RADIOSENSITIZING EFFECTS OF *OROXYLUM INDICUM* EXTRACT ON HELA CELLS

By

NURIN AFIQAH BINTI CHE LONG

Dissertation submitted in partial fulfilment of the requirements for the degree of

Bachelor of Health Science (Honours) (Medical Radiation)

June 2017

CERTIFICATE

This is to certify the dissertation entitled

Radiosenstizing Effects of *Oroxylum Indicum* Extract on HeLa Cells

Is the bona fide record of research work done by

NURIN AFIQAH BINTI CHE LONG

During the period September 2016 until June 2017

Under my supervisions:

Supervisor

_____

DR. WAN NORDIANA BINTI ABDULLAH

WAN ABD RAHMAN

Medical Radiation Programme

Malaysia,

School of Health Sciences

Health Campus,

Universiti Sains Malaysia,

16150 Kubang Kerian,

Kelantan.

Co-supervisor

_____

MR. REDUAN

Science Officer

Universiti Sains

16150 Kubang Kerian,

Kelantan.

DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated and duly acknowledge. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at Universiti Sains Malaysia or other institutions. I grant Universiti Sains Malaysia the right to use the dissertation for teaching, research and promotional purpose.

.....

(Nurin Afiqah Binti Che Long)

Date: .....

ACKNOWLEDGEMENTS

First of all, Alhamdulillah all praise to Allah S.W.T for empowering me with determination and fortitude to complete this study entitled 'Radiosensitizing Effects of *Oroxylum Indicum* Extract on HeLa Cells'.

I am ineffably indebted to my respected supervisor Dr. Wan Nordiana Binti Wan Abdul Rahman for dedicated guidance and encouragement to accomplish this research. Her enthusiasm in conducting research has truly been an inspiration for me to achieve my goals in the future.

I am extremely thankful and pay my gratitude to my co-supervisor, Mr. Reduan Bin Abdullah for his valuable guidance and knowledge during the experimentation phase which eased me to complete this project. To Ms Raizulnasuha bt Abd Rashid, thank you so much for your help in the laboratory works which required in this project. Their active guidance and cooperation has made me more competent in the new area of expertise which is cell culture.

This project will not be possible without funding, therefore I would like to thanks Malaysian Ministry of Higher Education or financial support through FRGS grant, No:203/PPSK/6171160.

I also acknowledge with a deep of reverence, my gratitude towards my parent, Sofiah Binti Samad and member of my family, who has always supported me morally as well as economically throughout my four years of study to complete this degree. Last but not least, gratitude goes to all my friends who directly or indirectly helped and supported me throughout this study. It would be impossible to mention each and every one of you, but all of you have been a blessing to me in your own ways. Thank you.

TABLE OF CONTENTS

Contents

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

cm	Centimeter
e^-	Electron
g/l	Gram per liter
MV	Megavolt
MeV	Megaelectron volt
mm	Millimeter
nm	Nanometer
mg/ml	Milligram per millilitre
ml	Millilitre
rpm	Revolutions per minute
µM	Micrometer
α	Alpha
β	Beta
CO_2	Carbon dioxide
CT-26	Colon tumour-26
DEF	Dose Enhancement Factor
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle's medium

DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
Gy	Gray
HL-60	Human promyelocytic leukemia cell-60
H_20	Dihydrogen monoxide
LQ	Linear Quadratic
Linac	Linear accelerator
MEOIL	Methanol extract Oroxylum Indicum leaves
MGDG	Monogalactosyl diacyglycerol
OI	*Oroxylum Indicum*
PB	Prestoblue
PBS	Phosphate buffer saline
RF	Radiofrequency
S	Surviving cell
SF	Survival fraction
SSD	Source to surface distance
Std. Dev	Standard Deviation

ABSTRACT

Introduction: The main aims of radiotherapy to optimally eradicate tumours while sparing the normal tissue could be achieved with the combination treatment using natural products based medicinal plants. *Oroxylum Indicum (OI)* is a Malaysian consumable plant with a variety of health attributes that contains useful biocompound that serves as anticancer agent. In this study, the potentials of *OI* extracts to increase cells radiosensitivity towards radiotherapy were investigated.

Methods: The cytotoxic effects of *OI* extracts were initially determined *in-vitro* using HeLa cells. The cells with non-toxic concentration of *OI* extracts were irradiated with 6 MV and 10 MV photon beams. The cell survivals were evaluated using colorimetric and clonogenic assay. The cell survival curves were then analysed using Linear Quadratic model.

Results and Discussion: The *OI* extracts with the tested concentration are found non-toxic to HeLa cells. The combination of *OI* extracts and irradiation are found to reduce the cells survival which indicates radiosensitization effects. The radiosensitization observed and calculated are found to be more than 3 folds. Radiobiological analysis shows inconsistent results with increase alpha value for most samples with *OI* extracts and random beta value.

Conclusion: The *OI* extracts are found to induce radiosensitization in HeLa cancer cells when irradiated with megavoltage photon beams. This study indicates the potential of plant based natural product to be applied to increase therapeutic efficiency in radiotherapy.

