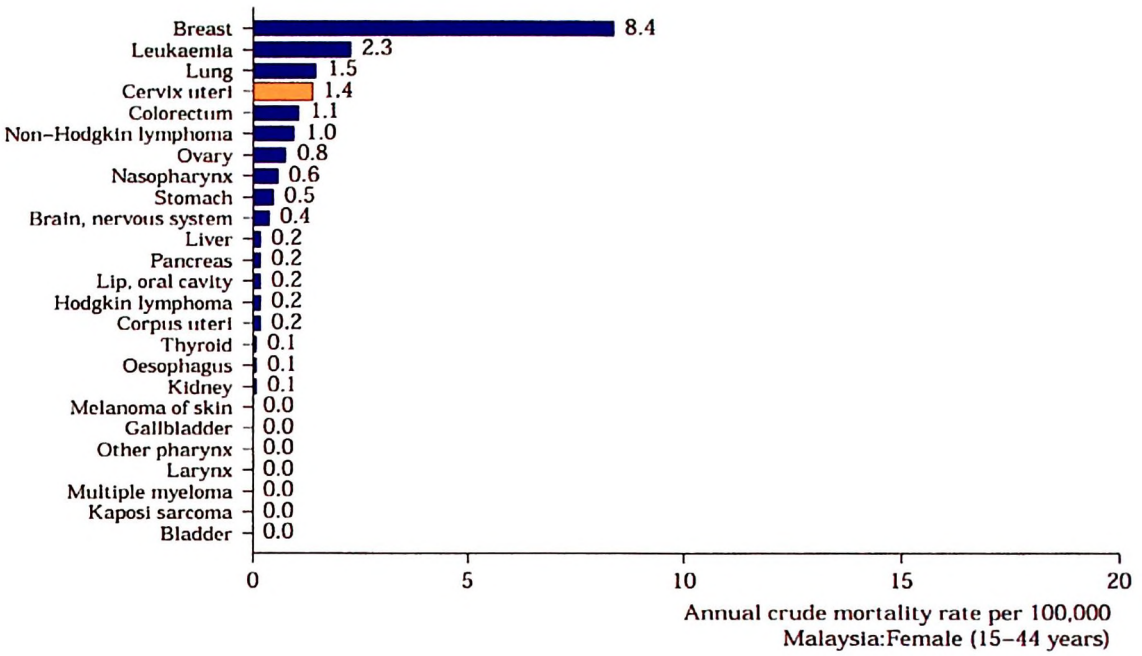

Figure 2.3 Age specific cervical cancer ncidence compared to age specific incidence of others cancers among women 15-44 years of age in Malaysia (estimation for 2012) (HPV Centre, 2015)

Population			
Women at risk for cervical cancer (Female population aged >= 15 yrs)			11.34 millions
Burden of cervical cancer and other HPV-related cancers			
Annual number of cervical cancer cases			2,145
Annual number of cervical cancer deaths			621
Crude incidence rates per 100,000 population and year ‡:		Male	Female
	Cervical cancer	-	14.8
	Anal cancer	0.0-0.3	0.2-0.3
	Vulvar cancer	-	0.2-0.7
	Vaginal cancer	-	0.2-0.7
	Penile cancer	0.1-0.7	-
	Pharynx cancer(excluding nasopharynx)	1.4	0.6
Burden of cervical HPV infection			
Prevalence (%) of HPV 16 and/or HPV 18 among women with:			
	Normal cytology		1.0
	Low-grade cervical lesions (LSIL/CIN-1)		30.4
	High-grade cervical lesions (HSIL/CIN-2/CIN-3/CIS)		49.3
	Cervical cancer		88.6
Other factors contributing to cervical cancer			
Smoking prevalence (%), women			2.6
Total fertility rate (live births per women)			2.4
Oral contraceptive use (%)			13.4
HIV prevalence (%), adults (15-49 years)			0.4 [0.4-0.5]
Sexual behaviour			
Median age at first sexual intercourse among men (25-54 years) and women (25-49 years)			-/-
% of young people (15-24 years) who have had sex before the age of 15 (men/women)			-/-
Cervical screening practices and recommendations			
Cervical cancer screening coverage, % (age and screening interval, reference)	23.0% (All women aged 18-69 yrs screened every 3yrs; WHS Malaysia) 23.6% (Urban women aged 18-69 yrs screened every 3yrs; WHS Malaysia) 21.9% (Rural women aged 18-69 yrs screened every 3yrs; WHS Malaysia)		
Screening ages (years)			20-65
Screening interval (years) or frequency of screens			3
HPV DNA test			No HPV DNA testing
VIA			No visual inspection program
HPV vaccine			
HPV vaccine licensure			
	Bivalent Vaccine (Cervarix)		Yes
	Quadrivalent Vaccine (Gardasil/Silgard)		Yes
HPV vaccine introduction			
	HPV vaccination program		National program
	Date of the HPV vaccination routine immunization programme start		2010
	HPV vaccination target age for routine immunization		13
	Delivery for primary target group		Schools
	Catch-up age group		13-18
	Estimated 3-dose HPV vaccination coverage for routine immunization: % (calendar year)		-

