UNIVERSITI PUTRA MALAYSIA

IMMUNOMODULATORY EFFECTS OF *RHAPHIDOPHORA KORTHALSII* METHANOL EXTRACT ON NATURAL KILLER CELL ACTIVATION AND CYTOLYTIC ACTIVITY

YEAP SWEE KEONG
FBSB 2010 2

IMMUNOMODULATORY EFFECTS OF *RHAPHIDOPHORA KORTHALSII* METHANOL EXTRACT ON NATURAL KILLER CELL ACTIVATION AND CYTOLYTIC ACTIVITY

By

YEAP SWEE KEONG

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

January 2010

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

IMMUNOMODULATORY EFFECTS OF *RHAPHIDOPHORA KORTHALSII* METHANOL EXTRACT ON NATURAL KILLER CELL ACTIVATION AND CYTOLYTIC ACTIVITY

By

YEAP SWEE KEONG

January 2010

Chairperson: Noorjahan Banu Mohamed Alitheen, PhD

Faculty: Biotechnology and Biomolecular Sciences

Rhaphidophora korthalsii (Araceae) is a root-climber plant which has been previously identified as splenocyte immunostimulator. The purpose of this study was to examine the *in vitro* and *in vivo* immunomodulatory effect of *R. korthalsii* methanol extract on immune cell proliferation, cytokine expression and cytotoxicity. More specifically, immunomodulatory effects of *R. korthalsii* methanol extract on the stimulation of NK cells activity and cytotoxicity against HepG2 monolayer and spheroid culture were determined. Immune cells [peripheral blood mononuclear cells (PBMC) and mice splenocytes] treated with this extract resulted in stimulation of cell proliferation, cytokine expression and cytotoxicity in dose and time dependent manner. For the *in vivo* immunostimulatory effect study, unlike rIL-2 which degraded rapidly, the stimulatory effect from the extract managed to last until day 15. In order to understand the activation of NK cells by *R. korthalsii* methanol extract, NK cells were treated directly or indirectly. Both direct and indirect stimulated NK cells showed high-level expression of cell surface FasL, NKG2D, 16B4 and extra-cellular IFN-γ and TNF-α. These activations contributed to the killing of NK cells against HepG2

monolayer cells through Granzyme B mitochondria caspases dependent secretory apoptosis pathway where DNA fragmentation, phosphatidylserine (PS) externalisation, caspase 3, caspase 8, caspase 9 up-regulation and XIAP, Bid down-regulation were observed. Apart from that, extract stimulated NK cells which caused cell death on the HepG2 spheroid and inhibited the HepG2 cell invasion, suggesting that *R. korthalsii* methanol extract was a potential agent to inhibit liver tumour metastasis. Our findings indicated a potential IL-2 free immunotherapy through direct and indirect *R. korthalsii* activation on NK cells which can further induce apoptosis on the HepG2 monolayer and spheroid culture.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

KESAN PEMODULASI-IMUN OLEH *RHAPHIDOPHORA KORTHALSII* PATI METANOL TERHADAP PENGAKTIFAN DAN AKTIVITI SITOLITIK SEL PEMBUNUH SEMULAJADI

Oleh

YEAP SWEE KEONG

Jun 2009

Pengerusi : Noorjahan Banu Mohamed Alitheen, PhD

Fakulti: Bioteknologi and Sains Biomolekul

Rhaphidophora korthalsii (Araceae) adalah sejenis tumbuhan pemanjat akar yang terdahulunya dikenali sebagai perangsang sel limpa. Tujuan kajian ini adalah untuk memeriksa kesan perangsangan-imun *in vitro* dan *in vivo* oleh *R. korthalsii* pati metanol terhadap pembiakan, ekspresi sitokin dan sitotoksisiti pada sel imun. Lebih khusus, kesan perangsangan-imun pada *R. korthalsii* pati metanol terhadap pengaktifan dan sitotoksisiti sel pembunuh semulajadi (NK cells) untuk menentang sel monolapisan dan sferoid HepG2 juga ditentukan. Sel imun (PBMC dan sel limpa tikus) yang dirawati dengan pati tersebut memberi kesan perangsangan terhadap pembiakan, ekspresi sitokine dan sitotosiksiti yang bergantung kepada dos dan tempoh perangsangan. Untuk ujikaji kesan perangsangan-imun secara *in vivo*, kesan yang terbaik dapat dicapai dengan kepekatan 350 and 700 μg/tikus. It berbeza dengan rIL-2 yang cepat mendegradasi di mana kesan perangsangan daripada pati dapat kekal sehingga hari ke-15. Bagi memahami pengaktifan sel NK oleh *R. korthalsii* pati metanol, sel NK telah dirawat dengan pati secara langsung dan secara tidak langsung. Kedua-dua sel NK yang dirawat secara langsung atau tidak langsung didapati

