PHYTOCHEMICAL ANALYSIS, ANTICANCER AND ANTIANGIOGENIC ACTIVITIES OF *BARRINGTONIA RACEMOSA* EXTRACTS AND ITS FRACTIONS

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by

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LIST OF ABBREVIATIONS

µg/mL	Microgram per millilitre
µL	Microlitre
Abs	Absorbance
$AlCl_3$	Aluminum chloride
ANOVA	Analysis of variance
ARSC	Animal Research and Service Centre
ATCC	American Tissues Culture Collection
BHT	Butylated hydroxytoluene
COX-2	Cyclooxygenase-2
CO_2	Carbon dioxide
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2'-Diphenyl-1-picrylhydrazyl
EA.hy926	Human vascular endothelial cell line
EC_{50}	Median effective concentration
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionization
Fe^{2+}	Ferrous cyanide
Fe^{3+}	Ferric complex
$FeCl_3$	Ferric chloride
FBS	Fetal Bovine Serum
FT-IR	Fourier transform infrared
GAE	Gallic acid equivalents
GC	Gas chromatography
H_2SO_4	Sulphuric acid
HCT-116	Colorectal carcinoma cells
HIFBS	Heat inactivated fetal bovine serum
HPLC	high performance liquid chromatography
IC_{50}	Half maximal inhibitory concentration
LC	Liquid chromatography
LCMS	Liquid chromatography mass spectrometry

LE	Linalool equivalents
mg	Milligram
mg/mL	Milligram per milliliter
min	Minutes
mL	Milliliter
MCF-7	Breast cancer cell line
MCP-1	Monocyte chemoattractant protein-1
METLIN	Metabolomics Database
MS	Mass spectrometry
MTT	3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide
$NaHCO_3$	Sodium bicarbonate
NADH	Nicotinamide adenine dinucleotide
$NaNO_2$	Sodium nitrite
NaOH	Sodium hydroxide
NH_3	Ammonia
nm	Nanometre
PBS	Phosphate-buffered saline
QE	Quercetin equivalents
Q-TOF	Quadrupole time of flight
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
SEM	Standard error mean
TFC	Total flavonoid content
TLC	Thin layer chromatography
TPC	Total phenolic content
TTC	Total terpenoid content
UV	Ultra violet

LIST OF SYMBOLS

ºC	Degree celsius
%	Percentage
β	Beta
ʎ	Lambda

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ANALYSIS FITOKIMIA, AKTIVITI ANTIKANSER DAN AKTIVITI ANTIANGIOGENIK TERHADAP EKSTRAK *BARRINGTONIA RACEMOSA* DAN FRAKSINYA

ABSTRAK

Barringtonia racemosa Roxb. (Lecythidaceae) ataupun dikenali sebagai putat sungai telah digunapakai dalam perubatan tradisional untuk merawat batuk, asma, ulser dan cirit-birit. Dalam penyelidikan ini, aktiviti antikanser ekstrak buah *B. racemosa* dan ekstrak biji *B. racemosa* beserta fraksi-fraksinya telah dikaji secara *in vitro*. Ekstrak dan fraksi yang aktif juga diselidik kesannya ke atas aktiviti antiangiogenik dan antioksidan. Buah dan biji *B. racemosa* diekstrak menggunakan teknik pemaseratan dan dipisahkan kepada fraksi-fraksi n-heksana, kloroform, etil asetat dan n-butanol menggunakan teknik pelarut-pelarut. Kesan aktiviti antikanser dikaji secara *in vitro* pada sel kanser payudara (MCF-7) dan sel kanser kolorektal (HCT-116) menggunakan asai MTT. Bagi mengkaji mekanisma apoptosis yang menyebabkan kematian sel kanser, asai fragmentasi DNA telah digunakan. Fraksi bioaktif yang berpotensi ke atas kesan antikanser juga telah digunakan bagi mengkaji kesan antiangiogenik secara *ex vivo* menggunakan teknik asai cerakin gelang aortik tikus. Kesan aktiviti antioksidan telah dikaji menggunakan asai DPPH, β-karotene dan kuasa penurunan. Hasil kajian ini mendapati fraksi n-butanol *B. racemosa* memberi kesan aktif sitotoksik ke atas sel MCF-7 (IC_{50} 11.12 ± 1.05 μg/mL). Bagi aktiviti antiangiogenik pula, ia menunjukkan perencatan pembentukan salur darah mikro pada cerakin gelang aortik tikus sebanyak 50 % pada 34.55 ± 1.34 μg/mL. Dalam asai antioksidan, fraksi n-butanol menunjukkan kesan yang sederahana secara keseluruhannya. Komposisi kimia yang hadir di dalam fraksi yang aktif, iaitu fraksi

n-butanol telah dikesan menggunakan teknik LCMS. Tiga kumpulan triterpenoid telah dikesan kehadirannya, iaitu cucurbitacin O, pristimerol dan flaccidin B. Penyelidikan ini telah menujukkan bahawa fraksi n-butanol biji *B. racemosa* menonjolkan kesan aktif sebagai agen antikanser dan antiangiogenik. Malah, penyelidikan ini telah berjaya menyokong kenyatan mengenai kegunaannya dalam perubatan tradisional bagi menentang penyakit seperti kanser berserta komposisi kimianya yang masih belum pernah direkodkan terdahulu.

