UNIVERSITI PUTRA MALAYSIA

APOPTOSIS INDUCED BY DAMNACANTHAL FROM MORINDA ELLIPTICA IN T-LYMPHOBLASTIC LEUKAEMIA CELL LINE (CEM-SS)

HISYAM BIN ABDUL HAMID

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By

HISYAM BIN ABDUL HAMID

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Chairperson : Dr. Latifah Saiful Yazan, PhD

Faculty : Faculty of Medicine and Health Sciences

Current chemotherapy drugs showed adverse side effects towards the patients such as vomiting, nausea, constipation, fatigue, muscle pain and other pains. With the high risk of these side effects, majority of cancer patients prefer on finding better cure and lesser side effects. This circumstance somehow urged the scientists to search for alternative medicine, which is natural products that is believed can cure cancer patients with lesser side effects. Such natural product, damnacanthal which is an anthraquinone has been reported to possess inhibitory effects on the cancer promoting *ras* gene and *p*561 gene. In a previous study, damnacanthal from the root of *Morinda elliptica* was found to induce apoptosis towards the human acute T-lymphoblastic leukaemia cell line (CEM-SS). This study was conducted to determine the mechanisms involved in apoptosis induced by damnacanthal. The cells were treated with 1, 3, 10 and 30 μg/ml of damnacanthal and incubated for 24, 48 and 72 hours. Untreated cell were included as control. Following treatment, the samples were subjected to several assays such as cytotoxic study, morphological study, cell

cycle analysis and determination of p53, multi caspases (caspase 2, 3, 6, 8 and 9) and Bcl2 and Bax proteins activities, The percentage of viable cell at the highest concentration of the compound (30 µg/ml) decreased significantly ($p < 0.05$) after 72 hours to 40.4%. Cells treated with damnacanthal (10 and 30 µg/ml) showed characteristics of apoptosis such as condensation of cytoplasm, membrane blebbing and apoptotic bodies. At the highest concentration of damnacanthal (30 µg/ml), the cell cycle was found to be arrested at the G2/M phase after 72 hours incubation. Apoptosis induced by damnacanthal was found to be related to the caspase 2 and caspase 6 activation possibly through p53-independent pathway. In the treatment at the higher concentrations of damnacanthal (10 and 30µg/ml), the expression of Bcl-2 protein was downregulated whereas Bax protein was upregulated yet insignificant. Nevertheless, there was a significant correlation of activities between these two proteins. In conclusion, this study indicates that damnacanthal induced cell cycle arrest at G2/M phase most probably through p53-independent pathway and later involved in the activation of caspase 2 and caspase 6, and the downregulation of Bcl-2 and upregulation of Bax proteins which are believed drive the cell to undergo apoptosis.

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

PENCETUSAN APOPTOSIS OLEH DAMNACANTHAL DARI *MORINDA ELLIPTICA* KE ATAS TITISAN SEL AKUT T-LIMFOBLASTIK LEUKEMIA (CEM-SS)

Oleh

HISYAM ABDUL HAMID

September 2009

Pengerusi : Dr. Latifah Saiful Yazan, PhD

Fakulti : Fakulti Perubatan dan Sains Kesihatan

Rawatan kemoterapi kini menunjukkan banyak kesan sampingan terhadap pesakit seperti muntah, loya, sembelit, keletihan, sakit otot dan kesakitan pada bahagian badan yang lain. Kadar risiko kesan sampingan yang tinggi ini menyebabkan para pesakit mencari rawatan yang lebih elok dan kurang kesan sampingan. Keadaan ini memaksa para saintis mencari rawatan alternatif, iaitu hasilan semulajadi yang dipercayai berkemampuan untuk merawat penyakit kanser dan kurang risiko kesan sampingan. Sebagai contoh, damnacanthal iaitu sejenis antrakuinon yang telah dilaporkan mempunyai berkebolehan untuk menghalang fungsi gen penggalak kanser *ras* dan *p*561. Mengikut kajian sebelum ini, damnacanthal yang diekstrak daripada akar *Morinda elliptica* didapati mencetuskan apoptosis ke atas titisan sel akut T-limfoblastik leukemia (CEM-SS). Kajian ini dijalankan untuk menentukan mekanisme yang terlibat dalam proses apoptosis yang dirangsang oleh damnacanthal. Sel tersebut dirawat dengan damnacanthal berkepekatan 1, 3, 10 and 30 μg/ml dan dieram selama 24, 48 dan 72 jam. Sel yang