mengekspresskan paras yang tinggi untuk FasL, NKG2D, 16B4 pada permukaan sel dan IFN-γ, TNF-α di luar sel. Pengaktifan ini menyumbang kepada pembunuhan sel NK terhadap sel monolapisan HepG2 melalui laluan apoptosis granzim B rembesan mitokondria berdasarkan caspase di mana peningkatan pengawalaturan penyepihan DNA, pengeluran phosphatidylserine (PS), caspase 3, caspase 8, caspase 9 dan penurunan pengawalaturan XIAP dan Bid telah diperhatikan. Selain daripada itu, sel NK yang dirangsang oleh pati juga menyebabkan kematian sel pada sferoid HepG2 dan merencatkan invasi sel HepG2 mencadangkan potensi rawatan ini untuk merencatkan metastasis tumor hati. Keputusan yang didapati kami mengesyorkan potensi rawatan imun tanpa IL-2 melalui pengaktifan NK sel secara langsung dan tidak langsung oleh *R. korthalsii* yang dapat merangsangkan apoptosis pada kultura monolapisan dan sferoid HepG2.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to my supervisor Dr. Noorjahan Banu Mohamed Alitheen and co-supervisors Professor Dr. Abdul Manaf Ali and Professor Dr. Abdul Rahman Omar for their valuable advice, technical guidance, encouragement and support for conversion and thesis improvement.

I wish to acknowledge the guidance and support from Dr. Kiew Li Chin and his father for supplying ideas and plant materials for this project; Mr. Lim and Uncle Chan from FRIM for plant identification; MKT staff Mr. Karim and my lab seniors; Mr. Fahmi and Mr. Teo. My gratitude also goes out to my colleagues; Miss Ho, Mr. Lam, Miss Ng, Miss Chuah and Miss Jane Liew who are always appreciated. I am indeed indebted to Miss Ho, Dr. Kiew, Mr. Loh, Mr. Kye, Mr. Thuc, Mr. Beh for sharing their knowledge and experience with me and my parents and my family for their unconditional sacrifice and love.

I wish to extend my appreciation to everyone, although not individually named here, who had contributed directly or indirectly to my project and thesis. Finally, I would like to thank the Ministry of Higher Education (MOHE) for providing FRGS grant no. 5523013 and University Putra Malaysia for providing RUGS grant no. 91194 for supporting this study. Without all of you, it would not be possible for me to complete my project and thesis. Thank you all for your support.

I certify that an Examination Committee met on 7th January 2010 to conduct the final examination of Yeap Swee Keong on his Doctor of Philosophy thesis entitled "Immunomodulatory Effects of *Rhaphidophora korthalsii* Methanol Extract on Natural Killer Cell Activation and Cytolytic Activity" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

Muhajir Hamid, Ph.D
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Tan Wen Siang, Ph.D
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Shuhaimi Mustafa, Ph.D
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Mustafa Ali Mohd, Ph.D
Professor
Faculty of Medicine
Universiti Malaya
(External Examiner)

_____
BUJANG KIM HUAT, Ph.D
Professor/Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfill ment of the requirements for the Degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Noorjahan Banu Mohamed Alitheen, PhD
Senior Lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairperson)

Abdul Manaf Ali, PhD
Professor
Faculty of Agriculture and Biotechnology
Universiti Darul Iman Malaysia
(Member)

Abdul Rahman Omar, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

_____
HASANAH MOHD GHAZALI, Ph.D
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 8 April 2010

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree in UPM or other institution.