PHYTOCHEMICAL ANALYSIS, ANTICANCER AND ANTIANGIOGENIC ACTIVITIES OF *BARRINGTONIA RACEMOSA* EXTRACTS AND ITS FRACTIONS

ABSTRACT

Barringtonia racemosa Roxb. (Lecythidaceae) or locally known as putat sungai have been used in the treatment of cough, asthma, ulcer and diarrhea as traditional remedy. This study investigated anticancer activity of *B. racemosa* fruit extract, seed extract and all its fractions, *in vitro.* The crude extract and its potent bioactive fraction as anticancer agent were also been investigated for their antiangiogenic and antioxidant activity. The extraction of fruits and seed were carried out by maceration method and further separated into fractions by solvent-solvent partitioning starting from n-hexane, chloroform, ethyl acetate and n-butanol. The anticancer activity was investigated by *in vitro* cytotoxic on human breast cancer cell lines (MCF-7) and human colorectal cancer (HCT-116) by MTT assay. DNA fragmentation assay was used to identify the apoptosis mechanism of cell cancer death. The *ex vivo* antiangiogenic properties of potent bioactive anticancer fractions were determined using rat aorta ring assay. The antioxidant activities were assayed using 1, 1- diphenyl-2- picrylhydrazyl (DPPH), β-carotene linoleic acid bleaching and reducing power. The results demonstrated that *B. racemosa* n-butanol fraction exhibited an active cytotoxic effect on MCF-7 cell line (IC_{50} 11.12 ± 1.05 µg/mL). The antiangiogenic activity of *B. racemosa* n-butanol fraction also showed 50 % inhibition of microvessels outgrowth in rat aortic rings at 34.55 ± 1.34 µg/mL. In antioxidant assay, the n-butanol fraction showed overall moderate activity among all assay tested. The chemical constituents present in the most bioactive faction, n-

butanol was detected by LCMS method. Three triterpenoid groups namely cucurbitacin O, pristimerol and flaccidin B were found to be present in n-butanol fraction. This present study indicated that the n-butanol fraction of *B. racemosa* seed extract exhibited active anticancer agent and possessed antiangiogenic properties. Thus, this study supported the ethnomedicinal use of the *B. racemosa* plant against cancer like diseases and the compounds identified in this plant have not been reported previously.

CHAPTER 1: INTRODUCTION

1.1 Cancer

Cancer can affect everyone including a woman and a man, young and old, the rich and the poor and today, each of us know at least a family member or a friend that has been diagnosed with cancer. So what is cancer? What are the differences between a cancer and a tumour? Cancer is a class of diseases characterized by rapid proliferation of abnormal cells which can then invade other parts of the body and spread out to other organs (National Cancer Institute, 2015). The cancer cells grow excessively in the body and a recent study has suggested it can also be considered a metabolic disorder (Coller, 2014).

Tumour is an abnormal mass of tissue, also known as neoplasm. A tumour is not necessarily a cancer, it may be benign (not cancer), pre-malignant (pre-cancerous), or malignant (cancer). Benign tumours do not spread or invade the nearby tissues while cancer can spread or invade the nearby tissues and travel to other body parts through the blood or the lymph system (National Cancer Institute, 2015).

Cancer cell can be characterised by biopsy or surgery where the tissue or fluid will be withdraw and sliced into thin layer for examination under microscope. This procedure is known as histological examination and it is the best way of cancer detection. This pathology report will reveal the patient information, cell morphology, microscopic observation, diagnosis, cancer types and cancer stage (National Cancer Institute, 2010).

1.2 Epidemiology of cancer

An estimated 12.7 million people were diagnosed with cancer across the world in 2008, and 7.6 million people died from the disease (WHO, 2011). The National Cancer Registry of Malaysia (NCR, 2004) reported a total of 21,464 cancer cases diagnosed in Peninsular Malaysia alone and the cases increased from 32,000 in 2008 to 37,400 in 2012 (The Star, 2014). In 2013, the Ministry of Health Malaysia have released the latest Health Facts 2013 and they reported that cancer is the one of the top five causes of death and one of the top ten causes of hospitalisation in both government and private hospitals. In the year 2014, cancer has overtaken heart disease as the number one killer disease in Malaysia.

The National Cancer Registry (NCR) report in 2011 showed the top ten leading cancers among the general population in Malaysia (Figure 1.1). The highest percentages of cancer were breast cancer (18.1 %), colorectal (12.3 %), lung (10.2 %), nasopharynx (5.2 %), cervix uteri (4.6), prostate gland (3.4 %) and leukaemia (4.1 %), ovary (3.6 %), stomach (3.5 %) and liver (3.3 %). In the United State America, the American Cancer Society has reported the list of the most common type of cancer diagnosed in the United States in 2015. Table 1.1 showed the number of new cases and deaths from the most common cancer types. The cancer rates are also seen in developing countries and breast cancer is the frequent diagnosed among females.