Keywords: *Oroxylum Indicum* extracts, radiosensitization, radiotherapy

ABSTRAK

Pengenalan: Tujuan utama radioterapi adalah untuk mematikan tumor secara optimum sementara mengekalkan tisu normal boleh dicapai dengan menggunakan rawatan kombinasi tumbuhan perubatan yang berasaskan produk semulajadi. *Oroxylum Indicum (OI)* adalah tumbuhan yang boleh dimakan dengan pelbagai ciri-ciri kesihatan yang mengandungi biokompaun yang berguna sebagai ejen anti-kanser. Dalam kajian ini, potensi ekstrak *OI* dikaji untuk meningkatkan radiosensitiviti sel-sel terhadap radioterapi.

Kaedah: Kesan sitotoksik daripada *OI* ekstrak pada mulanya ditentukan secara *in vitro* dengan menggunakan HeLa sel. Sel-sel dengan kepekatan ekstrak *OI* yang tidak toksik telah diradiasi dengan 6 MV dan 10 MV pancaran foton. Penghidupan sel-sel telah dinilai dengan menggunakan penilaian kolorimetrik dan penilaian klonogenik. Lengkung penghidupan sel dianalisis menggunakan model linear kuadratik.

Keputusan dan perbincangan: Kepekatan ekstrak *OI* yang diuji didapati tidak toksik kepada sel-sel HeLa. Gabungan ekstrak *OI* dengan radiasi dapat mengurangkan penghidupan sel yang menunjukkan kesan radiosensitiviti. Radiosensitiviti telah diperhatikan dan dikira bahawa ia melebihi daripada tiga kali ganda. Analisis radiobiologi menujukkan keputusan yang tidak konsisten dengan peningkatan nilai alfa untuk kebanyakan sampel dengan ekstrak *OI* dan nilai beta adalah rawak.

Kesimpulan: Ekstrak *OI* didapati mempengaruhi radiosensitiviti dalam sel-sel kanser HeLa apabila diradiasikan dengan pancaran megavoltan foton. Kajian ini menunjukkan potensi tumbuhan berasaskan produk semula jadi boleh digunakan untuk meningkatkan kecekapan dalam radioterapi.

Kata kunci: Ekstrak *Oroxylum Indicum*, radiosensitiviti, radioterapi

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF STUDY

Uterine cervix is the second most frequent cancer among women in Malaysia even though it is potentially preventable (ICO Information Centre on HPV and Cancer, 2017). Radiotherapy is an important treatment modality for cervical cancer, particularly for locally advanced tumours and is known to mainly induce cell death in tumours. Radiotherapy is limited by the total dose that can be given without damaging normal tissue. Any compound that increases the therapeutic efficacy of radiation without dose escalation (that is higher treatment response at lower doses) will therefore be beneficial for cancer treatment. Hence the study of such biological response modifiers is a prime requirement in cancer therapeutics (Sreekala et al., 2008). With the use of radiosensitizer agents, the sensitization of tumour cells can be improved in radiotherapy treatment respectively. These compounds apparently promote fixation of the free radicals produced by radiation damage at the molecular level, in which biochemical reactions in the damaged molecules prevent repair of the cellular radiation damage (Jayam et al., 2014).

In recent times, natural products has drawn considerable research interest in the study of anti- cancer agents as it contains flavonoids which can trigger apoptosis to the cancer cells (Sonia, 2006). In this regard, *Oroxylum Indicum (OI)* which also known as 'beka' in Malaysia has drawn the most attention as it can easily found in Asian country. *OI* is a medicinally active plant rich source of flavonoids and their glycosides act as inhibitors of tumor cells proliferation and malignancy (Laloua et al., 2013). It has been

reported that *OI* contains chrysin, baicalein, oroxylin B, baicalin (Yuan et. al, 2008) and dihydrooroxylin A-7-O- methylnglucuronide (Hari Babu et al., 2010). The evidence shown by methanol extract of *OI* leaves was able to inhibit the proliferation of HeLa cells by inducing apoptotic cell death (Khaizil et al., 2013). Regardless to this, plant *OI* with known anticancer activity may provide an important source (Laloua et al., 2013).

Therefore, the subject of this study is to investigate the potential use of *OI* leaves in the *in vitro* anti-proliferative as a radiosensitizer on HeLa cells for radiotherapy.

1.2 RADIOTHERAPY

Radiotherapy is the most important non-surgical modality for the curative and palliative treatment of cancer. High-energy rays are often used to kill cancer cells and prevent them from growing and dividing. It damages genetic materials including DNA of cells, as a consequence obstructing their ability to further proliferate and divide. In spite the radiation can damages both cancer cells and normal cells, the aim of radiation therapy is to allow more precise deposition of radiation dose to abnormal cancer cells while minimizing exposure to surrounding normal cells, which is proximate to cancer cells or in the path of radiation. Normal cells can retain to its normal function status as it can be repaired by themselves efficiently. Cancer cells normally are incompetent to repair the damage caused by radiation treatment resulting in differential cell killing.

The way to deliver radiation to the location of cancer can be categorized into two ways which are external beam and internal beam. External beam radiation which is the most approach in clinical setting is delivered by high energy rays (photons, protons or particle radiation) focus to the location of tumour from outside of the patient's body. While internal radiation is delivered from inside the patient's body by radioactive sources that is seed in the catheters or seeds directly into the tumour site. Radiation treatment can be combined with other treatment such as surgery, chemotherapy and immunotherapy. The combination therapy with radiation is to ensure the effectiveness of cancer treatment either used radiation to shrink the tumour before surgery (neoadjuvant therapy) or to destroy the microscopic tumour cells after the surgery (adjuvant therapy) that may be left behind (Rajamanickam, 2012).

