DNA FRAGMENTATION IN HeLa CELLS TREATED WITH *Brucea javanica* FRUITS EXTRACT

by

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CERTIFICATE

This is to certify that the dissertation entitled "DNA Fragmentation in HeLa Cells Treated with *Brucea javanica* Fruits Extract" is the bonafide record of research work done by Ms Noor Hidayu binti Pathni during the period from July 2008 to October 2008 under my supervision.

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LIST OF ABBREVIATIONS

ACS	American Cancer Society
AIF	Apoptosis Inducing Factor
ATCC	American Type Culture Collection
BJF	*Brucea javanica* fruit
BrdU	5-bromo-2'-deoxyuridine
BrdUTP	5-bromo-2'-deoxyuridine 5'-triphosphate
cm^2	Square centimeter
CO_2	Carbon dioxide
dH_2O	Distill water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonuclic acid
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
g	gram
HCl	Hydrochloric acid
HPV	Human Papilloma Virus

hr	hour
Hz	Hertz
IC_{50}	Median concentration that causes 50% inhibition
IUPAC	International Union of Pure and Applied Chemistry
KCl	Potassium chloride
m	Meter
mg	Milligram
mg/ml	Milligram per milliliter
ml	Milliliter
mm	Millimeter
M	Mole
MBA	Methylene Blue Assay
nm	Nanometer
N	Normal
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NCI	National Cancer Institute
OD	Optical Density
PBS	Phosphate Buffer Saline
rpm	Round per minutes
SDS	Sodium Dodecyle Sulfate
TdT	Terminal Deoxynucleotidyl Ttransferase

TUNEL	Terminal Deoxynucleotide Transferase dUTP Nick End Labeling
V	Volt
WHO	World Health Organization
µg/ml	Microgram per milliliter
µl	Microliter
µm	Micrometer
v/v	Volume per volume
°C	Degree celcius
%	Percentage

DNA FRAGMENTATION IN HeLa CELLS TREATED WITH *Brucea javanica* FRUITS EXTRACT

ABSTRACT

Fruits of *Brucea javanica* (*B. javanica*) have been used as traditional medicine in China as it has been shown to have antimalarial, antiplasmodial, antitumor or anticancer and antiamoebic. In this study, methanol extracts of *B. javanica* fruit (BJF) were treated on human cervical cancer cell (HeLa). Antiproliferative activity of BJF methanol extracts on HeLa cells showed IC_{50} value of 13.34 μg/ml. After that, TUNEL assay (Terminal Deoxynucleotide Transferase dUTP Nick End Labeling) was done to detect the apoptosis rate of BJF methanol extract on HeLa cells. The apoptosis rate for 24 h, 48 h and 72 h were 13.8%, 17.8% and 22.9% respectively. The percentage of apoptosis rate was time dependent. These results proposed that BJF methanol extracts exhibits antiproliferative effect on HeLa cells by inducing apoptosis. Therefore, it may be a potential candidate for antiproliferative agent for the treatment of human cervical cancer cells.

FRAGMENTASI DNA TITISAN SEL KANSER HeLa YANG DIRAWAT DENGAN EKSTRAK BUAH *Brucea javanica*

ABSTRAK

Buah *Brucea javanica* (*B. javanica*) telah digunakan dalam perubatan China sebagai ubatan tradisional kerana mempunyai ciri-ciri antimalaria, antiplasmodia, antitumor atau antikanser dan antiamoebik. Titisan sel kanser serviks (HeLa) dirawat dengan ekstrak metanol buah *B. javanica.* Aktiviti antipoliferasi ekstrak methanol buah *B. javanica* menunjukkan nilai IC_{50} (kemampuan ekstrak membunuh 50% sel kanser) sebanyak 13.34 μg/ml terhadap titisan sel kanser HeLa. Asai TUNEL (Terminal Deoxynucleotide Transferase dUTP Nick End Labeling) dilakukan untuk menentukan kadar apoptosis ekstrak buah *B. javanica* terhadap titisan sel kanser. Kadar apoptosis bagi 24 j, 48 j dan 72 j masing-masing adalah 13.8%, 17.8% dan 22.9%. Kadar apoptosis bergantung kepada masa rawatan. Keputusan ini mencadangkan buah *B. javanica* mempamerkan kesan antiproliferasi ke atas titisan sel kanser serviks melalui induksi apoptosis. Oleh yang demikian, ia mungkin berpotensi sebagai calon kepada antikanser.