Figure 2.4 Key statistic of cervical cancer on Malaysia (HPV Centre,2015)

Mortality cases of cervical cancer in Malaysia is about 621 new cervical cancer death occur annually which ranked as 5th of female cancer death in Malaysia. Other than that cervical cancer is the 4th leading causes of cancer deaths in women aged among 15-44 years in Malaysia.

2.3 Chemotherapy

Chemotherapy is a method of treatment that use of drugs to destroy cancer cells by stopping the cancer cells growth and proliferation. Chemotherapy treatment is given for the advance cancer stages if surgery is not successive or effective. Chemotherapy is oftenly been delivered through systemic system which is bloodstream to the targeted cancer cells throughout the body. There are two ways chemotherapy ways which is with intravenous (IV) way and oral ways by consume pill or capsule according to the specific number of cycle given over a set period of time by receiving one drug at time or combination of different drugs at the same time.

Chemotherapy treatment is often treated along with radiation therapy to increase the effectiveness of the radiation treatment. According to the National Cancer Institute (n.d) has listed the cancer drugs approved by the Food and Drug Administtration (FDA) that tthe most common chemotherapy drugs given is Cisplatin alone or with combination with other chemotherapy drugs. Sometimes it may be used before surgery or radiotherapy to shrink the cancer and to make the treatments more effective and this way is called neo-adjuvant chemotherapy. Others drugs that most often used in chemotherapy other than Cisplatin are Paclitaxel, Topotecan, Ifosfamide and 5-FU.

However, there are side effect of chemotherapy for cervical cancer such as loss of appetide, brushing, diarrhea, fatigue, hair loss, increased chance of infections, mouth sores after taking orally, nausea and vomiting. Other than that, chemotherapy can damage blood producing cells of the bone marrow which can increased the chance of infection, prolong bleeding or shortnee of breath due to low red blood cell counts. The concurrent

chemoradiation give more severe adverse effect which effect menstrual changes of women and can effect pregnancy in the future, neuropathy such peripheral neuropathy which can lead to numbness, pain, burning , high sensitivity to temperature or weakness and might be cause leukemia because certain chemo drugs can damage permanently the bone marrow and may cause blood cancer such myelodysplastic syndromes or acute myeloid leukemia (American Cancer Society, n.d.).

2.4 HPV E7 and p53

E7 oncoprotein are pleiotropic which bind to the pRb and retinoblastoma protein and enhancing by binding with ubiquination which resulting in phosphorylation of the protein (National Cancer Institute, 2012). According to the Boyer *et al*, (1996) Rb protein is known as tumor suppressor protein that play to control the length of the G_1 phase of cell cycle and being cellular targeted protein that specifically bind by the E7 and hyperphosphorylated which resulting to the uncontrolled cell cycle progression. p53 is posses growth inhibitory and transformation suppressor functions by inducing G1 growth arrest and apoptosis followed by DNA damage (Kastan *et al.*, 1992).