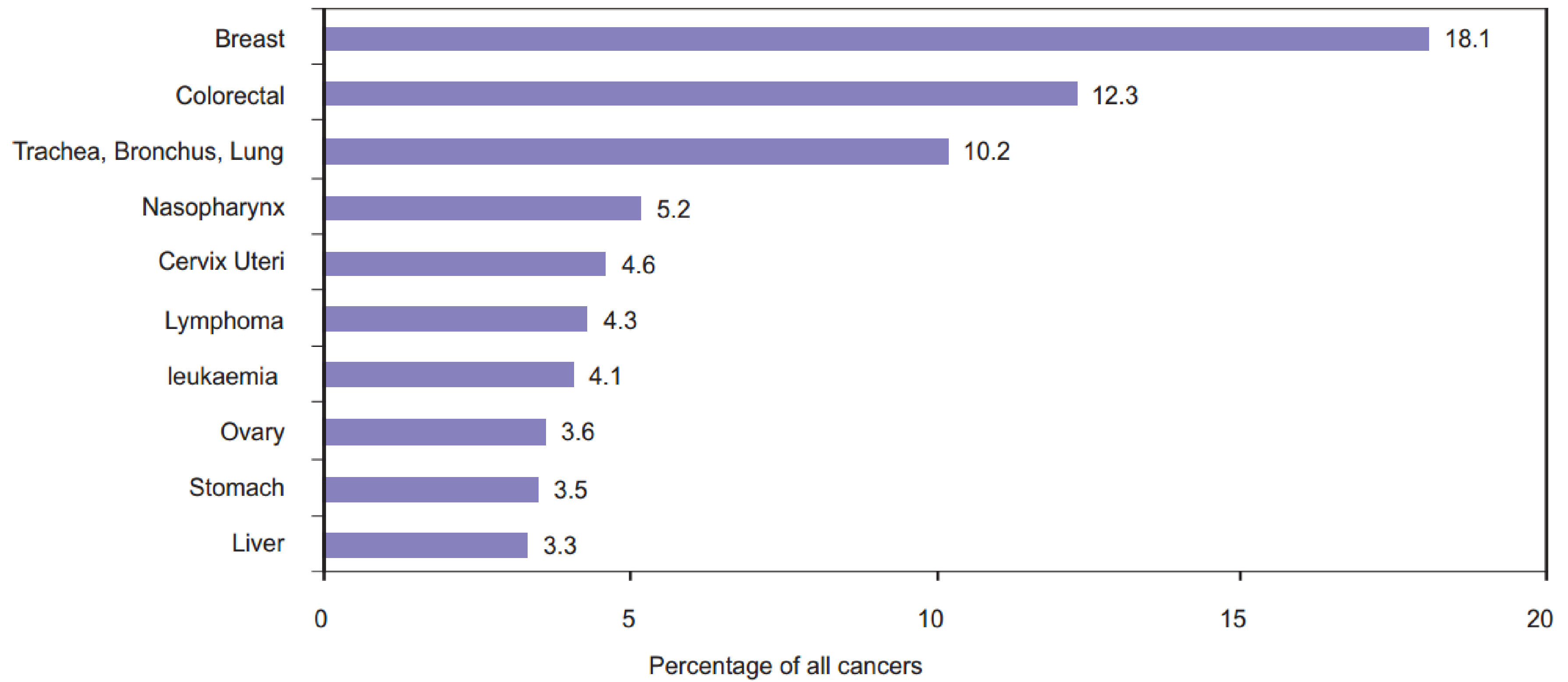

Figure 1.1: Summary statistics of cancers among the general population of Malaysia in 2011 (NCR, 2011).

Table 1.1: An estimated number of new cases and death caused by most common cancer types in America.

Cancer Type	Estimated New Cases	Estimated Deaths
Bladder	74,000	16,000
Breast (Female – Male)	231,840 – 2,350	40,290 – 440
Colon and Rectal (Combined)	132,700	49,700
Endometrial	54,870	10,170
Kidney	61,560	14,080
Leukemia (All Types)	54,270	24,450
Lung (Including Bronchus)	221,200	158,040
Melanoma	73,870	9,940
Non-Hodgkin Lymphoma	71,850	19,790
Pancreatic	48,960	40,560
Prostate	220,800	27,540
Thyroid	62,450	1,950

1.3 Types and treatment of cancer

Types of cancer come from the name of tissue or organ where the cancers originate. For example, breast cancer starts from breast cells, and prostate cancer starts in cells of the prostate. Besides, the cancer name also can be described by the types of cell that form cancer cell such as an epithelial cell or a squamous cell National Cancer Institute, 2010).

Majority of cancer patients will use surgery, chemotherapy and radiotherapy as treatment for cancer and many cancer survivors must cope with the long-term effects of treatment, as well as psychological concerns (Siegel *et al.*, 2012). Thus, integration of natural product-based drug in cancer treatment may be able to decrease the side effects caused by harsh and invasive conventional medical treatment. Consequently, in the last few years, the identification and development of natural

product-based drug has become a major area in cancer research. In fact approximately 74 % of anticancer drugs developed today originated from medicinal plants (Lopes *et al*., 2009).

1.4 Angiogenesis and cancer

What makes the tumour becomes a malignant tumour or another word cancerous? There is some element which helps the cancer cell growth and spread outside of its organ of origin. The key factor of tumour growth and distant metastasis is angiogenesis. Angiogenesis is the formation of new blood vessels from pre-existing vessels. It play a critical role in the progression of a cancer since the new blood vessels will deliver all the required nutrient and oxygen to cancer cell, thus allowing cancer to spread in other body parts (Folkman, 2002). Therefore, the process of angiogenesis is an important target in order to inhibit the cancer growth and slowly kill the cancer cell. Nowadays, antiangiogenesis targeting drugs were beginning to be the alternative treatment to cure cancer along with application of toxin delivery molecules of cytotoxic drugs.

1.5 Potential of Herbal Medicines

Plant-based traditional medicine has played a major role in the therapy of a spectrum of diseases. The plant medicinal value lies in the its selective chemical substances or secondary metabolites, that are capable of producing specific physiological action on the human body (Hassan *et al*., 2009). The use of medicinal plants as traditional medicines is important in rural areas of many developing countries (Sandhu and Heinrich, 2005; Gupta *et al*., 2005). Use of herbal extracts and nutritional supplements either as alternative or complementary medicine for the

treatment of cancer is well documented in various cultures (Dahanukar and Thatte, 2000). Natural product can be the most prolific source of biologically active compounds and play a role in the discovery and development of effective anticancer drugs. The rich and diverse plant resources of Malaysia are likely to provide effective anticancer agents where in this study, *Barringtonia racemosa* have been selected.

1.5.1 The use of herbal medication in cancer

A range of clinical studies have investigated that a spectrum of anticancer activities from various herbal medicines can be detected. A study reported that among 65 new drugs registered for cancer treatment, 48 drugs were obtained from natural products including vincristine and doxorubicin (Safarzadeh *et al*., 2014). Nowadays, traditional Chinese medicines (TCM) have discovered a number of anticancer agents, although most of their mechanisms of action have not yet been elucidated. Lin *et al*., (2007) showed that *Wedelia chinensis* herb containing various compounds such as indole-3-carboxylaldehyde and luteolin which capable of suppressing prostate cancer activity. In addition, a poplar herbal medicine such as turmeric was commonly used by cancer patients to either treat cancer or reduce the chemotherapy or radiotherapy effect. It contains 3.14 % by weight of curcumin which act as natural anticancer agent and was proven in a few clinical trials (Bar-Sela *et al*., 2010). With the various herbal medicines studies ongoing, systematic effort to expedite the discovery and development of new phytomedicines should be done intensively.

1.6 *Barringtonia racemosa*

Barringtonia racemosa (Figure 1.2) is an evergreen tree found East Africa and South East Asia. This plant is widely found in eastern Africa, India, Myanmar, Malaysia, Indonesia and southern China. The complete taxonomic classification of this plant is listed in Table 1.2. *B. racemosa* is a woody tree and is able to reach 20 m height. The leaves are about 40 cm long and 15 cm wide. The flowers are arranged in long spikes and have four white petals with white filament. The fruit is fleshy and become fibrous when ripe (Ong, 2004).

