CHARACTERISATION OF *ASPERGILLUS* CONTAMINANTS OF MILLED RICE AND ANTITOXIC EFFECTS OF MALAYSIAN ETHNOMEDICINAL PLANT EXTRACTS ON GROWTH SUPPRESSION

By

DARAB YAZDANI

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

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FP 2011 10

DEDICATION

I would like to dedicate this dissertation to:

My beloved mother, father and my dear family for their invaluable love, understanding, tolerance, sacrifice and moral support.

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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Chairman: Assoc. Prof. Zainal Abidin Mior Ahmad, PhD

Faculty: Agriculture

Contamination of food by mycotoxins is an important food safety concern for grains and other agricultural products. *Aspergillus* species are responsible for production of most of these toxins including aflatoxin and ochratoxin A (OTA). Results achieved by present study revealed that 65% of the tested rice samples collected from different region in Malaysia was infected by *Aspergillus* and/or *Eurotium* species. *A. flavus* and *A. niger* were the predominant species and were the major contaminants (account for 17%) in rice. 17 *Aspergillus* and *Eurotium* isolates were obtained and identified based on morphological characteristics and molecular techniques.

This study also confirmed that only *A. flavus* starin A2 was able to produce aflatoxins B1 and B2 in culture media. Compared with other techniques, thin layer chromatography was concluded to be a favorable method as it is simple, sensitive and reliable for detecting the aflatoxins and OTA production by *Aspergillus* isolates.

In screening of antifungal activity of Malaysian medicinal plants, it was found that both *A. galanga* and *P. betle* significantly inhibited the production of aflatoxins by *A. flavus*. Compared with other extracts, the chloroform extract of *A. galanga* exhibited the best antifungal effect and with MIC value 25 µg/mL. *P. betle* extract at 500 µg/mL demonstrated a complete inhibition on the production of aflatoxins and at 100 µg/mL, the same extract was able to inhibit 69.4% of the aflatoxin production in culture media. LC-MS analysis showed that the chloroform soluble fraction of methanolic extract from *A. galanga* possess 10 compounds, which were mainly cinnamic acid and related aromatic acids such as coumaric acid, cafeic acid and ferulic acid, whereas in *P. betle* extract 11 compounds was obtained, they were mainly dihydrochalcones.

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

PENCIRIAN PENCEMAR *ASPERGILLUS* PADA BERAS DAN KESAN ANTITOKSIK EKSTRAK TANAMAN UBATAN MALAYSIA TERHADAP PERENCATAN TUMBESARAN

Oleh

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.

Pencemaran makanan oleh mikotoksin adalah satu kebimbangan dalam keselamatan makanan yang penting bagi bijirin dan hasil pertanian yang lain. Spesies *Aspergillus* adalah bertanggung jawab dalam pengeluaran kebanyakan toksin termasuk aflatoksin dan okratoksin A (OTA). Keputusan yang didapati dalam kajian ini mendapati 65% daripada sampel beras diuji yang dikumpul dari kawasan berbeza di Malaysia dijangkiti oleh *Aspergillus* dan / atau spesies *Eurotium*. *A. flavus* dan *A. niger* adalah spesies yang terbanyak dan adalah pencemar utama (menyumbang 17%) pada beras. Sebanyak 17 pencilan *Aspergillus* dan *Eurotium* telah didapati dan dikenalpasti berdasarkan ciri morfologi dan teknik molikul. Kajian ini juga

mengesahkan hanya *A. flavu*s strain A2 boleh menghasilkan aflatoksin B1 dan B2 dalam medium kultur. Berbanding dengan teknik lain, kromatografi lapisan tipis disimpulkan cara yang disukai oleh kerana ia ringkas, sensitif dan dipercayai bagi mengesan penghasilan aflatoksin dan OTA oleh pencilan *Aspergillus*. Dalam penyaringan aktiviti antikulat oleh tanaman ubatan Malaysia, *Alpinia galanga* dan *Piper betle* telah didapati merencat dengan bererti penghasilan aflatoksin oleh *A. flavus*. Berbanding dengan ekstrak lain, ekstrak kloroform *A. galanga* menunjukkan kesan antikulat terbaik dengan nilai MIC 25µg/mL. Ekstrak *P. betle* pada 500 µg/mL menunjukkan perecatan sepenuh penghasilan aflatoksin dan pada 100µg/mL ekstrak yang sama boleh merencat 69.4% penghasilan aflatoksin pada medium kultur. Analisa LC-MS menunjukkan bahagian kloroform terlarut ekstrak metanolik dari *A. galanga* mempunyai 10 sebatian yang kebanyakkannya terdiri dari asid sinamik dan asid aromatik yang berkaitan seperti asid kumarik, asid kafeik dan asid ferulik, sementara pada ekstrak *P. betle* 11 sebatian didapati kebanyakkannya adalah dihidrochalkon.