Goodwin *et al.* (2000) reported that the activities of p53 and pRb are inactivated by their degradation by E6 and E7 which accumulates an excess of abnormalities in their genome that cannot be tolerated by p53 which determined that E6 and E7 oncogenes are good therapeutic targets for treatment of cervical cancers. Thus the expression of the E2

protein that repress E6 and E7 leads to reactivation of the p53 and Rb tumor suppressor pathways and cell proliferate (Kastan *et al.*, 1992)

2.5 *Quercus infectoria*

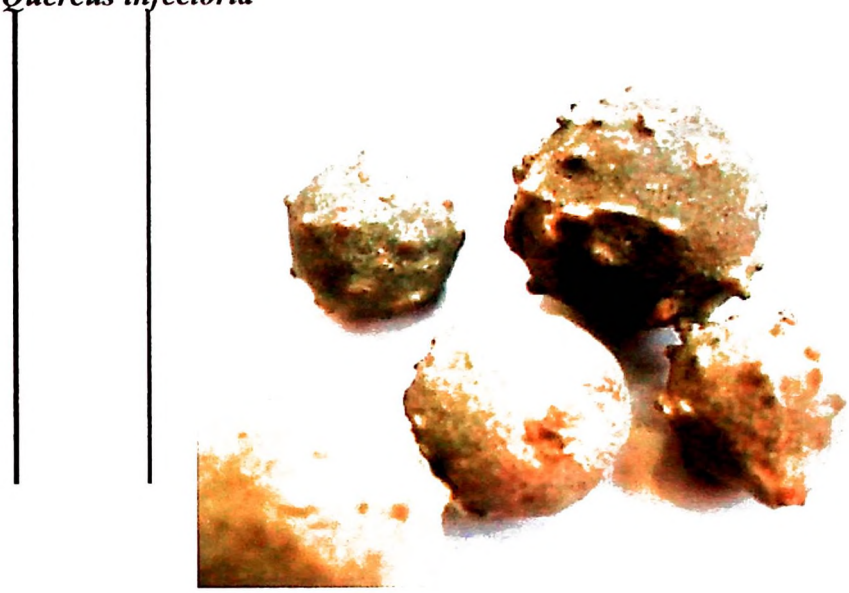

Figure 2.5 The structure of the *Q.infectoria* (manjakani) (Rainforest Herbs, n.d)

Q.infectoria (locally known as manjakani in Malaysia) is the tree of subtropical climates belongs to the Fagaccae family that can be found in Mid Meditterranean, Balkans, Anatolia and Iran. Besides that, this plant can grown in all types of soil even in the range from light acid to light alkali and they called gall and also used in leather industry since it contains of tannin (Goodwin & DiMaio, 2000). In Malaysia, the galls are combined with the other herbs that been used by the women after childbirth to regain the elasticity of the uterine wall (Efe *et al.*, 2011). This plant has been used as home remedy since long time ago by Indian culture to treat sore throat and chronic diarrhea and also used as an ingredient in Ayuverdic preparation. On the other hand, this gall oak also used in tradisional medicine as

"kurkatasringi" as treatment for coughs, phthisis , asthma , skin disease, intestinal hemorrhage, eczema, impetigo, hemorrhage and trichomoniasis.

Besides that, *Q.infectoria* also has been documented as posses anti inflammatory, antiviral, anti diabetic, larvicidal , anti bacterial, anti ulcerogenic and gastroprotective activity (Muhammad & Mustafa, 1994). Gurpreet *et al.* (2004) and team research have done research which shown that the gall extract inhibited the release of lytic enzymes: β-glucoronide, lysozyme from fMLP stimulated neutrophil which influence on the function of neutrophilwhich also be an important part of mechanism of anti-inflammatory activity. The study done by the Gupreet Kaur et al shown that this plant extract exhibit potential a potent free radical scavenging and antioxidant activities which capable of protecting against oxidative damage to the lipids and protein as well as chelates metal ions and also protect cellular systems from oxidative damage (Kaur *et al.*, 2004).