Linear accelerator (linac) is the main device that uses high radio-frequency (RF) electromagnetic waves to deliver the external radiation beam to the tumour site. All

linacs generate high x-rays and electron energy that are carefully aimed at the tumour area and can treat all area of the body from head to toe. The high energy photon is aimed into deep seated tumour for example prostate, colon, bladder, breast and ovary. The electron is used to treat the skin surface or tumours which close to the surface as electron beams are less penetrating as compared to photon.

The event in the gantry of linac generates using electrical fields to speed up the electrons to almost the speed of light (186,000 miles a second). As the electron is escalated, its kinetic energy is increased until it collides with the target (tungsten) and the energy is released as a photon (Bremsstrahlung radiation). The tungsten is removed to produce electron beam depending on the type and location of the tumour. The high energy photons are then enter patient's body and aim to break the DNA cells within the treatment area. Components of linac are shown as in figure 1.

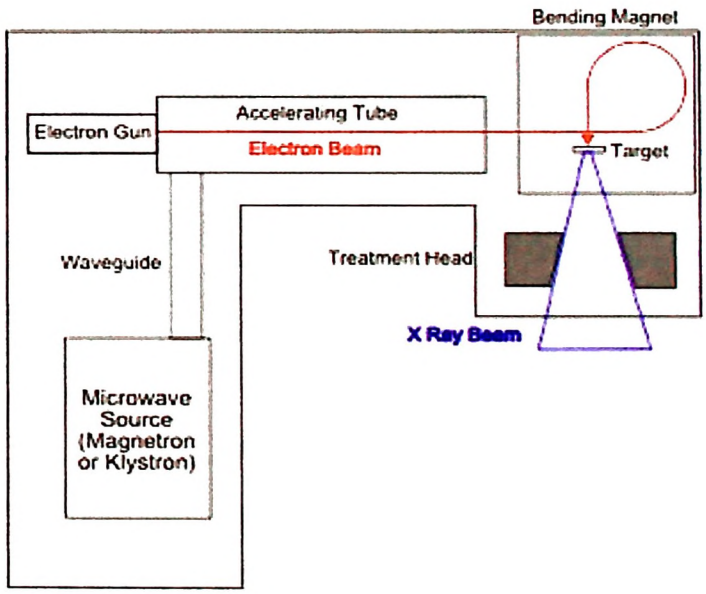

Figure 1: Block diagram of a typical medical linac in radiotherapy (Peninsular Cancer Center, n.d).

1.3 RADIOBIOLOGY

In radiotherapy, the interaction of radiation with matter, including biologic tissue is described as ionization and excitation. Ionization occurs when the incoming radiation ejects an electron from the shell of the atom, thus the atom starting to be charged (ionization). Meanwhile, excitation happens when an electron in the outer shell of the atom is said to be excited (vibrating) but is not ejected from the shell. Radiobiology is defined as the study of sequence of events following the absorption of energy from ionizing radiation, the exerts of the organism to compensate and the damage to the organism that may be produced.

When radiation initially interacts with a cell, the interaction can be classified into two which are direct or indirect ionization. When a living tissue is incident by a beam of charged particles such as alpha particles, protons or electrons, direct ionization is highly anticipated due to relatively dense ionizing nature of most particulate radiations. Furthermore, direct effects can be predominant when neutrons compose the primary beam because the secondary particle produced such as protons, alpha particles or heavy nuclear fragments from the neutron's interaction with the nucleus of the atom may cause damage directly to the deoxyribonucleic acid (DNA) or other important macromolecules in the cells.

The other form of interaction is indirectly ionization caused by the effects of specific secondary particles on the target. This mechanism can be predominant when uncharged particles such as x-rays, gamma rays or neutrons with high energy, charged secondary particles is directly or indirectly cause ionization in the critical target. Indirect effect occurs mainly when primary beam of x-rays or gamma rays is formed, thus producing fast electrons as the secondary particles that interact with the most

abundant cellular medium which is water (H_20). Indirect effect is followed by the series of reactions known as radiolysis (splitting) of water (Washington & Leaver, 2010).

The initial event in radiolysis involves the ionization or ejection of an electron from a water molecule, thus producing a charged molecule (water ion) as below:

$$H_2O \rightarrow H_2O+ + 1e^-$$

The ejected electron (e-), act as fast electron because of its high energy, may be absorbed by a second water molecule forming another water ion (H_2O^-) as below:

$$e^- + H_2O \rightarrow H_2O^-$$

The pair of water ions produce are chemically unstable and tend to instantly break down or dissociate into another ion and a free radical which is a highly reactive species with an unpaired valence electron:

$$H_2O+ \rightarrow H^+ + OH\cdot \text{ and } H_2O^- \rightarrow H\cdot + OH^-$$

The free radical symbolized by a dot ($H\cdot$ or $OH\cdot$). The ion pair (H^+ and OH^-) may recombine, thus forming normal water molecule with no net damage to the cell. Meanwhile, free radicals may also recombine like the previous ion pair thus forming normal water molecule:

$$H\cdot + OH\cdot \rightarrow H_2O$$

Free radicals may also combine with other nearby free radicals, thus forming a new molecule such as hydrogen peroxide which is toxic to the cell:

$$OH\cdot + OH\cdot \rightarrow H_2O_2 \text{ (hydrogen peroxide)}$$

Several other reaction involving normal cellular components can be participated by the free radicals including DNA. Due to majority of cells consist of water (80%), the probability of damage occurring through the indirect action is greater than the probability of damage through direct action (Washington & Leaver, 2010).