tidak dirawat disediakan sebagai kawalan. Selepas rawatan, sampel-sampel tersebut diagihkan kepada beberapa ujian seperti ujian viabiliti, ujian morfologi, analisa kitar sel, dan pengenalpastian aktiviti p53, aktiviti kaspase 2, 3, 6, 8 dan 9 dan aktiviti protin Bcl2 dan Bax. Setelah diperlakukan selama 72 jam pada kepekatan damnacanthal yang tertinggi (10 dan 30 µg/ml), peratusan sel yang hidup mengalami penurunan secara signifikan ($p < 0.05$) kepada 40.4%. Sel yang dirawat dengan damnacanthal (10 dan 30 µg/ml) menunjukkan ciri-ciri apoptosis seperti kondensasi sitoplasma, pembenjolan membran dan pembentukan badan apoptosis. Pada kepekatan damnacanthal tertinggi (30 µg/ml) kitar sel didapati mengalami perencatan pada fasa G2/M setelah dieram selama 72 jam. Apoptosis yang disebabkan oleh damnacanthal dipercayai berkaitan dengan pengaktifan kaspase 2 dan kaspase 6 berkemungkinan menerusi tapak jalan bebas-p53. Selepas didedahkan dengan kepekatan damnacanthal yang tinggi (10 dan 30 µg/ml), didapati berlaku penurunan tahap aktiviti protein Bcl-2 manakala berlaku peningkatan tahap aktiviti protein Bax tetapi dalam kadar yang tidak signifikan. Walau bagaimanapun, terdapat korelasi aktiviti yang nyata di antara kedua-dua protin tersebut. Kesimpulannya, kajian ini menunjukkan bahawa damnacanthal mencetus perencatan kitar sel pada fasa G2/M berkemungkinan melalui laluan bebas-p53 dan membabitkan pengaktifan kaspase 2 dan kaspase 6 dan penurunan Bcl2 dan peningkatan Bax di mana situasi ini dipercayai mendorong sel untuk memasuki apoptosis.

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I certify that an Examination Committee has met on **date of viva voce** to conduct the final examination of Hisyam Bin Abdul Hamid on his Master of Science thesis entitled "**Apoptosis Induced by Damnacanthal from *Morinda Elliptica* in T-Lymphoblastic Leukaemia Cell Line (CEM-SS)"** in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981. The Committee recommends that the student be awarded the Master of Science.

Members of the Examination Committee were as follows:

Assoc. Prof. Dr. Sabrina Sukardi, PhD
Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Assoc. Prof. Dr. Chong Pei Pei, PhD
Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Dr. Noorjahan Banu Mohamed Alitheen, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Assoc. Prof. Dr. Suzana Makpol, PhD
Professor
Name of Department and/or Faculty
Faculty of Medicine
Universiti Kebangsaan Malaysia
(External Examiner)

BUJANG KIM HUAT, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Latifah Saiful Yazan, PhD
Lecturer
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Raha Abdul Rahim, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

HASANAH MOHD GHAZALI, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

DECLARATION

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

HISYAM BIN ABDUL HAMID

Date :

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LIST OF ABBREVIATIONS

AIF	apoptosis inducing factor
ARF	alternative reading frame
Bcl-2	B-cell lymphoma 2
CARD	caspase recruitment domain
Cdk	cyclin dependent kinase
Chk	checkpoint kinase
DED	death effector domain
DR	death receptor
Endo-G	endonuclease G
FADD	Fas-associated death domain
GAPs	GTPase activating proteins
GEFs	guanine nucleotide exchange factors
ICE	interleukin-1β-converting enzyme
K18	keratin 18
MG63	osteosarcoma cell line
MPR	M-phase promoting factor
NPC	nuclear pore complexes
PI	propidium iodide
ROS	reactive oxygen species
^{3}S	triplet state sensitizer
STAT	signal transducer and activators of transcription
rpm	rate per minute
TNF-α	tumour necrosis factor α

CHAPTER 1

INTRODUCTION

The fact about cancer that feared most people is that anybody can get it without any discrimination when it comes to age, sex and race. Uncontrollably proliferation and division, ability to infiltrate and destroy normal body tissue become the main characteristics of this fearsome cancer. Interference to human body function occurs due to production of certain chemicals from the cancer cells. In U.S alone, approximately 1.44 million new cancer cases were estimated to occur in 2008 (Jemal *et al.*, 2008).

