UNIVERSITI PUTRA MALAYSIA

ANTI-LEUKEMIC EFFECTS OF TYPHONIUM FLAGELLIFORME ON HUMAN LYMPHOBLASTOID CELLS (CEMss) AND MURINE LEUKEMIC (WEHI-3) MODEL

MURALI MOHAN SYAM MOHAN

IB 2010 3

ANTI-LEUKEMIC EFFECTS OF *TYPHONIUM FLAGELLIFORME* ON HUMAN LYMPHOBLASTOID CELLS (CEMss) AND MURINE LEUKEMIC (WEHI-3) MODEL

By

MURALI MOHAN SYAM MOHAN

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

October 2010

DEDICATION

THIS THESIS IS DEDICATED TO

MY BELOVED WIFE SUVITHA SYAM MOHAN
MY LOVELY SON ADITHYA MOHAN
PARENTS AND PARENTS IN LAW
ALL MY TEACHERS AND LECTURERS
ALL MY SOULMATES AND KINDHEARTED FRIENDS
AND
TO EVERYONE WHO BELIEVED IN MY ABILITIES AND ALWAYS INSPIRED ME IN MAKING SOME OF MY GOALS COME TRUE

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

ANTI-LEUKEMIC EFFECTS OF *TYPHONIUM FLAGELLIFORME* ON HUMAN LYMPHOBLASTOID CELLS (CEMss) AND MURINE LEUKEMIC (WEHI-3) MODEL

By

MURALI MOHAN SYAM MOHAN

October 2010

Chairman: Ahmad Bustamam Abdul, PhD

Faculty: Institute of Bioscience

To date, there has been no literature reported on the mechanism of *Typhonium flagelliforme* and its effects on leukemia. Hence, the anti-leukemia effect of *Typhonium flagelliforme* was investigated *in vitro* and *in vivo* leukemic model. Extraction and fractionation using organic solvents were applied to obtain fractions from *T. flagelliforme* and subsequently, chemical analysis was done using GC-MS. *In vitro* cytotoxic effects of extracts and fractions were tested in several human cancer cell lines including leukemia (CEMss cells) using MTT assay. Various microscopy techniques were used to study morphological changes occurring during treatment. The Annexin V assay, TUNEL assay, cell cycle analysis and DNA laddering were employed to detect apoptosis. Colourimetric assays for caspase-3 and 9, immunoblot analysis for cytochrome c, BcL-2, PARP, FasL

and β-actin were analysed. The *in vivo* model of leukemia was induced in male BALB/c mice using WEHI-3 cells. The DCM extract of the plant tuber was used for treatment at various doses. Amongst 8 plant extracts investigated, the dichloromethane (DCM) extracts of *T. flagelliforme* tuber demonstrated low and significant anti proliferative effect against both CEMss (6.5±0.4 µg/ml) and WEHI-3 cells (24.0±5.2 µg/ml) ($p<0.05$). Further fractionation of the DCM tuber extract resulted into 12 fractions. Seven of these 12 fractions showed significant cytotoxicity against CEMss, in which the DCM/F7, DCM/F11 and DCM/F12 fractions showed highest anti-cancer activities of 3.0, 5.0 and 6.2 µg/ml respectively. Further studies of these fractions towards non cancerous Peripheral Blood Lymphocytes (PBL) exhibited significant selectivity of DCM/F7 compared to other fractions. Phytochemical analysis using GC-MS revealed that the DCM/F7 fraction contains linoleic acid (51.20%), *n*-hexadecanoic acid (17.89%), 9-hexadecanoic acid (6.99%) and Stigmasta-5,22-dien-3-ol (6.06%). Cytological observations exhibited chromatin condensation, cell shrinkage, abnormalities of cristae, membrane blebbing, cytoplasmic extrusions and formation of apoptotic bodies, further confirmed using AO/PI, SEM and TEM analysis. The Annexin V and TUNEL assay revealed apoptotic induction in CEMss cells exposed to the DCM/F7 in a time-dependent manner, whilst DNA fragmentation of CEMss cells were detected using 1.0% agarose gel electrophoresis. The DCM/F7 fraction significantly ($p<0.05$) stimulated both caspases 3 and 9 activities. The immunoblot results revealed that DCM/F7 caused the release of mitochondrial cytochrome c and cleaved 116 kDa PARP into 85 kDa fragments. The Bcl-2 protein was found to decrease