In radiotherapy, the ionization and excitation interaction of biological system is affected by sufficiently strong ionizing radiation. The interactions mainly occur with orbital electrons, ejecting the electron from atom (ionization) and raising others to higher energy levels within an atom or molecules (excitation). These two interactions lead to the breakage of chemical bonds and the formation of broken molecules known as free radicals. Thus, enzymatic reaction may happen due to free radicals reaction where some rare lesions fail to repair and may lead to cell death (early effects). As cells take time to die, the cells may undergo a number of mitotic divisions before dying (late effects). Figure below showing the sequence of events of radiobiological effects in biological systems (Joiner & Kogel, 2009):

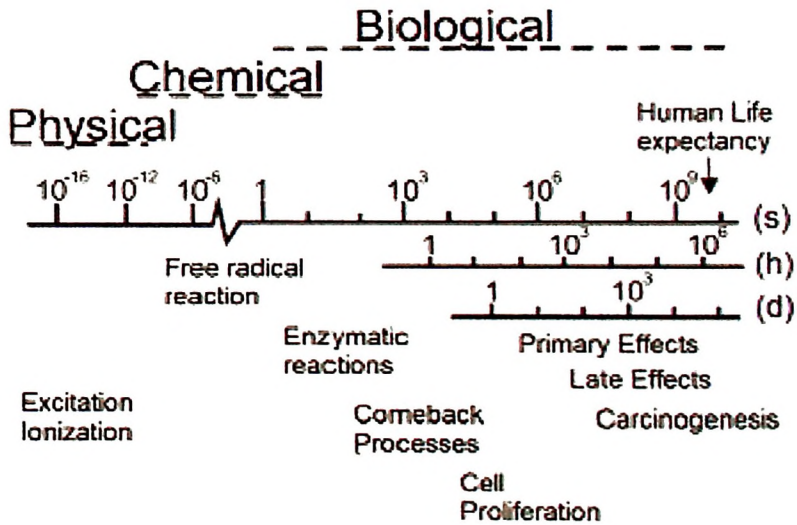

Figure 2: Timeline of the effects of radiation exposure on biological systems (Joiner & Kogel, 2009).

Radiation is an efficient breaker of DNA molecules which form genes and thousands of genes compose a chromosome by indirect or direct pathways. In direct pathway, the radiation interacts directly with the target in the cell. The ionization and excitation of the primary target may be happen through Coulomb interactions leading to physical and chemical events that finally produce the biological damage. In indirect pathway, the radiation interacts with other molecules or atoms which are mainly water as 80% of a cell is composed by water and free radicals are produced. The free radicals can be diffused in the cell and damage the primary target within the cell. This interaction of water and radiation produced extremely reactive free radicals such as H_2O^+ (water ion) and $OH\cdot$ (hydroxyl radical). The free radicals in turn may cause biological damage to the target within the cell (Nagalingam et al., n.d). Structural changes induced in chromosomes by radiation include single breaks, multiple breaks, and a phenomenon known as chromosome stickiness or clumping. Single breaks due to single event of ionization and caused little biologic significance which can be repaired readily using opposite strand as a template. Multiple breaks of DNA due to multi-ionization processes that involve several events of ionization DNA or water radiolysis. After irradiation, chromosome may appear to be "sticky" causing unequal division of chromatin. Thus, proliferation of daughter nuclei is interrupted (Beyzadeoglu et al., 2010).

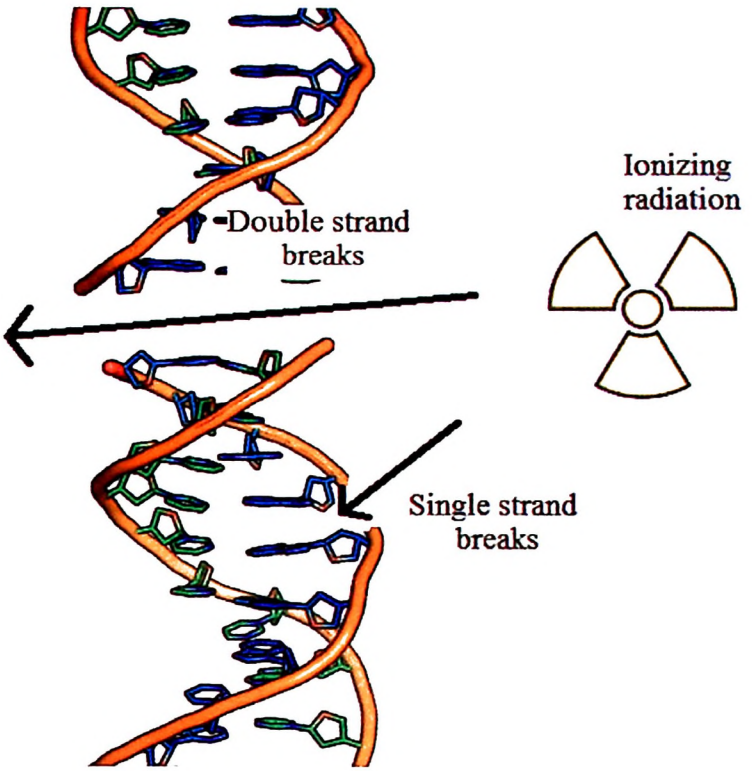

Figure 3: DNA breakage cause single strand break and double strand break by the ionizing radiation.