The main pytochemical compound found in the gall of *Q.infectoria* are tannin about (50-70%) and small amount of free gallic acid and ellagic acid (Kaur *et al.*, 2008). Basri *et al.* (2005) reported that the phytochemical *Q.infectoria* which is tannin has high potential as antibacterial agent which provides the insight of usage in the traditional treatment of wounds or burns associated with the bacterial infections(Muhammad & Mustafa, 1994). From the previous research done by Bushra and Dilger (2012) that investigate the cytotoxicity and possible damaging effects on genetic material on somatic and germ cells of Swiss albino mice have state that the effect of different doses of *Q.infectoria* extract on chromosomal abbreaviation such as chromatid breaks, chromosome breaks and centromeric breaks (Basri & Fan, 2005).

Another study done by Hasmah *et al.* (2010) revealed that this plant exhibit antioxidant activity which lead to the anti carcinogenic effect and they have found flavanols which reported that QI have high potential of antiproliferative agent towards cervical and ovarian carcinoma cell.

2.5 Apoptosis

Apoptosis is a pathway of cell death that is induced by regulated suicide program in which cell is destined to die by activated enzyme of degradation of cellular DNA and nuclear and cytoplasmic proteins. The apoptosis (meaning falling off) shown by the fragment of the apoptotic cells then break off giving the appearance. The plasma membrane of the apoptotic cell remain intact but the membrane already disturbed in such away and triggered the phagocytes to eliminate the cell. Thus, the damage cell is rapidly clear out before the content burst out and may avoid inflammatory reaction in the host (Kumar et al, 2007).

Apoptosis has been hallmark for the regulation of tumor formation and determinant of the treatment response. The activation of the apoptosis signal transduction pathways in cancer cells such as intrinsic and extrinsic pathways has been linked by most of anticancer strategies which currently used in clinical oncology. There are two pathways involve in the apoptosis mechanism which is first is extrinsic pathway which refer to the cytoplasmic pathway which triggered through the tumor necrosis factor (TNF) receptor (Zapata *et al*, 2001). Besides that, the second pathway is te intrinsic or mitochondrial pathway that when triggered resulting to the release of cytochrome-c from the mitochondria and thus activate of the death signal transduction (Hockenbery *et al,* 1990) . Both of the mechanism lead to

the final common pathway involving the activation of a caspase that cleave regulatory and structural molecules that culminating in the death of the cell.