CHAPTER 1

INTRODUCTION

1.1 Cancer

Nowadays, cancer prevalence has been increasing each day throughout the world. According to Cragg and Newman (2004), it is the second leading cause of death in the world while in Malaysia; it is the fourth leading cause of death (Lim *et al.,* 2002). According to American Cancer Society (2008), cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. If the spread is not controlled, it can result in death. Balasubashini *et al.* (2006) stated that in all types of cancer, genetic alterations give rise to changes in expression, activation or localization of regulatory proteins in the cells. They then affect the signaling pathways that alter their response to regulatory stimuli and allow the unrestricted cell growth.

Cancer can be caused by both external factors and internal factors as shown in Table 1.1. These causal factors may act together or in sequence to initiate or promote carcinogenesis. Ten or more years often pass between exposure to external factors and

detectable cancer. Cancer or tumor can also be differentiated into either benign or malignant as shown in Tables 1.2 (American Cancer Society, 2008).

Table 1.1 The causes of cancer.

External Factors	Internal Factors
Tobacco	Inherited mutations
Chemicals	Hormones
Radiation	Immune conditions
Infectious organisms	Mutations from metabolism

(Source: American Cancer Society, 2008)

Table 1.2 The differences between benign and malignant tumors

Benign Tumors	Malignant Tumors
▪ Not cancer	▪ Cancer
▪ Rarely life-threatening	▪ May be life-threatening
▪ Can be removed and usually do not grow back	▪ Often can be removed and sometimes grow back
▪ Cells from benign tumors do not spread to other part of the body.	▪ Cells from malignant tumors can spread to other parts of the body
▪ Cells from benign tumors do not invade the tissues around them.	▪ Cells from malignant tumors can invade and damage nearby tissues and organs.

(Source: National Cancer Institute, 2007)

The cancer treatments are either by surgery, radiation, chemotherapy, hormone therapy, biological therapy, and targeted therapy. The chemotherapeutic treatment

strategies usually attempt directly to inhibit proliferation of cancer cells or selectively remove transformed cells by inducing apoptosis or eliminating the cause of the growth advantage (Balasubashini *et al.*, 2006).

In 2008, it is expected that about 1,437,180 new cancer cases to be diagnosed. This estimate does not include carcinoma in situ (non-invasive cancer) of any site except urinary bladder, and does not include basal and squamous cell skin cancers. More than 1 million cases of basal and squamous cell skin cancers are expected to be diagnosed this year. This year, about 565,650 Americans are expected to die of cancer, more than 1,500 people a day. Cancer is the second most common cause of death in the US, exceeded only by heart disease. In the US, cancer accounts for 1 of every 4 deaths (American Cancer Society, 2008).

According to CS Market Research-Malaysia (2005), the incidence of cancer in Malaysia is estimated to be around 150 per 100,000 populations. It means that there are about 35,000 new cancer cases each year. As a result, the cumulative lifetime risk of getting cancer was 1 in 4, in Malaysia. The largest group of admissions was for cancer of the blood (leukemia) accounting for 13.6%, followed by: breast cancer (10%), lung cancer (9.3%), cervical cancer (6.6%) and lastly cancer of the mouth and throat (6.5%).

The definition for cervical cancer is it is a cancer that affects women of different ages and backgrounds in the United States and across the world as stated in Cervical Cancer Key Facts (2007). It begins in the cervix, which is the part of the uterus, or womb,

that opens to the vagina. This cancer typically develops slowly over time and can eventually spread into the surrounding areas. The prevalence of cervical cancer worldwide shows about 500,000 women are diagnosed with cervical cancer and more than 270,000 women die that is in every two minutes, a woman dies of cervical cancer somewhere in the world.

Most of the drugs used for the treatment of cancer are derived from plants and this was proved by US National Cancer Institute which has implemented a large-scale project of acquisition and screening of compounds isolated from medicinal plants. The screening of medicinal plants for potential anticancer properties has been increased greatly over the past five decades and there are worldwide efforts to discover new anticancer agents from plants. The medicinal plants are identified based on ethnopharmacological, chemosystemic and ecological information. There is still a need for more effective anticancer agents since the most common tumors of the adult are resistant to available anticancer drugs and the majority of the available drugs have limited antisolid tumor activity (Bungu *et al.,* 2006).

According to Cragg and Newman (2004), it is known that more than 3000 plant species have been reportedly used in the treatment of cancer as plants play an important role as a source of effective anticancer agents. Currently, there are over 60% of used anticancer agents are derived from natural sources including plants, marine organisms and microorganisms as stated by Val'ko *et al.* (2007). Extracts or residues of more than 500 plant species, used alone or in combination, are documented in the literature on Chinese

traditional medicine to have activity against helminth and micro-invertebrate pests of humans (Zasada *et al.,* 2002).

