UNIVERSITI PUTRA MALAYSIA

CONFIRMATION OF Trichophyton rubrum BY MICROMORPHOLOGICAL AND MOLECULAR TECHNIQUES AND IN-VITRO ANTIFUNGAL ACTIVITIES OF ALLICIN AND GARLIC EXTRACTS

MFARZAD AALA

FS 2011 10

CONFIRMATION OF *Trichophyton rubrum* BY MICROMORPHOLOGICAL AND MOLECULAR TECHNIQUES AND *IN-VITRO* ANTIFUNGAL ACTIVITIES OF ALLICIN AND GARLIC EXTRACTS

BY

FARZAD AALA

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

February 2011

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

CONFIRMATION OF *Trichophyton rubrum* BY MICROMORPHOLOGICAL AND MOLECULAR TECHNIQUES AND *IN-VITRO* ANTIFUNGAL ACTIVITIES OF ALLICIN AND GARLIC EXTRACTS

By

FARZAD AALA

February 2011

Chairman: Profesor Umi Kalsom Yusuf, PhD

Faculty: Science

Dermatophytosis caused by a group of pathogenic fungi namely, dermatophytes, is among the most prevalent infectious diseases worldwide. One of the most frequently isolated pathogenic dermatophytes is *Trichophyton rubrum*. Chemical drugs are widely used in the treatment of dermatomycosis, but can cause various side effects to the patients and drug resistance to the pathogens. Hence, alternative natural compounds should be assessed to solve this problem. Garlic can be considered as a good replacement due to its high level of sulfur compounds (e.g., allicin). Therefore, the main objective of this study was to evaluate allicin and garlic extract against various isolates of *T. rubrum*. These studies were divided into three parts. The first was to confirm ten different isolates of *T. rubrum* which were obtained from the Laboratory of Medical Mycology Department in

Tehran University of Medical Sciences, Iran by using both conventional and molecular methods. The second part was to evaluate the *in vitro* antifungal activity of allicin, aqueous garlic extract, ketoconazole and fluconazole and the combination of these azoles drugs with allicin and aqueous garlic extract against these ten isolate of *T. rubrum*. The third objective was to see the effect of allicin and garlic extract to the ultrastructure of *T. rubrum* using electron microscope. In this study, colony characterizations of all isolates of *T. rubrum* are varied and inconclusive. However the molecular study based on Internal Transcribed Spacer, 1 and 4 (ITS1 and 4) revealed that all the different isolates tested with reference from data base in Genbank (BLAST search) showing more than 95% similarity. *In vitro* antifungal study on the effects of allicin and aqueous garlic extract singly and in combination with ketoconazole and fluconazole against ten clinical isolate of *T. rubrum* were effective in inhibiting the fungal growth. The results showed that MICs for allicin ranged from 0.78 – 12.5 µg/ml, ketoconazole 0.25 – 8.0 µg/ml and fluconazole 1.0 - 32.0 µg/ml respectively. Combination of allicin or garlic extract with ketoconazole or fluconazole caused the synergistic or additive effect on dermatophytes, which may increase fungicidal effects, decrease toxicity, side effects and drug resistance. Besides, *in vitro* studies revealed that allicin and aqueous garlic extract alone and in combination with these two azoles drugs, has a good potential as antifungal drug based on the results of MICs (Minimal Inhibitory Concentration) and FICIs (Fractional Inhibitory Concentration Index). The SEM (Scanning Electron Microscopy) micrographs showed that allicin and garlic extract caused shrinkage, rough

and irregular- shaped hypha with expanded hyphal tip of *T. rubrum*. The TEM (Transmission Electron Microscopy) micrographs revealed that allicin and garlic extract caused cell wall thickening, disorganization of cytoplasmic contents and breaking down of cell membrane and cell wall of *T. rubrum*. SEM and TEM studies proved that allicin and garlic extract possessed antifungal activity by inhibiting the growth of *T. rubrum*, and can be considered to treat fungal infections. This study showed that although different isolates exhibited different morphological characteristics but molecular analysis proved that they belong to the *T. rubrum* species. Allicin and garlic extracts were effective in inhibiting dermatophytes growth. In addition, SEM and TEM studies demonstrated that allicin and garlic extract possessed antifungal activity which inhibits the hyphal growth of *T. rubrum.*

Abstrakt tesis yang dikemukakan kepada Senat Universiti Pura Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGENALPASTIAN *Trichophyton rubrum* SECARA TEKNIK MIKROMORFOLOGI DAN MOLEKULAR DAN KAJIAN *IN VITRO* ANTIFUNGI DARI ALLICIN DAN EKSTRAK BAWANG PUTIH