Figure 1.2: *Barringtonia racemosa* tree and fruit.

Table 1.2: Taxonomic classification of *Barringtonia racemosa* (L.) Roxb.

Taxonomic classification	
Kingdom	*Plantae*
Phylum	*Angiosperms*
Class	*Eudicots*
Order	*Ericales*
Family	*Lecythidaceae*
Genus	*Barringtonia*
Species	*B. racemosa*

1.6.1 Medicinal use of *Barringtonia racemosa*

Barringtonia racemosa locally known as putat sungai is a rich source of phytomedicine. In Malaysia, the fruit and seed are used to treat asthma, cough, abscess, ulcer and diarrhea (Ong, 2004). Of interest, ethnomedical survey has shown that the plants are traditionally used in certain remote villages of India to treat ulcer and cancer-like diseases (Thomas *et al*., 2002).

1.6.2 Bioactivities of *Barringtonia racemosa*

Extracts from different parts of the plant have also been reported to show various pharmacological activities such as antioxidant, antifungal and anti-tumour properties (Nurul Mariam *et al*., 2008; Hussin *et al*., 2009; Thomas *et al*., 2002). An anti-arthritic study done by Patil *et al*. (2011) demonstrated that a plant-derived triterpenoid from *B. racemosa* fruit extract, bartogenic acid appears to exert beneficial effects on multiple pathological manifestations of Complete Freund's Adjuvant (CFA)-induced arthritis in rats. Other studies demonstrated that the methanol extract of *B. racemosa* fruit showed high antioxidant activity compared to a well-established high antioxidant of another local plant, *H. sabdariffa* (Amran *et al*., 2016). Previous studies strongly proved that the extracts of *B. racemosa* have

strong anti-inflammatory activitiy through carrageenan-induced paw oedema (Shikha *et al*., 2010). It has long been studied that inflammation are related to cancer, and there is a strong correlations between the potential anti-inflammatory and antiangiogenesis capabilities (Rayburn *et al*., 2009; Tahergorabi *et al*., 2013). This relationship is due to the similarities of a key molecules involved in inflammatory (COX-2 and MCP-1) and angiogenesis (Cardenas *et al*., 2011). The COX-2 and MCP-1 expression are high in angiogenesis during cancer development.

1.6.3 Chemical constituents of *Barringtonia racemosa*

Previous phytochemical studies have revealed the presence of oleanane-type isomeric triterpenoids (isoracemosol A and racemosol A) from methanol extract of fruits (Gowri *et al*., 2009), and two neo- clerodane diterpenoids namely nasimaluns A and B from ethanol extract of roots of this plant (Hasan *et al*., 2000). In addition, two new triterpenoid sapogenins namely barringtogenic acid and barringtogenol have been isolated from the methanol fruit extract of *B. racemosa* (Anantaraman and Pillai, 1956). The chemical structures of isolated triterpenoid constituents were as shown as in figure 1.3. Indeed, triterpenoids are highly multifunctional and have been showed responsible for antitumor activity in their ability to block nuclear factor-kappa B activation, induce apoptosis and angiogenesis *in vitro* (Petronelli *et al*., 2009).

Quantitative phytochemical analysis of ethanol extracts of *B. racemosa* aerial parts has shown higher content of total flavonoids (Nurul Mariam *et al*., 2008). Flavonoids including polyphenols are secondary metabolites comprising the flavonols, flavones, flavanols, flavanones, anthocyanidins and isoflavonoid

subclasses (Kale *et al*., 2008). Indeed, the structurally related well-characterized flavonoid molecule, quercetin, is known to induce apoptosis in different types of cancer cells (Xavier *et al*., 2009). Of interest, the quercetin 3-O-rutinoside is known to be present in the methanol extract of fruits of *B. racemosa* (Samanta *et al*., 2010*).*

In vivo, Zhang *et al*. 2009 has also showed the significant relationship between cytotoxic effects and structurally related flavonols (quercetin, kaempferol, myricetin) as well as the molecular mechanisms responsible for the cytotoxic effects in a human esophageal squamous cell carcinoma cell line, KYSE-510. Even though a few compounds were isolated previously, no further research have been followed up using a systematic bioactivity-guided fractionation and purification of the plant fruit and seed bioactives to associate them with anticancer activities and the mode of action underlying the associated anticancer activities.

