



UNIVERSITI PUTRA MALAYSIA

**ANTI-LEUKEMIC EFFECTS OF TYPHONIUM FLAGELLIFORME ON
HUMAN LYMPHOBLASTOID CELLS (CEM_{ss}) AND MURINE
LEUKEMIC (WEHI-3) MODEL**

MURALI MOHAN SYAM MOHAN

IB 2010 3



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(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 fulfillment of the requirement for the degree of Doctor of Philosophy

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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 ($6.5 \pm 0.4 \mu\text{g/ml}$) and WEHI-3 cells ($24.0 \pm 5.2 \mu\text{g/ml}$) ($p < 0.05$). 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 $\mu\text{g/ml}$ 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-dependent 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

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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 ($6.5 \pm 0.4 \mu\text{g/ml}$) dan WEHI-3 ($24.0 \pm 5.2 \mu\text{g/ml}$) ($p < 0.05$). 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 $\mu\text{g/ml}$. 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 menunjukkan 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 pengawalan 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 undoubtedly 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 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.

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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.

MURALI MOHAN SYAM MOHAN
Date: 08.10.2010

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