during treatment. Meanwhile, FasL did not exhibit up or down regulation on treatment. Cell cycle analysis revealed that there is significant ($p<0.05$) G1 phase arrest in a time-depended manner. The DCM extract of *T. flagelliforme* tuber *in vivo* markedly inhibited the proliferation of WEHI-3 in male BALB/c mice as evidenced by reduction in the percentage of immature monocytes as well as granulocytes, liver weight, spleen weight and histopathological profiles of H&E stained spleen tissue. The DCM tuber extract of *T. flagelliforme* significantly decreased the spleen tumor size, which had dose-dependent effects. Sections of spleen tissue of the BALB/c mice treated with the extract. Treatment at 800 mg/kg dose showed evidence of apoptosis in comparison to the control groups. Collectively, results presented in this study demonstrate that *T. flagelliforme*, a local herbal medicinal plant in Malaysia inhibited the proliferation of leukemia *in vitro* selectively, leading to the programmed cell death, which was later confirmed to lead through mitochondrial pathways. Moreover, *in vivo* study on an orthotopic BALB/c mice model clearly shows that, *T. flagelliforme* tuber extract has inhibited the proliferation of leukemia via the induction of apoptosis.

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

KESAN ANTI-LEUKEMIA DARI *TYPHONIUM FLAGELLIFORME* PADA SEL LYMPHOBLASTOID MANUSIA (CEMss) DAN (WEHI-3) MODEL MURINE LEUKEMIA

Oleh

MURALI MOHAN SYAM MOHAN

Oktober 2010

Pengerusi: Ahmad Bustamam Abdul, PhD

Faculti: Institut Biosains

Sehingga hari ini, belum terdapat sorotan literatur yang melaporkan mekanisma *Typhonium flagelliforme* dan kesannya terhadap leukemia. Oleh itu, kesan anti-leukemia dari *Typhonium flagelliforme* dikaji menggunakan model leukemia *secara in vitro* dan *in vivo*. Kaedah ekstraksi dan fraksinasi menggunakan pelarut organik digunakan untuk mendapatkan fraksi dari *T. flagelliforme* dan seterusnya, analisis kimia dijalankan menggunakan kaedah GC-MS. Kesan sitotoksik *in vitro* ekstrak dan pecahannya telah diuji terhadap beberapa sel kanser manusia termasuk leukemia (sel CEMss) menggunakan asai MTT. Beberapa teknik mikroskopi telah digunakan untuk mengkaji perubahan morfologi yang berlaku semasa rawatan. Asai Annexin V, Asai TUNEL, analisis kitaran sel dan dan tetangga DNA telah digunakan untuk mengesan apoptosis. Asai kalorimetrik bagi caspase-3 dan 9, analisis