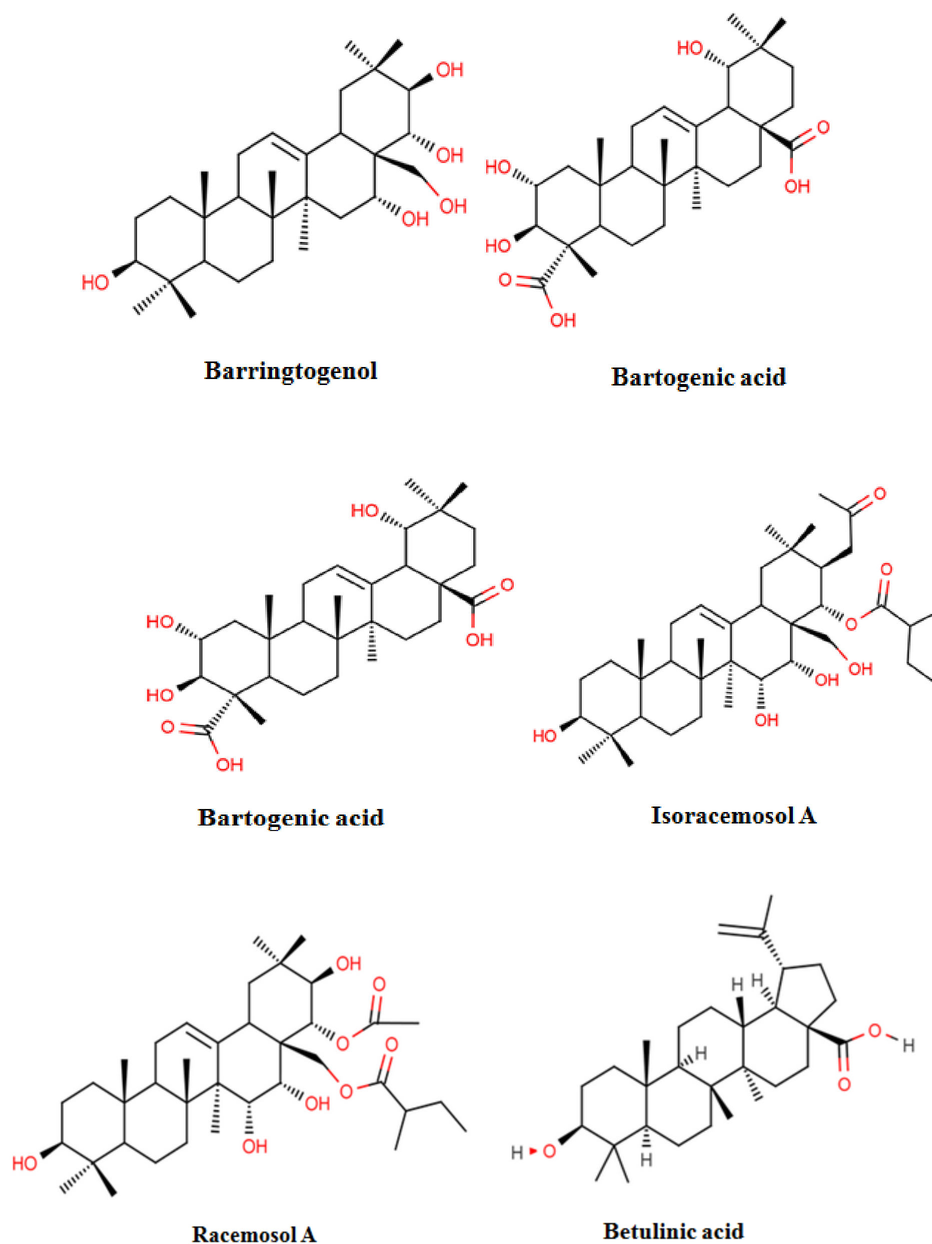

Figure 1.3: The isolated triterpenoid constituents of *B. racemosa.*

1.7 Problem statement and rationale of the study

The ethnomedicinal use of the fruits and seed of *B. racemosa* taken raw in traditional anticancer treatment is known (Ong, 2004). However, systematic and scientific investigations on the phytochemical moiety responsible for these desirable anticancer and antiangiogenic activity have yet to be explored and uncovered. The

present study is designed to determine the anticancer associated activity of *B. racemosa*; an in-depth preclinical pharmacological and biochemical studies will contribute significantly towards drug development in the prevention and/or treatment of cancer. These important plant extracts need to undergo complementary scientific methodology in order to transit from the traditional herbal medicinal source into medicinal products with effective levels of therapeutic value. We are also taking advantage of the unique pharmaceutical biodiversity in its constituents to discover new effective anticancer drugs from a Malaysian medicinal plant. In this study, local tropical plant *Baringtonia racemosa* is selected to identify for bioactive compounds associated with anticancer and antiangiogenic activity, as well as to identify what are the compounds present exert their potency against human cancer cells.

1.8 Research Objectives

The objectives of this study encompass the following:

1. To determine the anticancer activity of *Barringtonia racemosa* fruit extract, seed extract and all its fractions using *in vitro* model.
2. To determine the antiangiogenic activity of *Barringtonia racemosa* extract and its bioactive fractions associated with anticancer activity.
3. To determine the antioxidant activity of *Barringtonia racemosa* extract and its bioactive fraction.
4. To identify the bioactive chemical constituent(s) of *Barringtonia racemosa* most active fraction associated with anticancer and antiangiogenic activity.

CHAPTER 2: GENERAL METHODOLOGY

2.1 Introduction

This chapter delineates the methodology of this study which aimed to explain the process of extraction and fractionation from *B. racemosa* fruit and seed extracts. The present chapter covers starting from plant collection, extraction and separation into fractions.

2.2 Materials and methods

2.2.1 Chemicals and solvents

All chemical and solvents from various suppliers were of the highest purity needed for each application. The AR grade of methanol, n-hexane, chloroform, ethyl acetate and n-butanol were purchased from QReC, New Zealand.

2.2.2 Instrument

The plant extraction was evaluated using EYELA rotary evaporator N-1000 / N-1100 (USA), Memmert Oven (Germany) and LG refrigerator (Korea).

2.2.3 Plant material

The fresh whole fruit and seed of *B. racemosa* were collected from one source in Kepala Batas, Pulau Pinang, Malaysia (GPS coordinate: 5.471, 100.435). The plant species was authenticated by a botanist, Dr. Rahmad Zakaria with a voucher specimen number 11599. The specimen was preserved in the herbarium unit, School of Biological Sciences, Universiti Sains Malaysia.

2.2.4 Extraction of crude methanol of fruit and seed *B. racemosa* extracts

The solvent extraction was employed in order to separate the desired component using volatile organic solvent that can be removed by rotary evaporator. The fruits and seeds were washed, separated from each other and chopped into thin slices. The fruit and seed samples respectively were dried in an oven, at an average temperature of 40 ± 3 °C. They were then grounded into powder form. Dried powder of *B. racemosa* fruit and seed respectively were macerated with methanol at a ratio of dry weight: solvent of 1:10 (w/v) for 24 hours at room temperature. The extracts were filtered and evaporated on rotary evaporator to yield the crude methanol extract. The crude extracts were stored in amber vial and kept in refrigerator at 2 - 8 °C until further use.

2.2.5 Fractionation of crude methanol *B. racemosa* extract by solvent-solvent extraction

Solvent-solvent partitioning of the plant crude methanol extract was performed using separatory funnel to separate compounds based on their polarity and relative solubility in two different immiscible liquids. The crude methanol extracts of fruit and seed were dissolved in 95 % methanol AR grade and suspended with n-hexane at a mixture ratio of crude extract: solvent of 1:3 (v/v) respectively. The solution was mixed with gentle shaking to yield a brown coloured supernatant and the mixture was allowed to settle down for 24 hours. The suspension was extracted serially three times each with gradually increasing polarity solvent starting from n-hexane, chloroform, ethyl acetate to finally n-butanol. The extracts were evaporated on rotary evaporator to yield five fractions which are n-hexane fraction, chloroform fraction, ethyl acetate fraction, n-butanol fraction and aqueous fraction.

