

## **UNIVERSITI PUTRA MALAYSIA**

INDUCTION OF SELECTIVE CYTOTOXICITY AND APOPTOSIS IN HUMAN T4 – LYMPHOBLASTOID CELL LINE (CEMss) USING BOESENBERGIN A ISOLATED FROM BOESENBERGIA ROTUNDA L.

**NG KUAN BENG** 

IB 2013 36



### INDUCTION OF SELECTIVE CYTOTOXICITY AND APOPTOSIS IN HUMAN T4 – LYMPHOBLASTOID CELL LINE (CEMSS) USING BOESENBERGIN A ISOLATED FROM *BOESENBERGIA ROTUNDA L*.

By

NG KUAN BENG

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in fulfillment of the Requirements for the Master of Science

May 2013

# **DEDICATION**

# THIS THESIS IS DEDICATED TO

MY BELOVED PARENTS NG SING YAN AND KHOO SOO HOON MY BELOVED PARENTS IN LAW MY BELOVED BROTHER NG KWANG YONG MY BELOVED WIFE GOH YEE MEI ALL MY SUPERVISORS AND LECTURERS ALL MY SOULMATES AND FRIENDS AND

THOSE WHO CONTRIBUTE IN MY RESEARCH WORK TO MAKE MY GOAL BECOMES TRUE Abstract of thesis presented to the senate of Universiti Putra Malaysia in fulfilment of the requirement for the Master of Science

# INDUCTION OF SELECTIVE CYTOTOXICITY AND APOPTOSIS IN HUMAN T4 – LYMPHOBLASTOID CELL LINE (CEMSS) USING BOESENBERGIN A ISOLATED FROM *BOESENBERGIA ROTUNDA L*.

By

NG KUAN BENG

May 2013

Chairman: Ahmad Bustamam Abdul, PhD

#### **Faculty: Institute of Bioscience**

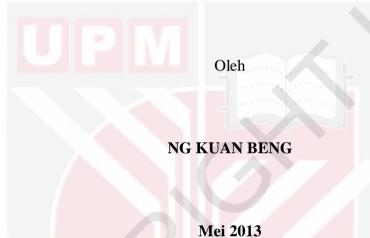
*Boesenbergia rotunda* (Roxb.) Schlecht (family zingiberaceae) is a rhizomatous herb that is distributed from north-eastern India to south-east Asia, especially in Indonesia, Thailand and Malaysia. Previous research has shown that the crude extract of this plant has cytotoxic properties. The current study examines the cytotoxic properties of boesenbergin A isolated from *Boesenbergia rotunda*. MTT assay was used to check the cytotoxicity of boesenbergin A. Boesenbergin A showed that it has the highest cytotoxicity towards human T4- lymphoblastoid cell line (CEMss)(IC<sub>50</sub> = 8.11 µg/ml). The morphological assessment of apoptosis was monitored using



normal and fluorescence microscopy. Evidence of apoptosis including blebbing and chromatin condensation was shown during the observation of microscopy analysis. The early and late phase of apoptosis was investigated using annexin V and DNA laddering assays, respectively. Annexin V assay revealed that early apoptosis is induced after treatment and DNA laddering confirmed that DNA fragmentation had occurred during late apoptosis. The mitochondrial membrane potential (MMP) was assessed by fluorescence microscopy and found that there is an increased in mitochondrial membrane potential in the treated CEMss cells. Human apoptosis proteome profiler assays were performed to investigate the mechanism of cell death. In addition, the protein levels of Bax, Bcl2 and Hsp 70 were also analyzed using western blot. Assays of caspase = -3/7, -8 and = -9 were carried out in order to test for induction during treatment. The activity of caspases -3/7, -8 and -9 was found to increase after treatment which indicates both intrinsic and extrinsic pathways are induced during apoptosis. Lastly, cell cycle progression was analyzed using flow cytometry and found that boesenbergin A is capable of inducing G2/M phase cell cycle arrest. The current findings warrant further research on boesenbergin A as a novel chemotherapeutic agent for leukemia intervention including studies in animal models.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

# INDUKSI SITOTOKSIK MEMILIH DAN APOPTOSIS DALAM SEL MANUSIA T4-LYMPHOBLASTOID (CEMSS) MENGGUNAKAN BOESENBERGIN A YANG DIASINGKAN DARIPADA *BOESENBERGIA ROTUNDA L*.