In this study, *Brucea javanica* is used as the plant of interest because it can be found in Malaysia and it is known to have anticancer property as stated by Ong (2004). It is a deciduous shrub with downy branches native to China, East India and to the south till Australia. It is also contain bitter alkaloid glycosides, yatanine and formic acid. Furthermore, it has been used traditionally for two centuries in Chinese medicine and as traditional medicine.

1.2 *B. javanica*

B. javanica is a tropical, 3 m high, woody shrub, clothed in yellow fluff. It is found from southern China to northern Australia. The seeds are used in Traditional Chinese Medicine for the treatment of dysentery, malaria and cancer while the leaves are used in folk medicine for poultices on boils, ringworm, scurf, centipede bites and enlarged spleens (Choi *et al.,* 2005). Figure 1.1 below show the *B. javanica* plant while Figure 1.2 shows the *B. javanica* fruits. Local names of *B. javanica* include kuwalot in Indonesia, embalau padang, kusum, lada pahit in Malaysia, balaniog, magkayapos, manongao-bobi in Philippines and rat-cha-dat in Thailand (Asia Medicinal Plants Database, 2008).

Taxonomy

Kingdom: Plantae

Division : Magnoliophyta

Class : Magnoliopsida

Order : Sapindales

Family : Simaroubaceae

Genus : *Brucea*

Species : *javanica*

Figure 1.1 *B. javanica* plant
(Source: http://www.fzrm.com/plantextracts/Java_Brucea_Fruit_extract.htm)

Figure 1.2 *B. javanica* fruits
(Source: http://jogjagarden.blogspot.com/2008/02/more-fruits-in-rainy-season.html)

The fruits appearance of *B. javanica* is ovoid. 6 to 10 mm in length and 4 to 7 mm in diameter. Externally black or brown with raised reticulate wrinkles, the lumen irregularly polygonal, obviously ribbed at both sides. Apex acuminate, base having a dented fruit stalk scar with hard and brittle shell. The seeds are ovoid with 5 to 6 mm in length and 3 to 5 mm in diameter. The external is yellowish white and reticulate with thin testa and milky white and oily cotyledons (Maslinda *et al.,* 2008).

The leaves and roots of B. javanica are depurative. The fruit is used for treatment of colic while the seed is used in the treatment of dysentery, coughs, fever, jaundice rheumatism and malaria. The decoction is used in the treatment of hemoptysis, inflammations, snakebite, stomachache, laryngitis and traumatic fractures (Maslinda *et al.,* 2008).

1.3 HeLa Cells

HeLa cell is an immortal cell which is derived from cervical cancer cells taken from an African American woman named Henrietta Lacks who died in 1951. The cancer cells are considered "immortal" as they can divide an unlimited number of times and have been grown in cell culture in an unbroken lineage ever since. These cells proliferate abnormally rapid than other cancer cell lines. HeLa cells have been reported to contain human papillomas virus (HPV-18) sequences and p53 expression was reported to be low.

1.4 Rationale of Study

Nowadays, most current therapies-drugs effectiveness is limited by the emergence of multidrug resistance. Studies showed that most chemotherapeutic agents exert their anticancer activity by inducing apoptosis which is required to eliminate damage and abnormal cell. If the cell is resistant to apoptosis, it will automatically resistant to anticancer agent (Noe'lia *et al.,* 2006). Apoptosis is important in cancer because it caused the abnormal cells to commit suicide and hence inhibit the cells proliferation of the cancer cells. This is to ensure that the abnormal cells are death and cannot cause more damage to the body and also increased the chance of recovery in the treatment of cancer patient. This study is done to ensure that the bioactive compound in *B. javanica* is a selective antiproliferative agent for chemotherapy and is not just accidentally occurred. According to Maslinda *et al.* (2008), HeLa cells treated with methanol extracts of BJF showed the best IC_{50} value compared to petroleum ether extracts and aqueous extracts so this is why only methanol extracts of BJF will be used in this study. The antiproliferative assay will be done by methylene blue to study the antiproliferative effects of BJF methanol extracts on HeLa cells while the determination of apoptosis cells will be done by TUNEL Assay.

1.5 Research Objectives

The purposes of conducting the research are:

1. To determine the antiproliferative activity of *B. javanica* fruit methanol extracts in inhibiting HeLa cells proliferation.
2. To determine the DNA fragmentation event in HeLa cells treated with *B. javanica* fruits (BJF) methanol extracts via TUNEL assay.