CHAPTER 3: *IN VITRO* CYTOTOXIC STUDIES OF CRUDE METHANOL EXTRACT OF *BARRINGTONIA RACEMOSA* AND ALL ITS FRACTIONS

3.1 Introduction

A Cancer cell is an abnormal cell which divides without control and is able to invade other healthy tissues or organ. To date, the development of resistance of cancer to some cancer drugs or treatments in the market has urged researchers in the field in search for a new anticancer drug (Mohammed *et al*., 2009). Moreover, the standard clinical treatments of cancer are often accompanied with severe adverse reactions in patients. Alternative anticancer drugs or compounds of lower toxicity with specific targeted action on cancer cell on normal cell are needed. There is always a need to find a potential and safer drug against cancer and traditional medicinal can offer such a source. World Health Organization (WHO) also has been taken the step on natural sources in order to work on active or greater activity of natural compounds (WHO, 2011).

Natural resources like plant contain many different phytochemicals which play an important role as active compounds in pharmaceutics, cosmetics and nutraceutics. The phytochemicals present in plant have been acting as a backbone for pharmaceutical industry and it was reported that 25 % of the molecules used in related industry were build up from natural plant origin (Payne *et al*., 1991). A superior example could be taxol (Paclitaxel) which comes from *Taxus brevifolia* extract that has displayed potent anticancer agent since its discovery in 1971. By employing purification of the plant extract and testing each purified fraction for anticancer bioactivity, the researcher was able to identify the pure substance responsible for the anticancer activity, taxol. This discovery led scientists to work on

the potential of plant as an alternative source to produce new compounds important for anticancer activity.

Cytotoxic assays are widely used in pharmaceutical research to screen for cytotoxic activity of chemical compounds or mixture of compounds as in natural product. The cell based assay is very important to determine if the plant extract have any effect on cell proliferation or cytotoxic effect which eventually lead to cell death (Riss *et al*., 2013). In developing a therapeutic drug that targets rapidly on dividing cancer cells, there are many methods employed for cytotoxic screening and one of the best known methods is 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay. The MTT assay is an established and best known colorimetric enzyme methods based on a reductive colouring reagent where colour changes in the presence of mitochondrial dehydrogenase activities in viable cells can be spectroscopically detected.

MTT a yellow water-soluble tetrazole is reduced by mitochondrial dehydrogenase in living cell to a purple coloured crystal formazan by NADH. The purple formazan formed acts as indicator and represents viable cell due to ability of mitochondrial dehydrogenase activities which is present in healthy cell. In turn, the dead cells will have lost their ability to reduce the yellow colour of MTT reagent to purple formazan. This method is superior because it is easy to perfom, safe, has good reproducibility and is widely used in both cell viability and cytotoxicity tests. Since reduction of MTT can only occur in metabolically active cells, the viability of the cells through colour changes can be measured easily.

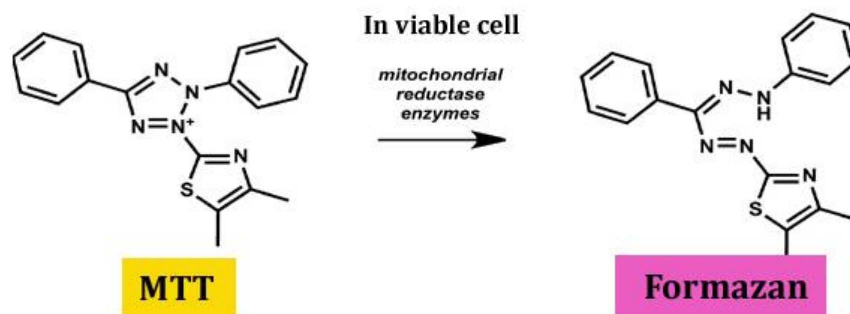

Figure 3.1: Viable cancer cells will reduce the yellow colour of 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) reagent to a purple formazan product.

Apoptosis is a programmed cell death and most of anticancer research has focused on apoptosis strategy as it does not induce an inflammatory reaction which can cause harm and stress to the normal tissues (Rock and Kono, 2008). The apoptosis mechanism can be detected at DNA level by DNA fragmentation and enzyme level by caspases activation (Krishnaraja *et al*., 2014). The DNA fragmentation assay allows the determination of DNA degradation upon cells treatment with a potential anticancer agent. In apoptotic cells, the ladder pattern of DNA fragment action becomes evident following an electrophoresis analysis.

In this chapter, the fruit and seed of *B. racemosa* were extracted by methanol. A further separation into fractions were done by serial extraction solvents of n-hexane, chloroform, ethyl acetate, n-butanol and aqueous respectively in the fractionation method. The aim is to investigate any potential anticancer properties of the different purified components of the plant extracts on two different cancer cell lines namely the breast cancer cell line (MCF-7) and colorectal cancer cell lines (HCT-116). The most active fraction was then selected and investigated in the DNA fragmentation

assay to observe if it exerts apoptosis mechanism of action associated with the observed cytotoxic activity.

3.2 Materials and Methods

3.2.1 Chemicals and solvents

Dulbecco's Modified Eagle Medium (DMEM) and Roswell Park Memorial Institute medium (RPMI) cell culture media, Fetal Bovine Serum (FBS), streptomycin/penicillin (PS) solution, trypsin-EDTA and trypan blue dye solution were purchased from Gibco Life Technology, UK. 5-fluorouracil (5-FU), tamoxifen, dimethyl sulfoxide (DMSO), sodium bicarbonate ($NaHCO_3$), 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) and phosphate-buffered saline (PBS) tablet were obtained from Sigma-Aldrich (Sigma Chemical Co., St Louis, MO, USA). All solvents were purchased from QReC, New Zealand.