imunoblot untuk sitokrom c, BcL-2, FasL dan β-actin telah dianalisa. Bagi model leukemia secara *in vivo* tikus jantan BALB/c diaruh menggunakan sel WEHI-3. Ekstrak DCM tanaman tuber telah digunakan untuk rawatan dalam beberapa dos. Diantara 8 ekstrak tumbuhan yang diuji, ekstrak tuber *T. flagelliforme yang* menggunakan diklorometana (DCM) menunjukkan kesan anti proliferasi terhadap kedua-dua sel; CEMss (6.5±0.4 µg/ml) dan WEHI-3 (24.0±5.2 µg/ml) ($p<0.05$). Fraksinasi lanjutan ekstrak tuber dengan menggunakan DCM telah menghasilkan 12 fraksi. 7 daripada 12 fraksi menunjukkan kesan sitotoksik yang signifikan terhadap CEMss, di mana fraksi DCM/F7, DCM/F11 dan DCM/F12 menunjukkan aktiviti anti-kanser paling tinggi dengan 3.0, 5.0 dan 6.2 µg/ml. Kajian lanjutan terhadap fraksi ini adalah pada Limfosit Darah Periferi (PBL) bukan kanser menunjukkan kesan pemilihan yang signifikan pada DCM/F7 dibandingkan terhadap fraksi lain. Analisis fitokimia menggunakan GC-MS mendedahkan fraksi DCM/F7 mengandungi asid linoleik (51.20%), asid n-hexadekanoat (17.89%), asid 9-heksadekanoat (6.99%) dan Stigmasta-5, 22-dien-3-ol (6.06%). Pemerhatian sitologi menunjukkan kondensasi kromatin, pengecutan sel, ketidaknormalan krista, penggelembungan membran, penonjolan sitoplasma dan pembentukan jasad apoptotik, Seterusnya pengesahan adalah menggunakan analisis AO/PI, SEM dan TEM. Asai Annexin V dan TUNEL menunjukkkan rangsangan apoptotik pada sel CEMss dikesan menggunakan 1.0% elektroforesis agarose gel. Fraksi DCM/F7 mendorong peningkatan aktiviti caspase 3 dan 9 secara signifikan pada ($p<0.05$). Keputusan imunoblot mendedahkan DCM/F7 menyebabkan pembebasan sitokrom c mitokondria dan pemecahan 116kDa PARP kepada 85 kDa

fragmen. Protein Bcl-2 didapati berkurang semasa rawatan. Sementara itu, FasL tidak menunjukkan peningkatan atau penurunan pngawalaturan terhadap rawatan. Analisis kitaran sel mendedahkan terdapat penahanan fasa G1 yang signifikan ($p<0.05$) dalam cara kebergantungan pada masa. Kajian ekstrak DCM tuber *T. flagelliforme in vivo* didapati merencatkan proliferasi WEHI-3 pada tikus jantan BALB/c seperti yang dibuktikan dengan penurunan dalam peratus monosit yang tidak matang dan juga granulosit, berat hati, berat hati, berat limpa dan profil histopatologi bagi H&E tisu limpa yang diwarnakan. Ekstrak tuber DCM *T. flagelliforme* mengurangkan saiz limpa dengan signifikan yang mempunyai kesan kebergantungan dos. Tisu bahagian limpa tikus jantan BALB/c dirawat dengan ekstrak. Rawatan pada dos 800 mg/kg menunjukkan bukti kejadian apoptosis dibandingkan dengan kumpulan kawalan. Secara kolektif, keputusan yang dibentangkan dalam kajian ini menunjukkan tumbuhan herba ubatan tempatan di Malaysia, *T. flagelliforme* telah menghalang proliferasi leukemia secara *in vitro*, membawa kepada kematian sel yang diprogramkan, yang mana kemudiannya disahkan melalui laluan mitokondia. Tambahan lagi, kajian *in vivo* pada model ortotopik *T. flagelliforme* jelas menunjukkan ekstrak tuber *T. flagelliforme* telah menghalang proliferasi leukemia melalui rangsangan apoptosis.

ACKNOWLEDGEMENTS

First and foremost, I would like to express tremendous gratitude, respect and admiration for my supervisor, Ahmad Bustamam Hj Abdul, PhD. Throughout my studies, I have learned from his wisdom and experience, and benefited from his continuous guidance and support. His enthusiasm and commitment to this research project is deeply appreciated and undoubtly invaluable. He has been the most wonderful mentor, confidant, and teacher. My utmost appreciation also goes to Prof. Dr. Mohd Aspollah Sukari and Prof. Dr. Rasedee Abdullah, that without their continuous support, encouragement, help, and advice, I was not be able to continue and complete this thesis. I truly do not know how to thank them enough for the invaluable help in making my experience as a doctoral student challenging, enlightening and meaningful for my future endeavors. I also wish to express deepest thanks to Prof. Dr. Abdul Rahman Omar for his timely advices.