CHAPTER 2

LITERATURE REVIEW

2.1 Secondary Metabolites from *B. javanica*

The water extract of *B. javanica* contain cytotoxic antileukaemic activities (Cassady and Stuffness, 1980). Animal and cell lines testing have shown that bruceatin is possibly a potent anticancer agent (Huang, 1992). The quassinoid glucosides in BJF show selective cytotoxicity in the leukaemia and non-small cell lung, colon, CNS, melanoma and ovarian cancer (Fukamiya *et al.,* 1992). Emulsion from BJF inhibited growth activity of cultured human carcinoma cells (Xuan *et al.,* 1994) and Bruceoside C is used against KB, A-549, RPMI and TE-671 tumor cells (Fukamiya *et al.,* 1992). Apoptosis inducing activity has been reported in more recent in vitro work (Lau *et al.,* 2005; Wang *et al.,* 2003).

Plants from Simaroubaceae family are known to contain compounds with highly oxygenated triterpenes and bitter taste quassinoids (Khosa *et al.,* 1985). Initially, the compounds of such chemical nature were known by the term "quassin" after the physician "Quassi" who used the bark of plants from this family for the treatment of fever (Polonsky, 1985).

According to their basic skeleton, quassinoids are categorized into five distinct groups, C-18, C-19, C-20, C-22 and C-25 types shown in Figure 2.1. Among them C-20 quassinoids have especially been the subjects of extensive investigations to dig their biological activities partially due to the discovery in the early 1970s by National Cancer Institute that some of these compounds possess marked antileukemic activity (Guo *et al.*, 2005). Many of these quassinoids display a wide range of biological activities *in vitro* and/or *in vivo,* including antitumor, antimalarial (Chulabborn *et al.,* 1994), antiviral (Polonsky, 1985), anti-inflammatory, antiamoebic (Duriez, 1962), antifeedant, insecticidal, antiulcer (Toma *et al.,* 2002) and herbicidal activities.

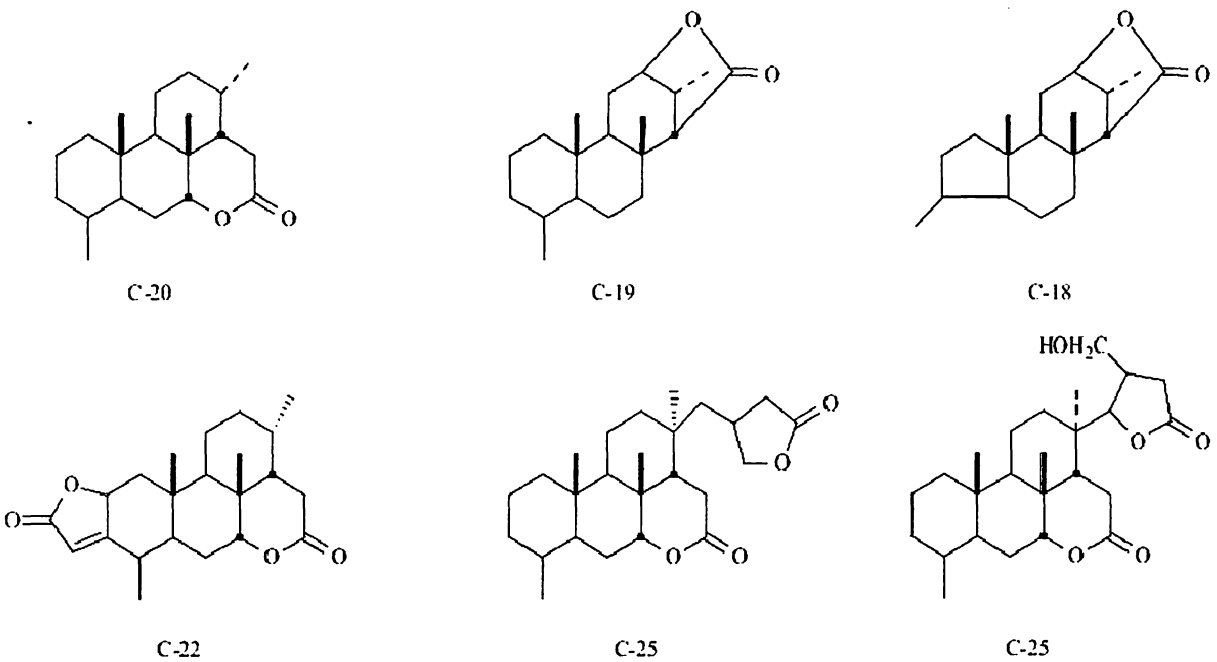

Figure 2.1 Skeleton of quassinoids

Semisynthesis of a natural product usually is an alternative source for biologically active compounds found in lower content in nature. The most successful example of semisynthesis of quassinoids is the conversion of bruceoside A, a compound easily