UNIVERSITI PUTRA MALAYSIA

EFFECTS OF DAMNACANTHAL, NORDAMNACANTHAL, BETULINIC ACID AND ZERUMBONE ISOLATED FROM LOCAL MEDICINAL PLANTS ON LEUKEMIA CELL LINES AND IMMUNE CELLS

MASHITOH BINTI ABD RAHMAN

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By

MASHITOH BINTI ABD RAHMAN

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

October 2007

DEDICATION

THIS THESIS IS DEDICATED TO

MY BELOVED HUSBAND KHAIRI BIN ABDUL RAHIM
MY LOVELY SON MUHAMMAD NAUFAL
PARENTS AND PARENTS IN LAW
ALL MY KINDNESS TEACHERS AND LECTURERS
ALL MY SOULMATES AND KINDHEARTED FRIENDS
And
TO EVERYONE WHO BELIEVED IN MY ABILITIES AND ALWAYS INSPIRES ME IN MAKING SOME OF MY GOALS COME TRUE

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of requirement for the degree of Master of Science

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Chairman : Noorjahan Banu binti Mohd Alitheen, PhD

Institute : Institute of Bioscience

The present study was to evaluate the toxicity and immunomodulatory effects of damnacanthal, nordamnacanthal, betulinic acid and zerumbone isolated from local medicinal plants towards leukemia cell lines and immune cells. Toxicity study was performed on HL-60 (Human acute promyelocytic leukemia), CEM-SS (Human T-lymphoblastic leukemia), WEHI-3B (Mouse myelomonocyte leukemia), 3T3 (Mouse embryo fibroblast) and human peripheral blood mononuclear cell (PBMC) by using MTT assay and cell cycle analysis. The results showed that damnacanthal significantly inhibited HL-60 cells, CEM-SS and WEHI-3B with the IC_{50} value of 4.0 µg/mL, 8.0 µg/mL and 3.3 µg/mL, respectively. Nordamnacanthal and betulinic acid showed stronger inhibition towards CEM-SS and HL-60 cells with the IC_{50} value of 5.7 µg/mL and 5.0 µg/mL, respectively. In contrast, Zerumbone was demonstrated to be more toxic towards those leukemia cells with the IC_{50} value less than 10 µg/mL. Interestingly, damnacanthal, nordamnacanthal and betulinic acid

were not toxic towards 3T3 and PBMC compared to doxorubicin which showed toxicity effects towards 3T3 and PBMC with the IC_{50} value of 3.0 µg/mL and 28.0 µg/mL, respectively. The cell cycle analysis exhibited that damnacanthal exerted its toxicity effect towards HL-60 cells by inducing apoptosis with value of 25% after 72 hours treatment.

Immunomodulatory study revealed that damnacanthal, nordamnacanthal, betulinic acid and zerumbone were able to stimulate the proliferation of mice thymocytes, mice splenocytes and PBMC in a time and dose-dependent fashion. Damnacanthal and nordamnacanthal were able to stimulate the proliferation of mice thymocytes, mice splenocytes and PBMC even at low concentration (0.46 µg/mL) and did not cause inhibition at higher concentration (30 µg/mL). In contrast, betulinic acid and zerumbone showed inhibition at higher concentration (30 µg/mL) and proliferate well at lower concentration (7.5 µg/mL) towards those immune cells. Results obtained from cell cycle analysis exhibited that the proliferation effect of those compounds on PBMC were corresponding with the MTT based lymphocyte proliferation assay. Moreover, those compounds were demonstrated to induce immunoregulatory cytokine production in highest degree of human IL-2 and in lower degree of human IL-12 upon stimulation of PBMC in a time dependent manner. Based on the result presented, the compounds damnacanthal, nordamcanthal, betulinic acid and zerumbone can act as cytotoxic and immunomodulatory agent which are very useful in treating cancer and enhancing the immune system.

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

KESAN SEBATIAN DAMNACANTHAL, NORDAMNACANTHAL, BETULINIC ACID DAN ZERUMBONE YANG DIPEROLEHI DARIPADA TUMBUHAN UBATAN TEMPATAN TERHADAP JUJUKAN SEL-SEL LEUKEMIA DAN SEL-SEL KEIMUNAN