3.2.2 Instrument

The cytotoxic assay was evaluated in ESCO biosafety cabinet (USA) using Fisher Scientific incubator (Germany), EVOS fluorescence microscope (USA) and Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer (USA).

3.2.3 Extraction of crude methanol of fruit and seed *B. racemosa* extract

The solvent extraction was employed in order to separate the desired component using volatile organic solvent that can be removed by rotary evaporator. The scheme used for the extraction of *B. racemosa* is shown in Figure 3.2.

3.2.4 Fractionation of crude methanol *B. racemosa* extract by solvent-solvent extraction

Solvent-solvent partitioning of the plant crude methanol extract was performed using separatory funnel to separate compounds based on their polarity and relative solubility in two different immiscible liquids. Fractionation was performed as in chapter 2.

3.2.5 Cytotoxic activity of *B. racemosa* extracts and fractions

3.2.5(a) Condition of cell lines and culture

3.2.5(a)(i) Media preparation

The breast cancer cell line (MCF7) and colorectal carcinoma cells (HCT-116) were purchased from the American Tissues Culture Collection (ATCC, USA). Both cells were evaluated in the investigation for cytotoxic activity of the plant crude extracts and fractions. The MCF-7 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) while the HCT-116 cells were maintained in Roswell Park Memorial Institute medium (RPMI). The media was prepared by dissolving 13.4 g (1 Packet) of DMEM/RPMI powder (Gibco) and 1.8 g of sodium bicarbonate ($NaHCO_3$, Sigma) with 1 L of deionized distilled water (18.2 Ω). Then, the media was filtered in sterile condition using a Nalgene rapid-flow Sterile Disposable Filter Unit (pore size 0.20 µm, 1000 mL, US). The filtered media was aliquoted into 50 mL conical centrifuge tube and kept at 2 - 8 °C. Both cells medium were supplemented with 10 % heat inactivated Fetal Bovine Serum (FBS) and streptomycin/penicillin (100 µg/ml) in culture flask at 37 °C in 5 % CO_2 incubator. Subculture was performed every 3 days.

3.2.5(a)(ii) Routine feeding and maintenance

The cells morphology and cell density of the breast cancer cell and colorectal cancer cell culture were examined regularly and routinely both macroscopically and microscopically for any presence of contaminants such as bacteria or fungi. The colour and turbidity of the medium were also regularly monitored to maintain the pH of medium and nutrients. The medium of the cell culture was changed every two or three days depending on growth rate of the cells (Macleod and Langdon, 2004).

3.2.5(a)(iii) Cell subculturing

Subculturing cell is a critical part in cell growth and proper sterile technique need to be implemented to make sure the cells are healthy and free from any contamination. The subculturing process is done once the cells become confluence (70 % - 90 %). In subculturing, the old medium was aspirated from the culture flask and the attached cells were washed with 5 mL phosphate-buffered saline (PBS). The PBS was then aspirated and 700 μL of trypsin was added and incubated in incubator for 4-5 minutes. The cells were observed under microscope to detect for detachment. After achieving 90 % detachment, a volume of 5 mL pre-warmed complete growth medium was added into the flask and the cells were dispersed by pipetting over the surface. The cells were then transferred to a 15 mL conical tube and centrifuged at 200xg for 4-5 minutes. The supernatant was discarded while pellet of the cells was resuspended with pre-warmed complete growth medium. About 1 mL of cells was transferred into a new flask containing 5 mL of pre-warmed complete growth medium. The cells were cultured at 37 °C in a 5 % CO_2 incubator. The passage number was recorded after subsequent subculture in maintaining the lifetime and achieving maximum passage number of the cells (Macleod and Langdon, 2004).

3.2.5(b) Cell counting

Cell counting was employed in determining the number of cells to calculate cell concentration that is required before undergoing any cell based assay. This process requires a counting chamber called hemacytometer, a device invented by Louis-Charles Malassez to perform blood cell counts in 19th century. One advantage of using the hemocytometer method is that it allows for a variation of technique involving the use of Trypan blue dye to differentiate between dead/damaged cells and the healthy viable cell of the population. The cells suspension was prepared by taking out 100 µL of cell suspension using sterile pipette into a 2.0 mL centrifuge tube. About 100 µL of 0.4 % Trypan Blue was added to stain the viable cell. A 10 µL aliquote of the mixtures were then transferred and filled into both chambers underneath the hemacytometer coverslip. The number of cells were viewed and counted under microscope at a magnification of 10X. The cells numbers inside four squares were counted using following formula (Eq.3.1):

$$\text{Cells numbers} = \text{Total cells counted} \times \frac{\text{dilution factor}}{\text{no. of squares}} \times 10\,000 \text{ cells/mL} \quad \text{Eq.3.1}$$

3.2.5(c) *In vitro* cytotoxic activity of crude methanol *B. racemosa* extracts and its fractions

The cytotoxic effect of extracts and fractions was evaluated by 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay according to the method developed by Mosmann, 1983 with modifications using human breast carcinoma cell line (MCF-7) and human colorectal carcinoma cell line (HCT-116). Briefly, after being harvested from culture flask, each MCF-7 and HCT-116 cells were seeded at 1 x 10^4 cells/mL in a 96-well plate containing 100 µL of fresh