#### Pengerusi: Ahmad Bustamam Abdul, PhD

#### **Fakulti: Institut Biosains**

*Boesenbergia rotunda* (Roxb.) Schlecht (keluarga *Zingiberaceae*) adalah herba rhizomatous yang boleh didapati dari utara-timur India sehingga ke selatan-timur Asia, terutamanya di Indonesia, Thailand dan Malaysia. Penyelidikan yang lalu telah menunjukkan bahawa ekstrak *Boesenbergia rotunda* mempunyai nilai antikanser. Kajian ini adalah untuk mengkaji sifat antikanser *Boesenbergin A* yang merupakan sebatian tulen yang diasingkan daripada *Boesenbergia rotunda*. Ujian saringan sitotoksik (MTT) telah digunakan untuk menyiasat sifat sitotoksik boesenbergin A. Boesenbergin A menunjukkan ketoksikan yang tertinggi terhadap sel kanser CEMss dengan IC<sub>50</sub> = 8.11  $\mu$ g/ml. Untuk memeriksa morfologi apoptosis, mikroskop biasa dan 'inverted' telah digunakan. Bukti di mana sel mengalami apoptosis seperti sel membengkak dan pemeluwapan chromatin telah didapati. Proses apoptosis samada peringkat awal ataupun akhir telah disiasat melalui Annexin V dan juga 'DNA laddering'. Annexin V telah membuktikan bahawa boesenbergin A telah menyebabkan berlakunya apoptosis awal semasa rawatan. 'DNA laddering' telah sahkan proses apoptosis kehadiran pecahan DNA semasa peringkat lewat apoptosis. Potensi membrane mitokondria didapati telah meningkat dalam sel CEMss yang dirawat apabila dikaji menggunakan mikroskop fluorescence. Untuk mengkaji mekanisme sel mati, kajian 'proteome profiler' manusia telah dijalankan. Selain itu, tahap protein Bax, Bcl2 dan Hsp 70 telah dianalisa menggunakan 'Western Blot'. Analisa terhadap caspase -3/7, -8 dan -9 telah dijalankan untuk mengetahui pasal induksi sel kanser semasa rawatan. Analisa tersebut telah mendapati bahawa kesemua caspase 3/7, 8 dan 9 telah meningkat semasa rawatan dan ini menunjukkan bahawa boesenbergin A mampu menggunakan kedua-dua laluan intrinsik atau ekstrinsik semasa apoptosis. Akhir sekali, analisis kitaran sel telah dijalankan menggunakan alat 'flow cytometry' dan mendapati boesenbergin A mampu untuk menghentikan pertumbuhan sel di fasa G2 / M terhadap sel-sel CEMss semasa rawatan. Keputusan positif daripada kajian ini menjadi asas yang kuat untuk kajian mendalam terhadap boesenbergin A sebagai ubat-ubatan untuk penyakit leukemia melalui kajian melalui haiwan.

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Without the people mentioned above, it will be impossible for me to finish this thesis in time. May Allah reward all of them now and in the here after. Amin I certify that a Thesis Examination Committee has met on 28 May 2013 to conduct the final examination of (Ng Kuan Beng) on his (or her) thesis entitled "Induction of selective cytotoxicity and apoptosis in human T4-lymphoblastoid cell line (CEMSS) using boesenbergin A isolated from *Boesenbergia rotunda L.*" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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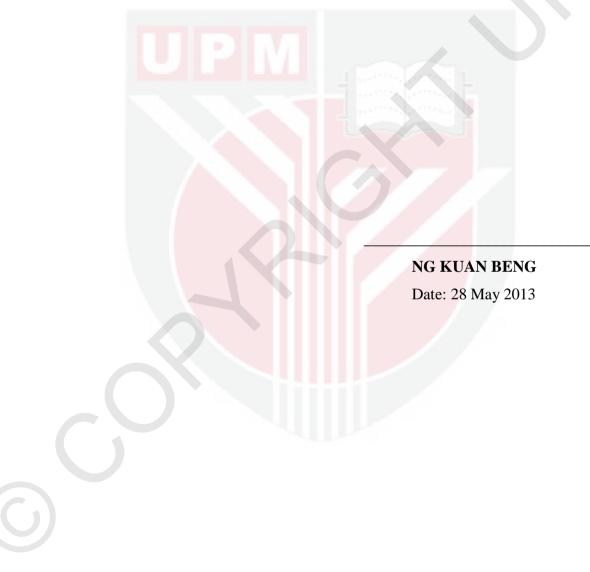
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## DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.