I gratefully acknowledge the School of graduate studies UPM for their financial support (GRF) during the course of PhD program.

It is worth to mention the name of Dr Siddig Ibrahim Abdulwahab, who stood with me as an elder brother as well as a co researcher and helps me to further my research in UPM. I also wish to express my heartiest gratitude to Dr Adel S Al Zubairi for his efficiency and availability. Also, immense thanks to Mr Sagi, Dr Gururaj, Dr Ibrahim for their availability and readiness to share

them. Special thanks to all my fellow colleagues and staffs of UPM Makna Cancer Research Laboratory.

I also wish to thank my parents, Mr. Murali Mohan and Mrs. Ushakumari for their unconditional love and support.

Thank God for giving me the opportunity to be a father for a lovely son, Adithya Mohan. To my wife, Suvitha Syam Mohan, thanks for being a true friend and a person who always inspired me to be smart in everything I do.

I certify that a Thesis Examination Committee has met on 8-10-2010 to conduct the final examination of Murali Mohan Syam Mohan on his thesis entitled "Anti-leukemic effects of *Typhonium flagelliforme* on human lymphoblastoid cells (CEMss) and murine leukemic (WEHI-3) model" 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 Doctor of Philosophy.

Members of Thesis Examination Committee were as follows:

ABDUL RAHMAN OMAR, PhD
Professor
Department of Veterinary Pathology & Microbiology
Faculty of veterinary medicine
Universiti Putra Malaysia
(Chairman)

ROZITA ROSLI, PhD
Associated Professor
Department of Obstetrics and Gynaecology
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

TENGKU AZMI TENGKU IBRAHIM, PhD
Professor
Department of Veterinary Preclinical Sciences
Faculty of veterinary medicine
Universiti Putra Malaysia
(Internal Examiner)

FANG-RONG CHANG, PhD
Professor and Director,
Graduate Institute of Natural Products (GINP)
College of Pharmacy
Kaohsiung Medical University (KMU)
Taiwan

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

Date: 15 December 2010

This thesis submitted to the Senate of University Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee are as follows:

Ahmad Bustamam Hj Abdul, PhD
Lecturer
Institute of Bioscience
Universiti Putra Malaysia
(Chairman)

Mohd Aspollah Bin Hj Md Sukari, PhD
Professor
Faculty of Science
Universiti Putra Malaysia
(Member)

Rasedee @ Mat Bin Abdullah, PhD
Professor
Faculty of Veterinary science
Universiti Putra Malaysia
(Member)

_____

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

Date: December 2010

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.