Oleh

MASHITOH BINTI ABD RAHMAN

Oktober 2007

Pengerusi : Noorjahan Banu binti Mohd Alitheen, PhD

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Kajian ini dilakukan adalah untuk menilai kesan ketoksikan dan pengawal imun daripada damnacanthal, nordamnacanthal, betulinic acid dan zerumbone yang diperolehi daripada tumbuhan ubatan tempatan ke atas jujukan-jujukan sel leukemia dan sel keimunan yang normal. Kajian ketoksikan telah dijalankan ke atas HL-60 (leukemia kronik promeilositik manusia), CEM-SS (leukemia T-limphoblastik manusia), WEHI-3B (Leukemia myelomonositik tikus), 3T3 (embrio fibroblast tikus) dan sel darah periferi manusia (PBMC) dengan menggunakan esei MTT dan analisis kitaran sel. Keputusan yang diperolehi secara signifikan menunjukkan damnacanathal telah merencat sel HL-60, CEM-SS dan WEHI-3B dengan nilai IC_{50} 4.0 µg/mL, 8.0 µg/mL dan 3.3µg/mL, masing-masing. Nordamnacanthal dan betulinic acid telah menunjukkan kesan perencatan yang tinggi terhadap CEM-SS dan HL-60 dengan nilai IC_{50} 5.7 µg/mL dan 5.0 µg/mL, masing-masing. Sebagai perbandingan, zerumbone telah mempamerkan kesan ketoksikan yang tinggi terhadap semua jujukan sel leukemia dengan nilai IC_{50} kurang daripada 10 µg/mL. Menariknya,

damnacanthal, nordamnacanthal dan betulinic acid tidak menunjukkan kesan ketoksikan ke atas 3T3 dan PBMC berbanding dengan doxorubicin yang menunjukkan kesan ketoksikan terhadap 3T3 dan PBMC dengan nilai IC_{50} 3.0 µg/mL dan 28.0 µg/mL, masing-masing. Kajian kitaran sel menunjukkan damnacanthal telah mempamerkan kesan sitotoksik ke atas HL-60 melalui pengaruhan apoptosis dengan nilai 25% selepas 72 jam rawatan.

Kajian terhadap pengawal imun mendedahkan sebatian damnacanthal, nordamnacanthal, betulinic acid dan zerumbone berkeupayaan merangsang proliferasi timus tikus, limfa tikus dan PBMC bergantung pada masa dan dos tertentu. Damnacanthal dan nordamnacanthal berkeupayaan merangsang proliferasi timus tikus, limfa tikus dan PBMC pada kepekatan yang rendah (0.46 µg/mL) serta tidak menyebabkan perencatan pada kepekatan yang tinggi (30 µg/mL). Berbanding dengan betulinic acid dan zerumbone, kedua-dua sebatian tersebut didapati merencat proliferasi timus tikus, limfa tikus dan PBMC pada kepekatan yang tinggi (30 µg/mL) dan berproliferasi dengan baik pada kepekatan (7.5 µg/mL) selepas 48 jam rawatan. Keputusan yang diperolehi daripada analisis kitaran sel mempamerkan kesan proliferasi daripada sebatian-sebatian tersebut terhadap PBMC adalah sejajar dengan esei MTT proliferasi limfosit. Malahan, sebatian-sebatian tersebut juga berkeupayaan mengaruhkan penghasilan immunoregulasi interleukin-2 manusia pada darjah yang tinggi dan penghasilan interleukin-12 manusia pada darjah yang rendah melalui stimulasi PBMC pada masa tertentu. Berdasarkan keputusan yang diperolehi sebatian-sebatian damnacanthal, nordamnacanthal, betulinic acid dan zerumbone berkeupayaan bertindak sebagai agen sitotoksik dan immunomodulator yang begitu penting dalam merawat kanser dan meningkatkan sistem keimunan.

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I certify that an Examination Committee has met on 1st October 2007 to conduct the final examination of Mashitoh Binti Abd Rahman on her Master of Science thesis entitled "Effects of Damnacanthal, Nordamnacanthal, Betulinic Acid, and Zerumbone Isolated from Local Medicinal Plants on Leukemia Cell Lines and Immune Cells" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the Master of Science.

Members of the Examination Committee were as follows:

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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:

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Date: 21 February 2008

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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MASHITOH BINTI ABD RAHMAN

Date: 20 January 2008

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LIST OF ABBREVIATIONS

APCs	Antigen-presenting cells
ºC	Degree celcius
BA	Betulinic acid
BSA	Bovine serum albumin
CDs	Cluster determinant
Con A	Concanavalin A
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
HBSS	Hank's Balance Salt solution
HCl	Hydrochloric acid
IFN-γ	Interferon gamma
Igs	Immunglobulins
IL-2	Interleukin 2
IL-12	Interleukin 12
KCl	Potassium chloride
LPS	Lipopolysaccharide
M	Molar
min	Minute
mg	Mili gram
mL	Mililiter
µ	Micron

µg	Microgram
µL	Microliter
µM	Micromolar
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium
bromide	
MW	Molecular weight
NaCl	Sodium chloride
NF-κB	Nucleur factor- κB
Nk	Natural killer
nm	Nanometer
Pi	Propidium iodide
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate buffer saline
PHA	Phytohemaglutinin
PWM	Pokeweed mitogen
RPMI	Roswell Park Memorial Institute
SAR	Structure-activity relationship
TNF	Tumour necrosis factor
U	Unit