1.4 CELL SURVIVAL CURVES

Cell survival curve usually used to describe relationship between radiation dose and the cell fractions which survive on that dose (Vandana, 2012). Cellular response to radiation can be evaluated by plotting the number of colonies formed on a semi-logarithmic graph. This cellular response evaluation was first introduced by Puck and Marcus in 1956, when they irradiated HeLa cells *in vitro*. Their result, termed as survival curve, was a plot of the radiation dose administered on the x-axis versus the survival fraction (SF) of cells on the y-axis as in Figure 4.

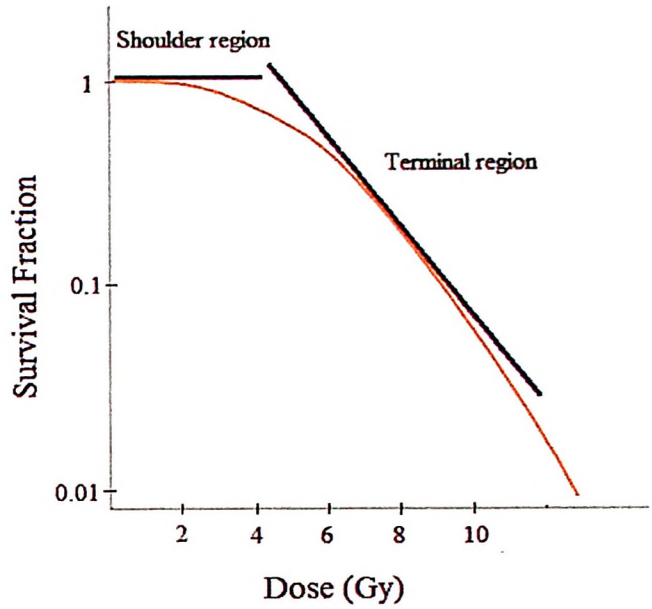

Figure 4: Representative typical shape of cell survival curve with fraction of cell survival plotted on logarithm scale versus dose on linear scale.

Initial portion has a shoulder and terminal region become straight line. A shoulder region which is in low dose region, certain radiation dose goes waste. Meanwhile, the terminal region describes as exponential relationship which means same dose increment result into equal reduction in surviving fraction. The results from the survival curve are observed as some cells are lethally damage, some cells are sublethally damaged and some cells are not damaged (Vandana, 2012).

The shape of cell survival is then described by using mathematical radiobiological model and the most frequently model used is linear quadratic model. There are two components in linear quadratic models, firstly, cell killing is proportional to dose. Secondly, cell killing is proportional to dose squared (Vandana, 2012).

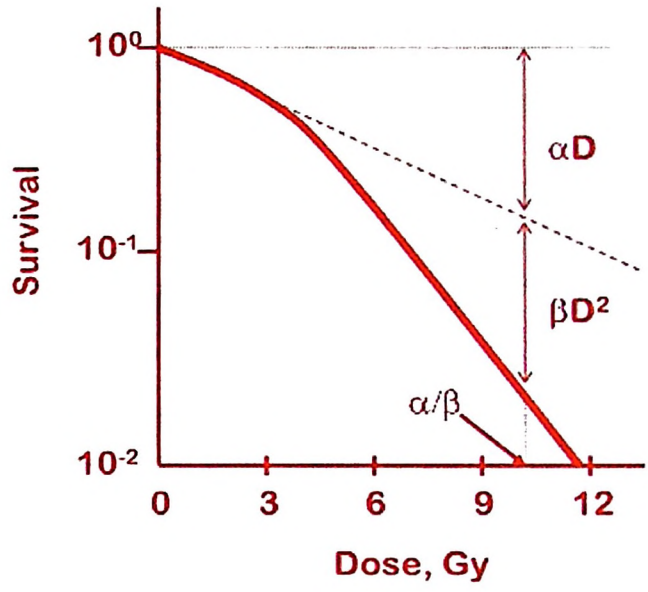

Figure 5: Typical model graph of survival fraction plotted logarithm scale versus dose with α and β characterisation (Vandana, 2012).

This model can be described by using equation as below:

$$S = e^{-(\alpha D + Bd2)}$$

S represents the fraction of surviving cells, D represents dose, alpha (α) and beta (β) are constant that characterize the slopes of the two portion of semi-log survival curve. Where α component representative of damaged cause by a single event (two chromosomes breakage proportional to dose) and β component representative by damaged cause by multiple events (two chromosomes breakage is the product of two separate electrons). The linear portion of the cell survival curve depict as α and quadratic components depict as β (Nagalingham et al., n.d).

1.5 EXTRACTS OF *OROXYLUM INDICUM*

Oroxylum Indicum is a small to medium sized deciduous tree measuring up to 12 metres in height and belongs to the family of Bignoniaceae. Leaves are large up to 1 – 5 m long, pinnate, bipinnate or tripinnate, leaflets are ovate or elliptic. They form enormous seed pods that hang down from bare branches (Ahad et al., 2012). The fruits are long and sword-like with capsules that have kidney-shaped, yellowish-green seeds that are surrounded by a light-brown papery wing. It is also known as beko, bonglai, kulai and it is called "Midnight Horror" because the flowers open at night, emitting a powerful stink that attracts bats to facilitate pollination (Dinda et al., 2014).