The problem of cancer in Malaysia rises gradually and it has been estimated that the annual incidence of cancer is 30 000. It is now the fourth leading cause of death among medically certified death after diseases of the circulatory system other than cerebrovascular disease, accidents, poisonings and violence and diseases of the respiratory system. Leukaemia stands at first place for the commonest diagnosed cancer among Malaysian children (National Cancer Registry, 2003). Until recently, majority of cancer are incurable even with the existence of a treatment using drugs called chemotherapy. Nevertheless, a lot of adverse drug effect arises from the treatment such as vomiting, nausea, constipation, fatigue, muscle pain and other pains (Andersen *et al.*, 2006).

Due to this unwanted results, a lot of patients are seeking for new methods such as natural products. Natural products are rapidly becoming a part of mainstream medicinal practice and one of the hottest topics among both healthcare providers and the general

public. Being the main part remedies in alternative medicine, the perfect natural plant product was searched throughout the past few years until today. Therefore scientists nowadays are attempting to extract out the biggest secret in plants which have been making them as a magical potion all these years. *Morinda elliptica*, or also locally known as "mengkudu kecil" for example is used as colourant and in traditional folk medicine for the treatment of diarrhea, cholera, headache, piles and to increase appetite (Chong *et al*., 2005). Eleven anthraquinones have been successfully extracted from *Morinda elliptica* including damnacanthal (Nor Hadiani *et al*., 1997). Damnacanthal was found to possess the immune enhancing activity (Hirazumi *et al*., 1996) and also inducing apoptosis in cancer cells (Hiramatsu *et al*., 1993).

In cancer, the therapeutic goal is to trigger tumour-selective cell death and apoptosis itself represents a universal and ecquisitely effiecient cellular suicide pathway which has mechanisms that are important in determining the efficacy of specific treatments (William and David, 1999). Apoptosis is an essential process that regulates many aspects of normal and pathophysiological development of both vertebrates and invertebrates (Bruno *et al*., 2001). This process occurs due to the initiation of a complex network of signaling pathways from both inside and outside of the cell (Majors *et al*., 2007). Apoptosis involves the big manufacturing process of the cell such as cell cycle (Aggarwal and Shishodia, 2006; Maddika *et al*., 2007), utilizing certain proteins such as caspases (Wang *et al*., 2005) and Bcl-2 family proteins (Schinzel *et al*., 2004), until to the small genes which regulates the cell cycle such as tumour suppressor gene *p53* (Fuster *et al*., 2007).

Cell cycle is a process of cell growth and replication (Foster, 2008) which is an ordered, tightly regulated process that involves multiple checkpoints that assess extracellular

growth signals, cell size and DNA integrity (Park and Lee, 2003). The transcription factor p53 has a key role in preventing DNA damage (Fuster *et al.*, 2007). The presence of DNA damage in the cells will execute cell cycle arrest and programmed cell death or apoptosis (Park *et al.*, 2004) and thus, leads to the activation of caspase pathway (Wang *et al.*, 2005). Following activation, caspases cleave a number of proteins including those involved in DNA synthesis, cleavage and repair (Turk and Stoka, 2007). In the cytochrome *c* pathway, cytochrome c is released from mitochondria and this event is mainly regulated by Bcl-2 family proteins (Zhonghua *et al.*, 2005). The release of cytochrome *c* is known to be as a "point-of-no-return" for the cell, which damages the mitochondria over time and leads to cytoplasmic acidification (Skommer *et al.*, 2007).

Loss of p53 functions may result from mutation, deletion or binding with other proteins which allows accumulation of oncogenic lesions in the genome and basically it gives birth to the purposeless clonal proliferation of cells in any tissue or organ of the body or also known as cancer (Foster, 2008). Apoptosis is required in treating cancer in order to reverse the behaviour of cancer cells that proliferate uncontrollably, therefore limiting the cells proliferation in natural way without any implications to other neighbouring cells. While the therapeutic goal is to trigger tumor-selective cell death or apoptosis, it represents a universal and exquisitely efficient cellular suicide pathway which has mechanisms that are important in determining the efficacy of specific treatments (William and David, 1999).

The general objective of this study was to determine the mechanisms of apoptosis induced by damnacanthal in CEM-SS cell line. The specific objectives were:

1. To investigate the cell cycle profile of the treated cells compared to the untreated cells
2. To determine the level of p53 expression and to elucidate the involvement of caspases 2, 3, 6, 8 and 9 in damnacanthal-treated CEM-SS cell line
3. To evaluate the effects of damnacanthal towards the level of expression of Bcl-2 and Bax

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