_____

MURALI MOHAN SYAM MOHAN
Date: 08.10.2010

TABLE OF CONTENTS

		Page
DEDICATION		li
ABSTRACT		iii
ABSTRAK		vi
ACKNOWLEDGEMENTS		ix
APPROVAL		xi
DECLARATION		xiii
LIST OF TABLES		xix
LIST OF FIGURES		xx
LIST OF ABBREVIATIONS		xxiv
CHAPTER		
1	**INTRODUCTION**	29
2	**LITERATURE REVIEW**	38
	2.1 Natural Products	38
	2.2 Drug Discovery from Plant-Derived Substances	39
	2.3 Plant Derived Anticancer Agents	40
	2.4 Natural Compounds from Local Medicinal Plants	41
	2.5 Typhonium Species	43
	2.6 Typhonium Flagelliforme	44
	2.6.1 Biological Activities of *T. flagelliforme*	44
	2.6.2 Chemical Constituents from *T. flagelliforme*	46
	2.7 Cancer	47
	2.8 Biology of Tumor	49
	2.9 Classification of Cancer	50
	2.10 Leukemia	51
	2.11 CEMss, an Acute Lymphoblastic Leukemia Cell Line	54
	2.12 WEHI-3, a Murine Monomyelocytic Cells	55
	2.13 Chemotherapy	56
	2.14 Terminology of Cell Death	57
	2.14.1 Necrosis	57
	2.14.2 Apoptosis	59
	2.14.3 Morphology of Apoptosis	61
	2.14.4 Distinguishing Apoptosis from Necrosis	64
	2.14.5 Mechanism of Apoptosis	66
	2.14.6 Biochemical Features	68
	2.14.7 Endonuclease Activation	68
	2.14.8 Phagocytosis	71
	2.14.9 Intracellular Signaling	73
	2.15 Molecular Regulation of Apoptosis	75

2.15.1 Bcl-2 Family 76
2.15.2 Tumor Suppressor Genes 80
2.15.3 Extrinsic Pathway 82
2.15.4 Perforin/granzyme Pathway 83
2.15.5 Intrinsic Pathway 84
2.16 Caspase 84
2.17 Apoptosis and Cancer 91
2.18 Cell Cycle 91
2.19 Polyunsaturated Fatty Acids 96
2.20 Currenty Proposed Mechanisms of PUFA Effects on Tumor Cell Growth 98
2.20.1 PUFAs Modulate Cancer Growth through Their Influences on Cell Membrane Properties 98
2.20.2 PUFAs Modulate Cancer Growth via Eicosanoid Formation 100
2.20.3 PUFAs Regulate Cancer Growth via Oncogenes and Cancer Suppressor Gene Expressions 102
2.20.4 PUFAs Modulate Cancer Cell Proliferation 103
2.20.5 PUFAs Modulate Cancer Cells Proliferation via Cell-Cycle Progression Control 104

3 **MATERIALS AND METHODS** 107
3.1 Extraction Procedure 107
3.2 *In vitro* Anticancer Properties of *T. flagelliforme* Crude Extracts 108
3.2.1 Preparation of Extracts 108
3.2.2 Cell Culture Condition 108
3.2.3 Cryopreservation 109
3.2.4 Thawing Cryopreserved Cells 109
3.2.5 Cell Growth Inhibition Assay 110
3.3 Fractionation of the Dichloromethane Extract 111
3.4 The Effect of Selected Fractions on Stimulated Primary Human Blood Lymphocytes. 112
3.5 Identification of Bioactive Fraction and Chemical Analysis Using GC–MS 113
3.6 Microscopic Observation of Cellular Morphology Using Phase Contrast Inverted Microscope 114
3.7 Quantification of Apoptosis Using Propidium Iodide and Acridine Orange Double Staining. 115
3.8 Scanning Electron Microscopy of CEMss Cells 116
3.9 Transmission Electron Microscopy of CEMss Cells 117
3.10 Annexin V Assay 118
3.11 ApoBrdU—TUNEL Assay 119