In Malaysia, the young leaves and fruits of *OI* is consumed raw or cooked as vegetables. *OI* have been applied traditionally to treat toothache, headache, stomach ache, ulcer and rheumatic pain which can be prepared by decoction of the *OI* leaves (Bhushan et al., 2013; Dinda et al., 2014) and have some biological activities such as anticancer (Zazali et al., 2013), antidiabetic (Kaldate et al. 2011), anti-oxidant (Tenpe et al., 2009) and antimicrobial (Phatalung et al., 2010).

Flavonoids and other phenolics are the major constituents of the different parts of the plant. The main compounds isolated from stem bark and root are flavonoids such as baicalein, oroxylin A, chrysin and baicalein naphthoquinone-lapachol and the phenolic ellagic acid. Among them, baicalein is the most abundant in both stem, bark and root (Ahad et al., 2014).

Flavonoid baicalein present in *OI* has anti-tumour effect on human cancer cell lines and inhibited the 50% proliferation of Human promyelocytic leukemia cells (HL-60) cell lines at a concentration of 25-30 μM (Prakash et al., 2013). Experiment carried out by Lalou et al. (2013) have shown that baicalein and oroxylin-A glycoside exhibited

potent anti-proliferative effects. Baicalein also blocked the furin-mediated cleavage of pro-h-VEGE-C proteins in Colon Tumour-26 (CT-26) tumour cells more efficiently than the other isolated flavonoids, oroxylin-A and chrysin. Baicalein is a major flavonoid constituent of both stem bark and fruit of plant. In leaves, the major constituents are baicalein, scuttelarein and their 7-O-glucuronides. Therefore, it may represent a new molecule for further exploration as a potential herbal anti-tumour agent (Lalou et al.,2013; Ahad et. al., 2014).

Apoptosis also known as programme cell death is characterised by distinct morphologic changes, including cell shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation, and the formation of apoptotic bodies (Gowhar et al., 2009). High accumulation of p53 protein caused by methanol extract *Oroxylum Indicum* Leave (MEOIL) in HeLa cells up to 88.5% during 72 hours treatment. Pressures such as oncogene stimulation, DNA damage, hypoxia, radiation and medication of the chromatin bodies can activate p53 protein (Elmore, 2007). Apoptosis is the main system for anticancer agents to kill tumour cells, thus the interaction of flavonoids of *OI* may increase p53 and Bax protein (apoptotic proteins) expression to induce apoptosis activity on HeLa cells due to activation of DNA damage. The results shown by Khaizil et. al. (2013) had proved that MEOIL was able to inhibit proliferation of HeLa cells by restoring sensitivity to apoptotic cell death.

1.6 AIM AND OBJECTIVES

Aim:

To study the radiosensitizing effects of *OI* extracts on HeLa cells.

Objectives:

I. To determine non-toxic concentration of *OI* extracts on HeLa cells without irradiation.

II. To quantify the radiosensitizing effects of different *OI* concentration on HeLa cells for photon beams.

III. To radiobiologically analyse the radiosensitizing effects of *OI* extracts.

1.7 SIGNIFICANCE OF STUDY

This study will be beneficial in term of investigating a radiosensitizing effects of *OI* extracts on cancer cells. The toxic concentration of *OI* extracts without radiation is determined initially by using serial dilution method on HeLa cells. The response of HeLa cells with and without *OI* extracts are evaluated using colorimetric assay and clonogenic assay interpreted with cell survival curve based on Linear Quadratic (LQ) model. The results of radiosensitizing effects of *OI* extracts can be analysed radiobiologically on HeLa cells at 0 to 6 Gy ranges of dose.

CHAPTER 2

LITERATURE REVIEW

2.1 Biomedical Application of *Oroxylum Indicum* Extracts

Natural products have the potential as useful drugs for humans to combat many types of diseases. Plants possess numerous phytochemicals with different bioactivities such as antioxidants, anti-inflammatory and anti-cancer. *OI* is one of the most medicinal tree widely used in the treatment of many ailments in folk medicine (Talari et. al., 2012). The search for anticancer agents from plant sources started in 1950s after the discovery of Yinca Alkaloids and the isolation of cytotoxic podophyllotoxins was carried out (Prakash et. al., 2013).

The tremendous potential natural products for medicinal has been proven as many herb become best-selling pharmaceutical as their strong biological properties such as phenolics, flavonoids and carotenoids which can be therapeutics role againts various ailments (Deka et al., 2013). The comprises of compounds in *OI* such as phenols, tannins, alkaloids, flavonoids and saponins makes *OI* as important therapeutic uses *Ayurveda* and *Unani* system (Deka et al., 2013). The extractions of bioactive molecules in leaves of *OI* indicate that it contains flavonoids namely chrysin, oroxylin-A and baicalein. The flavonoid Baicalein in *OI* showed the toxicity to tumour cell lines as it inhibits proliferation of cancer cell line *in vitro* via induction of apoptosis (Roy et al., 2006).

The anti-tumour properties such as flavonoid baicalein of methanolic extracts of *OI* showed that baicalein effectively inhibited the proliferation by 50% of a leukemic cell line at a concentration 25-30 μM, HL-60 by decreasing the number of cell by way

of apoptosis in hindering the cell cycle process. The apoptotic event of cells increased with the concentration of 10 μM baicalein exposure for 36 hours or 48 hours. Apoptosis cell death cause changes in cell proliferation and cells failing to progress to mitosis (Roy et al., 2006).