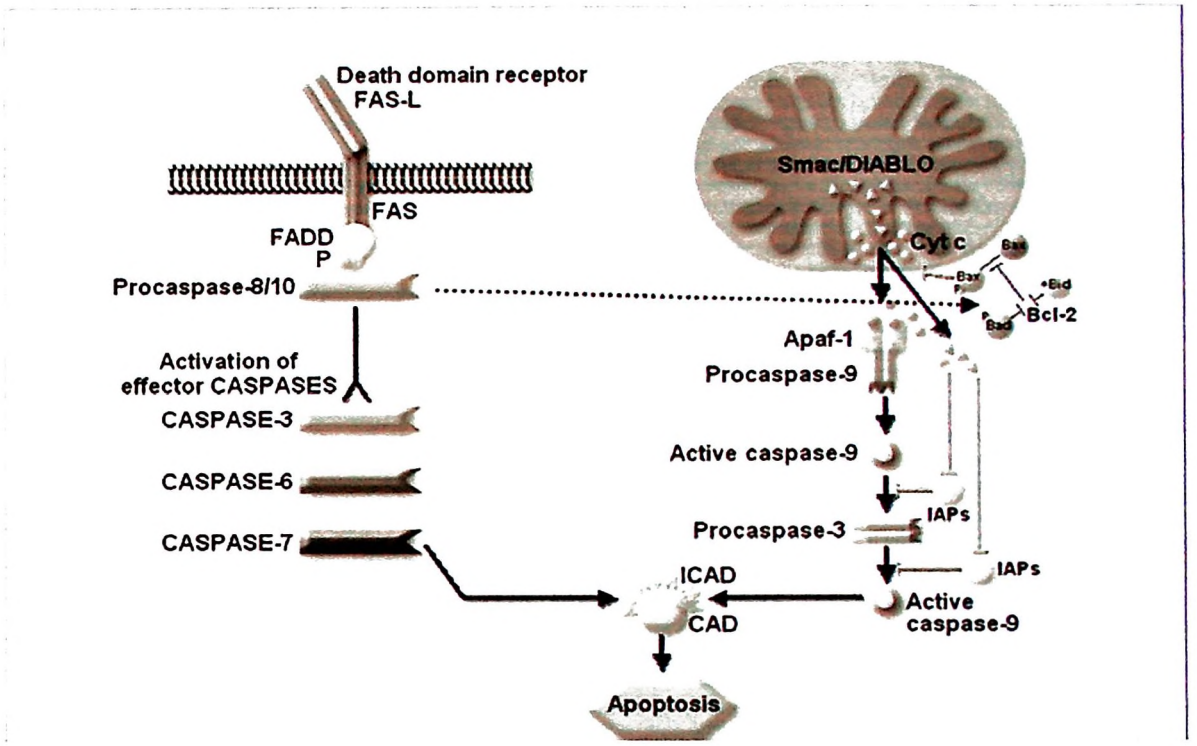

Figure 2.6 Apoptosis Occurs Through Two Main Pathways. The extrinsic or extrinsic pathway is triggered through the Fas death receptor. The second pathway is the intrinsic or extrinsic pathway that when stimulated leads to the release of cytochrome c from the mitochondria and activation of the death signal. Both pathways converge to a final common pathway involving the activation of the caspades that cleave regulatory and structural molecules and culminate in the death of the cell. (Ghobrial *et al.*, 2005)

2.6 HeLa cancer cell line

HeLa cancer cell lines is an epithelial human cervical cancer tissue that adherent culture properties that bought from American Type Cell Condition. This cell is obtained from the patient of age 31 years old adult female patient of adenocarcinoma from Black ethnicity. This cells should be conducted in the biosafety level 2 because it contain human papilloma virus and kept in liquid nitrogen vapor phase.

From the research done by Yee *et al.* (1985) have revealed that the HeLa cells lines contain HPV- 18 DNA which analysed by hybridizied with an HPV 18 DNA probe in under stringent condition and also contain HPV- 18 RNA which HeLa cells were analysed by slot-blot hybridization using HPV 18 specific radiolabeled probe and further analysed by fractionation in formaldehyde gel. According to the Scheffner *et al.* (1991), from immunoblot analysis shown that a very low level of p53 level detected in the HeLa cells compared to the HPV negative cervical carcinoma which indicates that E6 association with p53 does not cause an increase in HPV positive carcinoma cells.