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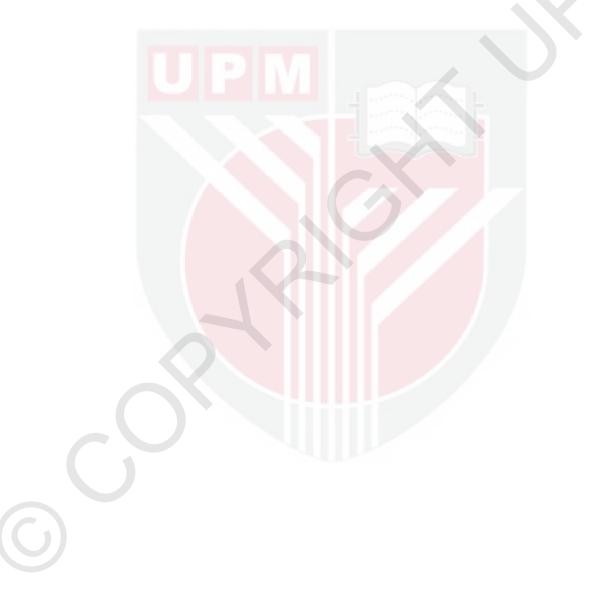
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regulatory proteins.



## LIST OF ABBREVIATIONS AND SYMBOLS

| AIDS           | Acquired immunodeficiency syndrome                       |
|----------------|--|
| ALL            | acute lymphocytic leukemia                               |
| AML            | acute myeloid leukemia                                   |
| ATP            | Adenosine triphosphate                                   |
| CD3            | Antigen  |
| Hela           | Cervical cancer cell line                                |
| CLL<br>CML     | chronic lymphocytic leukemia<br>chronic myeloid leukemia |
| HT-29          | Colon cancer cell line                                   |
| cdks           | cyclin dependent kinases                                 |
| caspase        | cysteine dependent aspartate-cleaving proteases          |
| DD             | death domains  |
| DED            | death effector domains                                   |
| DNA            | Deoxyribonucleic acid                                    |
| FADD           | Fas-associated via death domain                          |
| HPLC           | High Peformance Liquid Chromatographic                   |
| MDA-MB-<br>231 | Homo sapiens mammary gland                               |
| h              | hour   |
| MCF-7          | Human breast cancer cell line                            |
| HIV-1          | Human immunodeficiency virus type-1                      |
| PC3            | Human prostrate cancer cell line                         |
| DU145          | Human prostrate cancer cell line                         |
| CEMss          | Human T4- lymphoblastoid cell line                       |
|                |  |

| μΜ    | Micro Molar  |
|-------|--|
| μg    | Microgram  |
| mg    | Milligram  |
| ml    | Milliliter   |
| min   | Minute   |
| CaOV3 | Ovarian cancer cell line                                   |
| RAPD  | Random amplification of polymorphic DNA                    |
| rpm   | rotation per minute  |
| TCL   | T-cell lymphoma  |
| TNF   | Tumor necrosis factor                                      |
| TRAIL | Tumor Necrosis factor-Related Apoptosis-Inducing<br>Ligand |

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#### **CHAPTER 1**

#### **INTRODUCTION**

For a very long time, an important role has been played by natural product especially in health care and prevention of diseases. This is further strengthened by the written evidence of man's ingenuity in using plants for the treatment of a selection of diseases since the ancient civilizations. Examples are such as North Africans, Indians and Chinese (Phillipson 2001). Besides that, natural products have also numerous applications in the fields of biology, pharmacy and medicine. There has already been a number of a variety of essential novel commercialized drugs that has been discovered from natural sources through structural alteration of natural compounds, or by synthesizing novel compounds, and also designed according to a natural compound as a model (Gordaliza 2007). Eighty percent of drug substances were either from natural produce or stimulated by a natural complex and there are over a 100 new products which are currently undergoing clinical trials are compounds copied from natural products and there are also at least 100 similar projects are in preclinical advancement (Harvey 2008).

It is reported by Said (1999) that Malaysia is known to have about 20,000 species tropical plants of which around 1,300 are said to be medicinal. However, to date only around a hundred of these plant species have been investigated fully for their

therapeutic potential. Natural products which are often being used as main traditional health care agents over the past 20 years have generated interest from western countries to use it to sustain health and of alternative therapy (Wills, Bone, and Morgan 2000). One of the possible sources for screening of anticancer agents is plants from tropical regions. Thai people have been using various vegetables, herbs, and fruits for spice flavors, and condiments in their daily cuisine. Some of these items have been used as ancient medicines. Some of the plant extracts that were obtained are reported to have tendency to be developed as drugs (Manosroi, Dhumtanom, and Manosroi 2006). The explore for anti-cancer agents from plant resources has since took place in the 1950s with the finding and improvement of vinca alkaloids, vinblastine and vincristine, and later with the isolation and discovery of cytotoxic podophyllotoxins (Bickenbach et al. 2007).