Since plant is an endangered species, leaves also can be used instead of root, root bark and stem bark as antioxidant aspects. The study conducted by Mishra et al, evaluated that leaves extracts exhibits highest free radical scavenging activity other than the other parts of *OI* due to presence of polyphonic compounds. Phenolics compound have been related with anti-oxidative action in biological system which acts as free radical neutralizers of singlet oxygen (Mishra et al., 2010).

2.2 Application of natural product in radiotherapy

Some tumours does not affected well to the radiation treatment, thus, a very high dose is required to kill the cancer cells and causing radiation injury to the surrounding normal tissues which creating a serious limitation to the radiotherapy. Hence, an alternative approaches to minimize the dosage by selectively sensitize tumour cells to radiation treatment and thereby avoid the harmful effects of radiotherapy (Dest, 2006).

An extract of a major chloroplast membrane galactolipid from spinach, Monogalactosyl diacyglycerol (MGDG) has been reported as a strong DNA replication and repair inhibition. Therefore, Akasaka et al. (2015) were interested to test on human pancreas cell lines whether the MGDG could increase the cytotoxic effects of radiotherapy in vitro and in vivo. The results of the study showed the induction of apoptosis in human pancreatic cells as the combination between MGDG and

radiotherapy (21.6%) outstandingly increased the proportion of apoptotic effect to the cells than each alone (control: 2.6%, MGDG: 5.2% and IR: 8.8%).

Combination of ionizing radiation and curcumin, yellow pigment of tumeric has demonstrated a potent radiosensitizer of human cervical cells. Using a concentration of 10 μM of curcumin, showed 20% decrease in survival of HeLa cell after 8 hours pre-treatment at each of the radiation doses ranging from 2 Gy, 4 Gy and 6 Gy. Curcumin significantly increased the production of reactive oxygen species (ROS) that led to continue activated the protein kinases in cells. These activations of protein kinases caused decreased survival of Hela cells after irradiation (Prasanthi et al., 2008).

Diosprysin a plant derivative from the stem bark of Diospyros Montana Roxb. could enhance the radiation effects in breast carcinoma by regulating the gene expression involved in cell cycle and apoptosis. The cell viability reduced to -14% by 5 Gy radiation doses, whereas cell viability reduced to -33% by 5 μM concentration of Diosprysin alone. Anyhow, there were a great decreased to -64% of cellular viability when the treatment above were given in combination to each other (Binod et al., 2007).

One study showed that plant-derived polyphenols such as resveratrol, a compound from grapes can induced radiosensitization which using clonogenics cell survival assays, showed that pre-treatment HeLa cells and SiHa cell with resveratrol causes cell death at early S-phase cell cycle after the cells were exposed to ionizing radiation (Zoberi et al., 2002). Other investigations of combination plant derivative compound with ionizing radiation such as genistein (soy), epigallocatechin gallate (EGCG) (green tea) and flavoporidol have been studied for their potential to exert antitumoregenic effects through the inhibition of various mechanism of cell proliferation (Amit et al.,2005).

CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS

This chapter discusses the materials and experimental methodology used for determining the radiosensitizing effects of *OI* extracts on cancer cells. The experimental methods are divided into several sections. The first section describes the preparations of toxicity test of *OI* extracts, followed by a section which details cell culture protocols for sample preparations. The third section is the irradiation procedure. Last section is the method to obtain cell survival curves by using colorimetric assay and clonogenic assay is explained.

3.1.1 Preparation of samples

This section will explain the materials used for samples preparation procedure.

3.1.1.1 HeLa cells

HeLa cell is derived from cervical cancer cells taken from Henrietta Lacks, a patient who died of her cancer in 1951. HeLa cells were obtained from American Type Culture Collection (ATCC). HeLa cell have been distributed around the world and used in innumerable medical endeavors, including investigations into the nature of cancer, the development of vaccines, the mapping of genes, the treatment of diseases, and the mechanisms involved with programmed cell death (apoptosis).

HeLa cell was discovered from samples collected during a biopsy on Henrietta Lacks, a poor 31-year-old African-American patient suffering from a cervical tumour.

Therefore, the name of HeLa is derived from the first two letters of her first and last name (Potash, 2011). Typically, somatic cells will undergo cell aging and lose the ability to divide and replicate after a few dozen generations, however, the sample from HeLa cell behave abnormally and do not die from apoptosis. Thus, HeLa cell became the first human cell line to survive indefinitely *in vitro* and the most requests by other laboratories and researcher for samples that could be cultured (Importance of HeLa cells, 2014). The reason of using HeLa cells in this experiment because HeLa cells have the extraordinary ability to divide indefinitely.