	3.12 DNA Laddering	120
	3.13 Colourimetric Assay of Caspase-3	121
	3.14 Colourimetric Assay of Caspase 9	121
	3.15 Protein Detection by Western Blotting	122
	3.15.1 Extraction of Whole Protein from the Cell	122
	3.15.2 Western Blotting Analysis	124
	3.16 Flow Cytometric Analysis of DNA Cell Cycle	125
	3.17 *In vivo* Antileukemic Properties of *Typhonium flagelliforme*	126
	3.17.1 Determination of Maximum Tolerated Dose of DCM Crude Extract of *T. flagelliforme* Tuber in BALB/c Mice	126
	3.17.2 Antileukemia Model in BALB/c Mice and Drug Treatment	127
	3.17.3 Leukocyte Counting in Peripheral Blood of Treated Mice	128
	3.17.4 Tissues Samples (Liver and Spleen)	128
	3.17.5 Histopathology of Spleen by H&E Staining	128
	3.17.6 Detection of Apoptosis Using TUNEL Assay	129
4	**RESULTS**	134
	4.1 Extraction of *T. flagelliforme*	134
	4.2 Cell Growth Inhibition Assay	136
	4.3 Fractionation of Dichloromethane Crude Extract of *T. flagelliforme* Tuber.	138
	4.4 Cell Growth Inhibition Assay of DCM Tuber Fractions on CEMss	140
	4.5 The Anti Proliferative Effect of Selected Fractions Primary Human Blood Lymphocytes.	142
	4.6 Identification of Bioactive Fraction and Chemical Analysis of DCM/F7 Using GC–MS	144
	4.7 Microscopic Observation of Cellular Morphology Using Phase Contrast Inverted Microscope	149
	4.8 Quantification of Apoptosis Using Propidium Iodide and Acridine Orange Double Staining.	151
	4.9 Effects of DCM/F7 on Human T4 Lymphoblastoid Leukemic (CEMss) Using Scanning Electron Microscopy	154
	4.10 Effects of DCM/F7 on Human T4 Lymphoblastoid leukemic (CEMss) Using Transmission Electron Microscopy	157
	4.11 Annexin V Assay	161
	4.12 ApoBrdU—TUNEL Assay	163
	4.13 DNA Laddering	167
	4.14 Flow Cytometric Analysis of Cell Cycle and DNA Content	169

	4.15 Colourimetric Assay of Caspase-3 and Caspase 9	171
	4.16 Western Blot Analysis	174
	4.17 *In vivo* Antileukemic Properties of *Typhonium flagelliforme*	176
	4.17.1 Determination of Maximum Tolerated Dose of DCM Crude Extract of *T. flagelliforme* Tuber in BALB/c Mice	176
	4.17.2 *In vivo* Studies	178
	4.17.3 Tissues Sampling and Histopathology	181
	4.17.4 Detection of Apoptosis Using TUNEL Assay System	189
5	**DISCUSSION**	195
	5.1 Extraction, Fractionation and Phytochemical Identification of *T. flagelliforme*	195
	5.2 Cell Growth Inhibition Assay	198
	5.3 Antiproliferative Effects of DCM Tuber Fractions on CEMss and Primary Human Blood Lymphocytes.	200
	5.4 Microscopic Observation of Cellular Morphology Using Phase Contrast Inverted Microscope	202
	5.5 Quantification of Apoptosis Using Propidium Iodide and Acridine Orange Double Staining.	203
	5.6 Effects of DCM/F7 on Human T4 Lymphoblastoid leukemic (CEMss) Using Scanning Electron Microscope (SEM)	204
	5.7 Effects of DCM/F7 on Human T4 Lymphoblastoid leukemic (CEMss) Using Transmission Electron Microscope (TEM)	205
	5.8 Annexin V Assay	207
	5.9 ApoBrdU—TUNEL Assay	208
	5.10 DNA Laddering	209
	5.11 Colourimetric Assay of Caspase-3 and Caspase 9	209
	5.12 Western Blot	210
	5.13 Cell Cycle Analysis	212
	5.14 *In vivo* Antileukemic Properties of *Typhonium flagelliforme*	213
	5.14.1 Determination of Maximum Tolerated Dose of DCM Crude Extract of *T. flagelliforme* Tuber in BALB/c Mice	213
	5.14.2 *In vivo* Studies	215
	5.14.3 Tissues Sampling and Histopathology	218
	5.14.4 TUNEL Assay	219
6	**SUMMARY, CONCLUSION AND FUTURE RECOMMENDATIONS**	221
		229

REFERENCES
APPENDICES 256
LIST OF PUBLICATIONS 284
BIODATA OF THE STUDENT 286