CHAPTER 1

INTRODUCTION

Plants have been recognized as the greatest source in the treatment of wide a variety of diseases since ancient times (Balunas and Kinghorn, 2005; Adriana *et al*., 2002). More interestingly, some of these plants are believed to enhance the immune system and at the same time might contribute to a reduction in cancer incidence in human. There are experimental evidences showed that some of chemopreventive agents derived from natural products such as curcumin and betulinic acid modulate the immune system (Zuco *et al*., 2002; Gao *et al*., 2004). The capability of some natural products inhibiting cancer cells through modulation of the immune system have provided a greatest alternative in solving toxic side effects towards healthy cells and suppression of the immune system of current chemotherapeutical drugs available today. Therefore, it is a worth while effort to identify substances particularly from natural products which have potential therapeutical activity in treating cancer as well as might enhance the immune system.

The human immune system, despite having its own sophisticated defense mechanisms, is inferior to bacteria and viruses with respect to adaptability (Wagner, 1999). Generally, the immune system can be defined as an intricate network of specialized tissues, organs, cells and chemicals. The main functions of immune system are responsible for self-recognition and helps defend the body against a wide variety of pathogens (Abbas and Licthman, 2003). Among of them, lymphocyte is one of the most important immune cells which play a vital role in body's defence

mechanism. According to Anazzetti *et al*. (2003), when considering the chometherapy side effects, it is very important to verify whether or not the drug shows a harmful effects against dividing normal cells particularly in proliferating of lymphocytes. Therefore, the capability of a drug to proliferate the lymphocytes is one of the most important immune function markers to avoid toxic side effects towards healthy cells and immune system. Fortunately, there are some mitogens derived from natural products have been recognized to stimulate the proliferation of lymphocytes naturally which might useful in enhancing the immune system.

Beside lymphocytes, cytokines are soluble glycoproteins which are critically involved in the immune systems and showed to have diverse functions in normal humoral and T cell-mediated immune response. Changes in cytokines levels are accompanied by various pathological conditions which might cause unbalance in the immune system (Fulup *et al*., 2006). Interleukin-2 (IL-2) and interleukin-12 (IL-12) are some of the well known cytokines. IL-2, a T-cell growth factor play a pivotal role in activation and proliferation of most T cells, natural killer cells (NK) and B cells during certain phases of their responses (Bryan *et al*., 2006). IL-12 is a pleoitropic cytokines with important proinflammatory and immunoregulatory functions. The major biological activity of IL-12 is on T and NK cells in which it increases cytokine production, particularly IFN-γ production. Currently, both IL-2 and IL-12 are used as natural adjuvants for cancer immunotherapy to help in activating and enhancing the immune response (Parmiani *et al*., 2000; Eva *et al*., 2007).

A fully functioning immune system is one of the most important criteria for a healthy life. However, nowadays our immune system is increasingly exposed to detrimental effects due to immunosuppressive environmental consequences, unhealthy living, malnutrition, cancers and others chronic illness (Wagner, 1999; Keller *et al*., 2005). Moreover, current cancer therapeutic practices, such as chemotheraphy and radiotheraphy may also suppress the immune system. Therefore, this situation demands a compensatory mechanism in enhancing and maintaining our immune system. Presently, a wide variety of natural products from medicinal herbs were exhibited to have an immunomodulatory effects which are very useful in solving those problems.

Immunomodulator is any substances that capable of modifying or regulating one or more immune system (Stanilove *et al*., 2005). This regulation is a normalization process. It has been applied in cancer therapy and immunological diseases (Kumar *et al*., 2005). Immunomodulators may include some bacterial products, plant derived substances and lymphokines (Wagner, 1999). These fundamental fields of immunomodulators are currently receiving inadequate attention. For that reason, a number of plant products are being investigated concurrently for anticancer and immune response modifying activity (Uphayay, 1997).

Therefore, this present study was carried out to investigate the toxicity and immunomodulatory effects of natural pure compounds derived from our local medicinal plants. The compounds selected in this study were damnacanthal and nordamnacanthal which were isolated from *Morinda elliptica*, zerumbone which

was isolated from *Zingiber zerumbet* and betulinic acid which was isolated from *Melalueca cajuputi*. The objectives of this project were:-

1) to study the toxicity effects of damnacanthal, nordamnacanthal, betulinic acid and zerumbones towards cancerous and non-cancerous cell lines,
2) to investigate the immunomodulatory effects of damnacanthal, nordamnacanthal, betulinic acid and zerumbones on mice splenocytes, mice thymocytes and PBMC *in vitro* and
3) to evaluate the production of human IL-2 and human IL-12 upon stimulation of PBMC by damnacanthal, nordamnacanthal, betulinic acid and zerumbone.