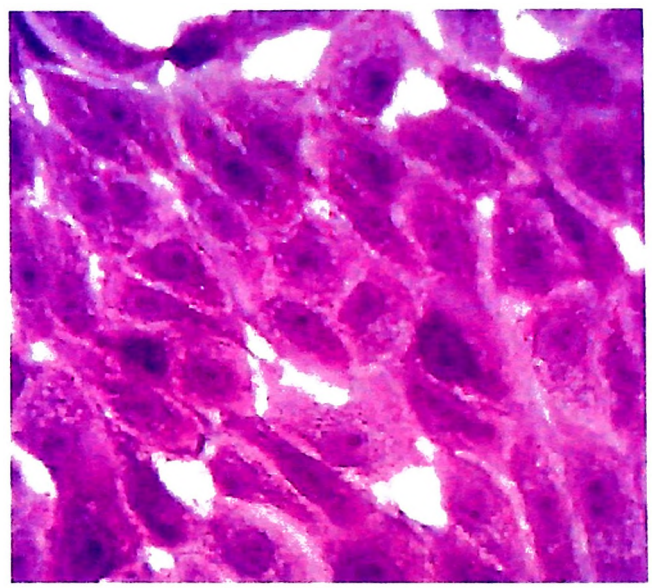

Figure 6: The HeLa cell lines

3.1.1.2 *Oroxylum Indicum* extracts

The leaves of *OI* as in Figure 7 were collected from Kelantan, Malaysia. The *OI* extracts were derived from a grinded dried plant leaves with methanol using soxhlet apparatus (Khaizil et. al., 2013). The concentration of *OI* extracts was diluted in a dimethyl sulfixide (DMSO) (10 mg/ml) and appeared in a dark green solution. The extracts were then stored at 4°C.

Figure 7: Leaves of *O. Indicum.*

Figure 8: 10 mg/ml of *OI* extracts concentration in centrifuge tube.

3.1.1.3 Cell counter

Cell counter is important to perform the kinetics of cell growth evaluation. The accuracy of cell counting and viability measurement including live, dead and total cells

can be achieved by using trypan blue stain technique. The Countess cell counter is more efficient and time effective than manual method as it only takes less than a minute per sample to read.

Figure 9: Cell counter used in culture lab, School of Health Science, USM Kelantan.

3.1.1.4 Dulbecco's Modified of Eagle's Medium (DMEM)

DMEM is a cell culture's basal medium in the form of red colour solution designed to support the growth of the cells. It is essential as the cell culture need to be grown under controlled conditions, generally outside of their natural environment. DMEM requires supplementation with 10% of FBS as it contains no proteins, lipids and growth factors. Furthermore, physiological pH can be maintained by DMEM as it requires 5-10% carbon dioxide (CO_2) environment using sodium bicarbonate buffer system (2.2 g/l).

Figure 10: DMEM from Gibco ® by Life Technologies.

3.1.1.5 Fetal Bovine Serum (FBS)

FBS is a media supplement that commonly practices to in vitro cell tissue culture. It contains element such as growth factors and hormones, vitamins, binding and transport proteins which are extremely essential to the cells (Gerhard, n.d).

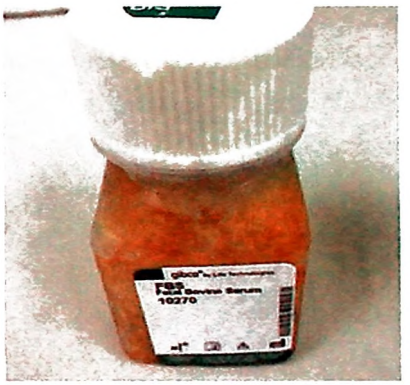

Figure 11: FBS from Gibco ® by Life Technologies.

3.1.1.6 PrestoBlue (PB) reagent

PrestoBlue is a quick, simple and the most efficient live assay in monitoring cytotoxicity and viability of cells. It is a resazurin based, membrane permeable reagents that can be visually measured using absorbance or utilising the fluorescent outputs of the reduced resorufin (Namrita et al., 2013).

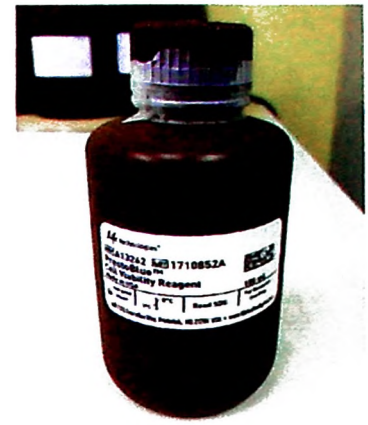

Figure 12: PrestoBlue reagent from Life technologies.

3.1.1.6 Eppendorf tube

Eppendorf tubes use as a benchmarking for small volume sample which ranging from several microlitres to several millilitres. Cultured cells with volume 0.2 ml is transferred in eppendorf tube for irradiation as it is more practical and safe to handle the sample from lab and fit to arrange the sample in the field size for irradiation.

Figure 13: Eppendorf tube (0.2 ml).

3.1.1.7 Biological safety cabinet

The needs to protect researcher, sample and environment from exposure to cross contamination that may affect experimental consistency during procedures are very

crucial. Therefore, biological safety cabinet is designed to keep safe from any harm that might be happen. A flow of inward air moving into the cabinet contains aerosols generated during microbiological manipulations. It then travels through a filtration filter that traps all airborne particles and contaminants. Clean and decontaminated air is depleted from the cabinet ultimately (ESCO, n.d)

Figure 14: Picture of biological safety cabinet in culture lab, School of Health Science, USM Kelantan.

3.1.1.8 Micropipette and micropipette tips

The micropipette is a highly precise instrument that is used to transfer small quantities (< 1 ml) of liquids conveniently. Micropipettes use a disposable pipette tips to aspirate liquids. The only part of the pipette that makes contact with the solution is the tip. To prevent cross contamination, a new tip is utilized for every sample (Pipette.com, 2017).