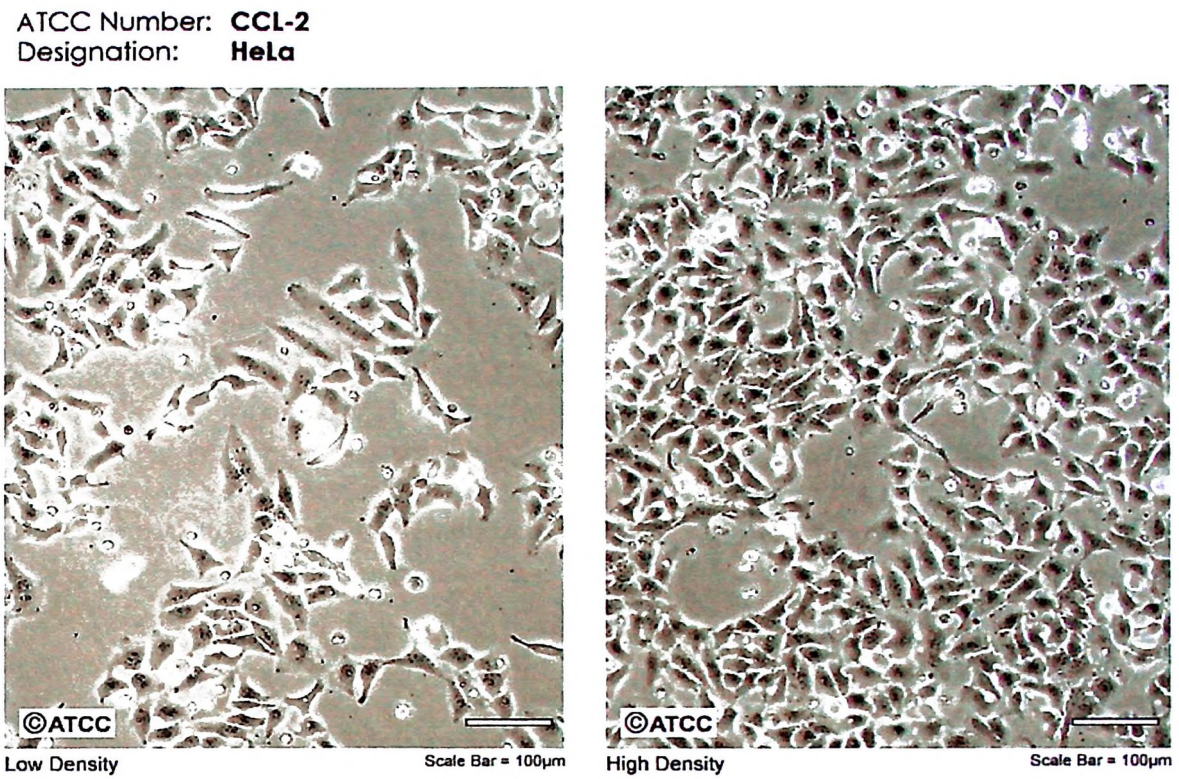

Figure 2.7 : The Morphology of HeLa cancer cell lines (American Type Culture Collection, n.d)

CHAPTER 3

METHODOLOGY

3.1 Materials

Manjakani extract, HeLa cells bought from American Type Culture Condition (ATCC), reagents such Dubellco's Modified Eagles Medium (DMEM), Fetal bovine serum (FBS), antibiotic mixture (penicillin- 10,000 units/ml, streptomycin-10000 µg/ml), Phosphate buffer saline (PBS). Trypsin – EDTA and Hoeschst 33342. 25 cm^3 cell culture flasks and 75 cm^3 culture flasks with filtered, 15 ml and 50 ml falcon tube were from BD Falcon, Dimethyl Sulfoxide (DMSO) and Cisplastin. Primary antibody for western blotting such HPV-E7 antibody from Novex , p53 antibody and β-actin from Santa Cruz Biotechnology and secondary antibody , anti mouse igG HRP from Santa Cruz Biotechnology.

3.2 Instrumentation

Instruments that were used throughout the laboratory work are Biosafety Cabinet Class II (LABCONCO Delta series Purifier, Kansas City, Missisouri), Carbon Dioxide Incubator (SHELL Lab, model IR2424), water bath, centrifuge machine (Hettich Zentrifugen, Universal 32R), Fluorescent microscope (Leica).

3.3 Methods

3.3.1 Plant Extract

Q.infectoria extract was obtained from Dr Hasmah previous group of student. The aqueous extract of *Q.infectoria* was done by the process of Soxhlet solvent-solvent extraction.

3.3.2 Cell Culture

a) Revival of cells

The HeLa cell lines in the vail were thawed rapidly (less than 1 minutes) in the water bath (37 °C). Five ml of media was added into the corning flask. The cells were added into the flasks that contain complete Dubellco's Modified Eagles Medium (DMEM) containing 1% penicillin-streptomycin and fetal bovine serum(FBS). The flask was labeled with the type of the cell, passage and also the date of revival. The cells were incubated for overnight in the CO2 incubator. The cell culture media was changed every 2-3 days until it reached confluent about 80-100%. The confluency of the cell was observed under an inverted microscope.