_____

YEAP SWEE KEONG

Date: 26th February 2010

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AICL	Activation-induced C-type lectin
ADME	administration, distribution, metabolism or excretion
ASCT	Autologous stem cell transplantation
BAT3	HLA-B-Associated Transcript 3
BH3	Bcl-2 homology domain 3
Bid	BH3 interacting domain death agonist
Bid	Bcl-2 interacting domain
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
cIAP	Cellular Inhibitor of apoptosis
CLL	Chronic lymphocytic leukemia
CML	Chronic myelogenous leukemia
Con-A	Concanavalin A
CTL	Cytolytic T lymphocyte
DHI	5,6-dihydroxyindole
DMEM	Dulbecco's modified eagle media
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Link Immunosorbent assay
erbB2	Erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)
FADD	Far associating protein with death domain
FasL	Fas ligand
FBS	Fetal bovine serum

FITC	Fluorescein
FLICE	FADD like interleukin-7 beta converting enzyme
FLT3	FMS-like tyrosine kinase 3
FRIM	Forest Research Institute Malaysia
g	Gravity
G	Gap
G1	Gap 1
G2	Gap 2
GLCD	Granzyme B leakage-induced cell death
GM-CSF	Granulocyte-macrophage colony stimulating factor
HBSS	Hanks balance salt solution
HCC	Hepatocellular carcinoma
HDL	High-density lipoprotein
IC_{50}	Inhibition concentration that reduces 50% of cell viability compared to control
ICAM-1	Inter-Cellular Adhesion Molecule-1
IFN	Interferon
IL	Interleukin
ILP	IAP like protein
ITAM	Immunoreceptor tyrosine-based activation motif
U	International unit
kDa	Kilo Dalton
KIR	Killer cell immunoglobulin-like receptors
KLF	Kruppel-like factor
LAK	Lymphokine-activated killer

LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LFA-3	Leukocyte function-associated antigen-3
LPS	Lipopolisaccharide
MAPK	Mitogen activated protein kinase
MCTS	Multicellular tumor spheroids
MHC	Major histocompatibility complex
MIC	MHC class I related chains
mL	Milliliter
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NKT	Natural Killer T
NPC	Nasopharyngeal carcinoma
OxyHb	Oxyhaemoglobin
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PE	Phycoerythrin
pg	Piko gram
PI	Propidium Iodide
PGE2	Prostaglandin E2
PMEA	9-(2-phosphonylmethoxyethyl) adenine
PS	Phospholipids phosphatidylserine
PVPF	Polyvinylpyrrolidone-free polycarbonate
PVR	Polyoma virus receptor

PWM	Pokeweed mitogen
Pyk2	Proline-rich tyrosine kinase 2
Rac1	Ras-related C3 botulinum toxin substrate 1
RBC	Red blood cell
rIL-2	Recombinant interleukin 2
S	Synthesis
SDS	Sodium lauryl sulfate
SSC	Side scatter
tBid	Truncated Bid
Th	T helper
TMB	3,3',5,5' tetramethylbenzidine
TNF	Tumor necrosis factor
TP53	Tumor protein 53
TRAIL	TNF related apoptosis inducing ligand
Tunel	Terminal dUTP nick-end labeling to detect apoptotic cells
ULBP	UL16-binding protien
UPM	University Putra Malaysia
UV	Ultraviolet
VCAM-1	Vascular cell adhesion molecule-1
VLA-4	Very late antigen-4
VLDL	Very low-density lipoprotein
WHO	World Health Organization
XIAP	X-link IAP
α	Alpha
β	Beta

γ	Gamma
μ	Micro
FcγRIII CD16	Fragment, crystallizable gamma region III
16B4 ITGAL	Integrin α2β1
^{51}Cr	Chromium-51

CHAPTER 1

INTRODUCTION

Immune system is one of the most important glossaries when the issue of health is discussed. It protects us from infectious diseases. Generally, immune system can be defined as a network of lymphoid organs, tissues and cells and also the products of these cells. The main function of immune system is to protect the body against infectious microbes or foreign substances (Abbas and Lichtman, 2005). Natural killer (NK) cells are the key component of the innate arm of the immune system which plays an important role in first line defense against tumor and viral infections (Titanji et al., 2008).

Malignant diseases are always associated with decreased immune competence (Ordemann et al., 2002). However, level of immune system is always reduced whenever influenced by anthropogenic factors such as polluted environment, malnutrition, seasonal changes and etc. (Keller et al., 2005). For such reason, people nowadays have increased their awareness towards building a healthy immune system. Guidelines such as reducing life stresses, maintaining balance diet and sufficient of exercise as well as obtaining adequate sleep and rest have been suggested to maintain individual's healthy and to protect them against pathogens (Nieman, 2000).