Oleh

FARZAD AALA

Februari 2011

Pengerusi: Profesor Umi Kalsom Yusuf, PhD

Fakulti: Sains

Dermatophytosis adalah penyakit kulit berjangkit yang biasa di seluruh dunia yang disebabkan oleh kulat pathogenik yang dikenali sebagai Dermatofit. Salah satu dari kulat penyebab Dermatofit adalah *Trichophyton rubrum*. Dadah kimia digunakan secara meluas sebagai rawatan untuk dermatomikosis tetapi menyebabkan berbagai kesan sampingan ke atas pesakit and ketahanan dadah ke atas peaskit. Jadi sebagai alternatif, kompaun asli dikaji untuk menyeleasikan masalah ini. Bawang putih boleh dijadikan sebagai alternatif disebabkan oleh kandungan sulfur yang tinggi (seperti allicin). Jadi, objektif utama kajian ini adalah untuk menilai allicin and ekstrak bawang putih ke atas isolat *T. rubrum*. Kajian in terbahagi kepada tiga bahagian. Bahagian pertama adalah untuk pengecaman sepuluh

isolat klinikal *T. rubrum* yang diperolehi dari Jabatan Perubatan Mikologi, Universiti Perubatan Tehran, Iran dengan menggunakan kaedah Konvesional dan molekular. Bahagian kedua adalah untuk menentukan kombinasi yang terbaik antara kumpulan azole dengan allicin (Alexis– Biochemicals Co, USA), dan Ekstrak Akueus bawang putih sebagai bahan antikulat. Bahagian ketiga untuk melihat kesan allicin dan Ekstrak Akueus bawang putih ke atas struktur ultra *T. rubrum* menggunakan mikroskop electron. Kajian ini mendapati kaedah konventional untuk pengesahan spesies dermatofit bergantung kepada perbezaan fenotip adalah tidak konklusif. Pendekatan kaedah molekular berdasarkan "Internal Transcribed Spacer" 1 dan 4 (ITS1 dan 4) menunjukkan keseluruhan isolat berbeza yang diuji dan dibandingkan dengan isolat rujukan dari pengkalan data "Genbank" (carian BLAST) menunjukkan persamaan melebihi 95%. Kajian antikulat secara *in vitro* kesan allicin dan Ekstrak Akueus bawang putih secara bersendirian atau digabungkan dengan ketonazole dan fluconazole terhadap 10 isolat klinikal *T. rubrum* adalah berkesan menghambat pertumbuhan kulat tersebut. Keputusan kajian mendapati MICs untuk allicin masing masing berjulat antara 0.78-12.5 µg/ml, ketoconazole 0.25-8.0 µg/ml dan fluconazole 1.0-32.0 µg/ml. Kombinasi allicin atau Ekstrak Akueus bawang putih dengan ketoconazole atau fluconazole menghasilkan kesan synergistik atau kesan penambahan terhadap dermatofit, seterusnya berkemungkin meningkatkan kesan antikulat, mengurangkan toksisiti, kesan sampingan dan kesan kerintangan terhadap bahan antikulat. Kajian *in vitro* juga menunjukkan allicin dan Ekstrak Akueus bawang putih secara bersendirian atau

digabungkan dengan dua bahan antikulat kumpulan azol berpotensi sebagai bahan antikulat berdasarkan keputusan MICs (Minimal Inhibitory Concentration) dan FICIs (Fractional Inhibitory Concentration Index). Analisis mikrofotograf menggunakan SEM (Scanning Electron Microscopy) menunjukkan allicin dan Ekstrak Akueus bawang putih menyebabkan hifa *T. rubrum* mengecut, permukaan hifa kasar, pertumbuhan hifa tidak menentu dan terdapat benjolan pada hujung hifa. Gambar mikrofotografi TEM (Transmission Electron Microscopy) menunjukkan allicin dan Ekstrak Akueus bawang putih menyebabkan dinding hifa menebal, kandungan sitoplasmik berkecelaru, membran sel dan dinding sel mereput. Kajian mikrofotograf menggunan SEM dan TEM membuktikan allicin dn Ekstrak Akueus bawang putih mengandungi bahan antikulat dan boleh dipertimbangkan untuk rawatan jangkitan kulat. Kajian ini membuktikan walaupun *T. rubrum* dari isolat yang berbeza dan memberikan ciri-ciri morfologi yang berbeza, tetapi kajian molekular membuktikan ia adalah dari spesies *T. rubrum* yang sama. Allicin dan ekstrak bawang putih sangat berkesan untuk menghalang pertumbuhan dermatofit. Kajian SEM dan TEM menunjukkan bahawa Allicin dan ekstrak bawang putih aktiviti antifungal dimana menghalang pertumbuhan hifa *T. rubrum.*

ACKNOWLEDGEMENTS

There are several people to whom I owe a great debt concerning this dissertation. First, I thank Professor Dr. Umi Kalsom Yusuf for being the chair of my committee and her significant role in this project. I also am extremely grateful for my first supervisor Professor. Dr. Faridah bt Abdullah that unfortunately passed away last year.

I also owe Professor Dr. Farida Jamal and Dr. Rosimah Nulit for being the members of my committee. I thank these two people due to their kind assistants in guiding me and being so patient with me.

My deep appreciation is extended to Dr. Sassan Rezaie, Associate Professor of Department of Medical Mycology in Tehran University of Medical Sciences, Iran for sending the isolates of dermatophytes used in this investigation.

I would like to express my sincere thank to the laboratory staff of Mycology, Department of Biology, Faculty of Science; Universiti Putra Malaysia.