b) Subculture and maintenance of the cell

The media from the flask was discarded into the waste beaker. The cells were rinsed by putting 5 ml of Phosphate Buffer Solution (PBS) into a flask and the flask was swirled gently and discarded into the waste container. The steps were repeated for two times. The cells were trypsinized by using Trypsin-EDTA to make sure

all cells are detached from the surface of the flask. The flask was incubated in the CO_2 incubator for 5 minutes. Then the cells were checked for detachment after the 5 minutes incubation period under inverted light microscope. The detached cell were appeared round, either as single cell form or in clusters and appear floating on the media. The media was added into the flask to inhibit the trypsin reaction that might damage cells and transfer into the 15 ml centrifuge tube and spinned under 1500 rotation per minutes (rpm) within 5 minutes. The supernatant was discarded, 1 ml of media was added into the pellet and resuspended slowly to homogenized the cell with media. Then the mixture was transferred into the new flask with new media and incubated for 2 days incubation. The cell was maintained by changing with new media for every two days and the confluenct were observed under inverted microscope.

c) Cell Counting and Cell Plating

The cells in the flask were rinsed twice with Phosphate Buffer Saline. The cells were trypsinized and incubated for 5 minutes in the CO_2 incubator. The cell counting is done by the mixed 1:1 of 10µl of cell and 10µl of tryphan blue. The cell counting was observed using cell counting chamber Countess device. The desired concentration for each treatment was 7.0×10^4 cell/ml.

d) **Cryopreservation**

The media from the flask was discarded into the waste container. The flask was rinsed 3 times with Phosphate Buffer Saline. Trypsin-EDTA was added into the flask to make sure all cells in the flask are detached. The flask was incubated for 5 minutes in CO_2 incubator. The freezing solution was 1 ml Dimethyl Sulfoxide, 7 ml DMEM medium and 2 ml Fetal Bovine Serum but if the media absent can be alternatively used 1:9 (1ml Dimethyl Sulfoxide + 9 ml Fetal Bovine Serum). The freezing solution was swirled gently to solubilise the reagent added. Equal volumes of media with trypsin was added into the flask and swirled. The cells suspension were transfered into the 15ml centrifuge tube. The centrifuge tube then went for centrifuged at 3000 rpm for 3 minutes and the supernatant was discarded. Freezing solution was added and the cells were mixed throughly. The mixed solutions were transfered into the cryovial tube (max 1.0ml) and kept in the -20°C refrigerator for overnights before transferred to - 80°C.

3.3.3 Nuclear Fragmentation Assay

Cell were harvested for overnight prior for attachment and treated with 17.92 ± 0.42 µg/ml aqueous extract of *Quercus Infectoria* based on the preliminiary findings by Norliyana Ismail. They were incubated for 24, 48 and 72 hours. The cells were centrifuged at 1500 rpm for 10 minutes and the cell pellets were re-suspended in 1 ml of PBS solution and centrifudged again. The suspended pellet was then spread on a clean Poly- L –lysine slide and left to dry. After dried out, the slide was fixed in the 70% ethanol for 15 minutes and left at 4 °C. The cell were then washed with PBS for to removes the fixatives and 0.2%

Triton was put on the slide for cell permeabilization and left for 2-3 minutes. The slide is then stained with 600 µl of Hoechst 33342 stain and incubated for 30 minutes in dark. The slides were then rinsed with PBS for three times and then observed under fluorescent microscope Leica and analysed by image analyzer QCapture.

3.3.4 Protein Expression Analysis by Western Blot

a) Protein extraction

The cells were harvested in the $75cm^3$ of flask and Quercus Infectoria aqueous extract with IC_{50} 17.92 ± 0.42 µg / ml was added into the flask with 20 ml of DMEM media and incubated within respective incubation time (24 , 48 and 72 hour). After incubation time, the media was discarded and washed with PBS about 3 times. The cell was trypsinized for 5 minutes for cell detachment and centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded and 250 µl of RIPA lysis buffer were added and re-suspended slowly and transferred into the 1.5ml appendorf tube. The cell lysate was agitated by 5 minutes and kept overnight in -20°C freezer. The cell lysate were centrifuged at 12000 rpm for 20-30 minutes. Then was transferred into the appendorf PCR tube and was kept in the -20°C for western blot analysis.

b) Protein concentration calculation

The protein extract and RIPA buffer were keep in the ice. The computer was turn on and Nanodrop™ 2000 software icon was opened. One drop of the distilled water was