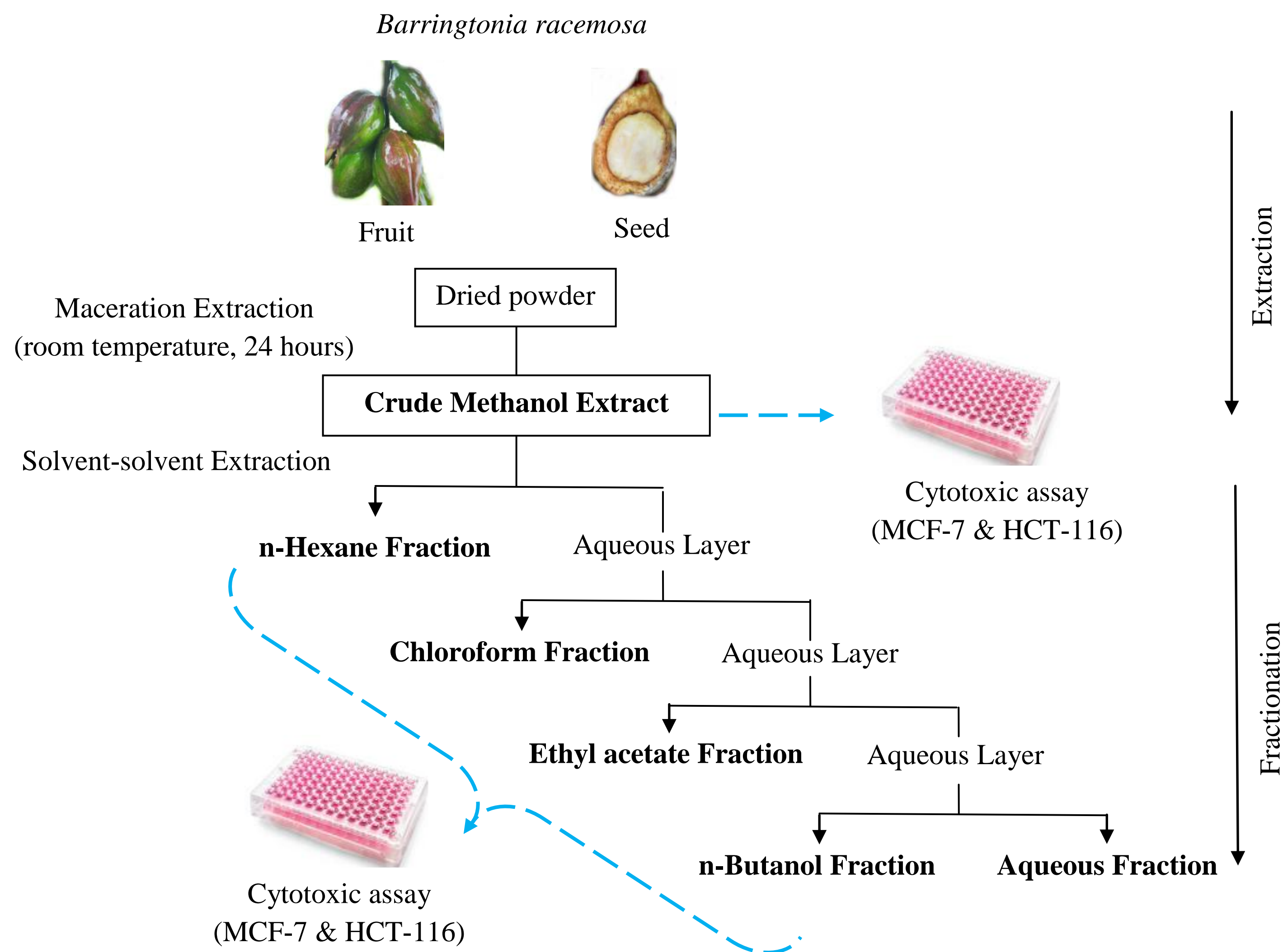

Figure 3.2: Schematic diagram showing the extraction and sequential fractionation of the dried fruit and seed of *B. racemosa* respectively. Cytotoxic assay was performed at crude extract level and each fraction to select the potent anticancer activity.

growth media per well. The plate was incubated at 37 °C in 5 % CO_2 for 24 hours. After incubation, 100 µl of medium containing extracts or fractions at six different concentrations (400 µg/ml, 200 µg/ml, 100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml and 6.25 µg/ml) respectively were added into 96 well plates and incubated for 48 hours. Positive control of tamoxifen and 5-Fluorouracil, blank containing medium only and negative control containing cell with medium in 1 % DMSO only were included. Then, the medium from each well was aspirated and replaced by 20 µL of MTT (5 mg/mL) and incubated for 3-4 hours. After that, the crystals were solubilized by adding 120 µl of DMSO. After 10 mins of incubation, the absorbance was measured at 570 nm ʎmax using a Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer. The inhibition percentages of the cells were calculated using following equation (Eq.3.2):

$$I\,(\%) = [1 - (A_t - A_0)/(A_1 - A_0)] \times 100 \quad \text{Eq.3.2}$$

Where, I (%) is an inhibition percentage of the cells. A_t is the absorbance of test samples, A_0 is the absorbance of a blank and A_1 is the absorbance of a negative control.

Tamoxifen (MCF-7) and 5-Fluorouracil (HCT-116) served as a positive control. The 50 % inhibition of each sample was then determined by plotting the values of inhibition percentage against respective samples concentration. Consequently, the logarithmic regression equations obtained were used to calculate the 50 % inhibition. The results were expressed as mean ± SEM (n=3). The plant fraction with lowest 50 % inhibition was selected for further apoptosis, antiangiogenic, antioxidant and phytochemical study.

3.2.6 Mechanism of action: Apoptosis studies

3.2.6(a) DNA fragmentation assay

Any sign of apoptosis involvement in the anticancer mechanism can be evidenced by a DNA fragmentation pattern. In this study, the potent fraction of plant fraction on selective cancer cell line was chosen for evaluating the apoptosis study. The breast cancer cell line, MCF-7 (5×10^4 cells per ml) was seeded in cultured flasks. After 24 hours incubation, a medium containing n-butanol fractions at IC_{50} concentrations (11.5 µg/mL) was added into the flask and incubated for 24 hours. The treated cells were collected by centrifugation after trysinization (1000 rpm, 10 min). The supernatant was discarded. The pellet was resuspended in 1 ml of PBS and centrifuged at 1000rpm for 10 minutes. Pellet was collected and extracted using Qiagen DNA extraction kit. The extraction of DNA was performed using protocol provided by Qiagen DNA extraction kit. The samples were analyzed by electrophoresis on a 1.5 % agarose gel and assessed under UV illumination.

3.2.7 Statistical analysis

Statistical analysis was carried out by SPSS software version 16.0. All experiments values were expressed as means ± SEM (standard error mean). Data were analyzed and the comparisons of the samples to positive control were done using one-way analysis of variance (ANOVA) followed by post-hoc test treatment of Dunnett's test. The differences were considered significant at $P<0.05$.