This study was supported by the Research University Grants Scheme (RUGS) from University Putra Malaysia. I really appreciate their assistance.

My family merited to acknowledge for their very important role in my project. To my parents, I thank you for helping me in any time of my life and study. I am also grateful for my wife and children for their support, love and encouragement that have sustained me around four years in Malaysia.

I certify that a Thesis Examination Committee has met on 22 February 2011 to conduct the final examination of Farzad Aala on his Doctor of Philosophy thesis entitled "In vitro Antifungal Activities of Allicin and Garlic Extracts, and Molecular and Micro Morphological Identification of *Trichophyton rubrum*", in accordance with 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 degree of Doctor of Philosophy

Members of the Examination Committee are as followes:

NORHANI ABDULLAH, PhD
Professor
Faculty of Biotecnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Sariah Meon, PhD
Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Internal Examiner)

Hishamudin Bin Omar, PhD
Senior Lecturer
Faculty of Science
Universiti Putra Malaysia
(Internal Examiner)

Stephen Rolfe, PhD
Senior Lecturer
Faculty of Animal and Plant Sciences
University of Sheffield, UK
(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 fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Umi Kalsom Yusuf, PhD
Professor
Faculty of Science
Universiti Putra Malaysia
(Chairman)

Farida Jamal, PhD
Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Rosimah Nulit, PhD
Senior Lecturer
Faculty of Science
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 it has not been previously, or is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

_____

FARZAD AALA

Date: 22 February 2011

TABLE OF CONTENTS

		Page
ABSTRACT		ii
ABSTRAK		v
ACKNOWLEDGMENTS		viii
APPROVAL		ix
DECLARATION		xi
LIST OF TABLES		xv
LIST OF FIGURES		xvi
LIST OF ABBREVIATIONS		xix
CHAPTER		
1	**GENERAL INTRODUCTION**	1
2	**LITERATURE REVIEW**	5
	2.1 Dermatophytes	5
	2.1.1 *Trichophyton rubrum*	7
	2.1.2 Pathophysiology	8
	2.1.3 Clinical manifestations	10
	2.1.4 Epidemiology	13
	2.1.5 Treatment	14
	2.2 Identification of dermatophytes	15
	2.2.1 Conventional method	15
	2.2.2 Trichophyton species	17
	2.2.3 Molecular methods	19
	2.3 Treatment of dermatophytosis and drugs	25
	2.3.1 The azoles group	26
	2.3.2 Classification of azoles	27
	2.3.3 Mechanism of action	27
	2.3.4 Ketoconazole	28
	2.3.5 Fluconazole	30
	2.3.6 The development of azole-resistance	31
	2.3.7 Dermatomycosis and azoles group	32
	2.4 Plant extracts	34
	2.4.1 Garlic (*Allium sativum*)	35
	2.4.2 Allicin	39
	2.5 Antifungal susceptibility testing	43
	2.6 Electron microscopy	46
3	**CONVENTIONAL AND MOLECULAR CHARACTERIZATION OF *Trichophyton rubrum***	50
	3.1 Introduction	50
	3.2 Materials and methods	51

3.2.1 Conventional method 52
3.2.2 Molecular method 52
3.2.3 DNA extraction 53
3.2.4 PCR Amplification 54
3.2.5 PCR purification 55
3.3 Results 55
3.3.1 Morphological characteristics of colonies *T. rubrum* 55
3.3.2 Isolation, identification and molecular characterization of ITS1 of *T. rubrum* 59
3.4 Discussion 60
3.5 Conclusion 62

4 ***IN VITRO* ANTIFUNGAL ACTIVITIES OF ALLICIN AND AQUEOUS GARLIC EXTRACTS AGAINST DERMATOPHYTIC FUNGI** 63
4.1 Introduction 63
4.2 Materials and methods 65
4.2.1 Experimental design 65
4.2.2 Isolates 66
4.2.3 Media 66
4.2.4 Preparation of aqueous garlic extract 67
4.2.5 Inoculum preparation 67
4.2.6 Antifungal compounds 69
4.2.7 Broth microdilution method (NCCLS M38-A protocol) for determination of MIC 70
4.2.8 Incubation time and temperature 71
4.2.9 Evaluation of the MIC, MFC and FICI 71
4.2.10 Time-kill studies 73
4.3 Results 74
4.3.1 Data analysis 78
4.4 Discussion 86
4.5 Conclusion 91

5 **SCANNING AND TRANSMISSION ELECTRON MICROSCOPY FOR EVALUATION OF THE 0 EFFECTS OF ALLICIN AND GARLIC EXTRACT ON *Trichophyton rubrum*** 92
5.1 Introduction 92
5.2 Materials and methods 94
5.2.1 Preparation of antifungal agents and fungus 94
5.2.2 Culture conditions for the microscopic observation 94
5.2.3 Specimen preparaiton for SEM and TEM 95
5.3 Results 97
5.4 Discussion 128

5.5 Conclusion 130

6 **GENERAL CONCLUSION** 131

RECOMMENDATIONS 133
REFERENCES 134
APPENDICES 144
BIODATA OF STUDENT 169
LIST OF PUBLICATIONS 170