Cancer affects all communities around the world and this disease have taken 10 of millions of life annually (Stewart and Kleihues 2003) and it is consider one of the leading cause of fatality (Farooqui et al. 2011). It is approximate that in 2008, 12.7 million cancer cases and 7.6 million cancer deaths occurred worldwide (Jemal et al. 2011). In Malaysia, cancer is also a major health problem and is increasingly significant as a public health anxiety due to expansion and growth that is happening (Lim 2002). In the year of 2007, an overall of 18,219 new cancer cases were diagnosed and registered, and from the total it comprised of 8,123 males and 10,096 females (Loo et al. 2013). It is also found in 2007 that cancer was the third most common cause of death after heart diseases and diseases of pulmonary circulation and septicaemia (Zainal Ariffin and Nor Saleha 2011).

Leukemia which is a malignant hematopoietic disease is capable of affecting organ for instance bone marrow and this result in the overproduction of unusual blood cells (Lee et al. 2007b). Leukemia refers to white blood cells cancer, which also called as leukocytes or white blood cell. The production of other blood cell such as red blood cells and platelets will be interrupted when leukemia continued (Rakel and Bope 2007). Basically, leukemia is a type of cancer of the blood or bone marrow that is categorized by an unusual increase of undeveloped white blood cells called "blasts". Leukemia is a very wide definition which coves a variety of diseases. Even though the overall incidence is rare compare to other cancer, but it is a common childhood cancer where 30% of all cancers diagnosed among children under age 15 years (Linet et al. 1999). Treatment of leukemia is an effort by multiple group of people. The treatment that is normally used include chemotherapy, radiotherapy, hormonal therapy, symptomatic, supportive therapy and immune therapy. The problem is that the current and most commonly used drug has severe side-effects.

Tropical plants are considered the richest sources of anti-cancer agents, in this research we have investigated an herbal plant, *Boesenbergia rotunda (L.)*, formerly known as *Boesenbergia* or *Kaempferia pandurata (Roxb). Schltr. (Zingiberaceae)* local name known as temu kunci, found in Southeastern Asian countries for example Indonesia, Thailand and Malaysia. It is being used in the treatment of a variety of diseases such as peptic ulcer, urinary disorders, oral diseases, colic, inflammation and dysentery (Saralamp et al. 1996). Numerous studies proposed this plant to be anti-inflammatory, neuroprotective, anti-mutagenic, chemopreventive, anticancer, anti-dermatophytic, anti-Helicobacter pylori and anti-dengue-2 virus NS3 protease (Bhamarapravati, Mahady, and Pendland 2003). This plant consists of both anti-

oxidant and also anti-cancer properties which can help to cure cancer. There is insufficient information currently accessible on the mechanism of *Boesenbergia rotunda* and also its effects on leukemia. Therefore, the present study was proposed to determine the potential biological activities of pure compound isolated from *Boesenbergia rotunda* on leukemia cells. In this study, *in vitro* (CEMss Cells) models are used to evaluate the anticancer properties of pure compound isolated from *Boesenbergia rotunda*.

*In vitro* methods were applied to the CEMss cells included the Cytotoxic (MTT) assay, fluorescent microscopy and also confocal microscope. Several analytical techniques are available to examine the cell biology of leukemic cells. Flow cytometry was utilized to study the progression of changes of leukemic cells under treatment. In this study, Annexin V assay and Cell cycle analysis had been preformed using flow cytometry to determine the drug effect on the distribution of cell cycle and DNA content. One of the easiest way to measure the characteristic of apoptotic cells is the breakage of the genomic DNA by cellular nucleases. Agarose gel electrophoresis DNA laddering was used to detect these fragments.

### **Objectives of the study**

#### Main objective:

To evaluate the anti-cancer activities of boesenbergin A from *Boesenbergia rotunda* in *vitro*.

### **Specific objectives:**

- To perform in vitro assessment on CEMss cell line using boesenbergin A.
- To determine and evaluate apoptosis induction of *Boesenbergin* A on CEMss cell line.
- To determine the effect of boesenbergin A on caspases and other apoptotic proteins in CEMss cells.

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### LIST OF PUBLICATION

 Title: Induction of selective cytotoxicity and apoptosis in human T4lymphoblastoid cell line (CEMss) by Boesenbergin A isolated from Boesenbergia rotunda rhizomes involves mitochondrial pathway, activation of caspase 3 and G2/M phase cell cycle arrest

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