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I certify that a Thesis Examination Committee has met on 29 July 2011 to conduct the final examination of Darab Yazdani on his thesis entitled "Characterisation of A*spergillus* contaminants of milled rice and antitoxic effects of Malaysian ethnomedicinal plant extracts on growth suppression" in accordance with the Universities and University College 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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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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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 institutions.

DARAB YAZDANI

Date: 29 July 2011

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LIST OF ABBREVIATIONS

µL	micro liter
µM	micro molar
λ_{max}	Lambda max is the wavelength at which the maximum fraction of light is absorbed by a solution
AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
ANOVA	analysis of variance
APCI	atmospheric-pressure chemical ionization
bp	base pair
CS20%	Czapek Dox agar +20% sucrose
CDA	Czapek Dox agar
CYA	Czapek yeast extract agar
DD	distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
FOC	flora Of China
GAP	good manufacturing practices
HACCP	hazard analysis and critical control point
HPLC	high performance liquid chromatography
HPTLC	high performance thin-layer chromatography
ITS	internal transcribed spacer
L	liter
LC-MS	liquid chromatography-mass spectrometry
M	molar; molarity
Mb	mega base pair

MEA	malt extract agar
MEGA	molecular evolutionary genetics analysis
MeOH	methanol
MESA	malt extract salt agar
MFC	minimum fungicidal concentration
MIC	minimum inhibitory concentration
mL	milliliter
mM	millimolar
MS	mass spectrophotometry
MS^2	Tandem mass spectrometry (MS/MS)
m/z	mass-to-charge ratio
N	nitrogen
NaOH	sodium hydroxide
ng	nanogram
nm	nanometer
O_5O_4	Osmium tetoroxide
OTA	Ochratoxin A
PCIA	phenol: chlorofom: isoamyl alcohol
PCR	polymerase chain reaction
PDA	potato dextrose agar
PDB	potato dextrose broth
rDNA	ribosomal deoxyribonucleic acid
Rf	retention factor
RT	retention time
SEM	scanning electron microscope
Taq polymerase	a thermostable DNA polymerase
TBE buffer	a mixture of Tris base, boric acid and EDTA
TLC	thin-layer chromatography
Tris	Tris (hydroxymethyl) aminomethane

UNiCC	Microbial Culture Collection Unit, UPM
UPM	Universiti Putra Malaysia
UV	ultraviolet radiation
YES	Yeast Extract Sucrose agar

CHAPTER 1

INTRODUCTION

Aspergillus is a diverse anamorphic genus consisting approximately 250 known species covering nine distinct teleomorphic genera. Genera are categorized into Subgenera and Sections according to anamorph and teleomorph characteristics and relationships (Geiser *et al.,* 2008). *Aspergillus* species are responsible for production of several toxins, including aflatoxins and ochratoxin A (OTA) (Varga and Samson, 2008). Contamination of foods by mycotoxins is an important food safety concern for grains and other agricultural products (Buzby, 2003). Rice (*Oryza sativa* L.) is one of the most important staple food crops in Malaysia. About 672 304 hectares of rice are grown in Peninsular Malaysia (FAO, 2010). Several *Aspergillus* species have been reported from rice including *Aspergillus niger, A. candidus, A. flavus, A. fumigatus* and *A. versicolor* (Udagawa, 1976). Traditional methods for identification of *Aspergillus* are being supplemented by molecular approaches. Although molecular methods continue to improve and the advantages of PCR based identification are obvious, microscopy and culturing remain the primary laboratory tools for detecting *Aspergillus* (Mc Clenny, 2005). However, morphological characters are not stable, because some morphological features are not always present in all isolates of a species, and also their presence can vary among cultures of the same isolate. Physiological characters may vary, some metabolites may be

absent altogether in some isolates. DNA sequence data are very useful for recognizing species, but there is not strict criteria as to where to draw the line between phylogenetic species. Therefore, there is no one method (morphological, physiological or molecular) that works perfectly in recognizing *Aspergillus* species (Geiser *et al.,* 2007).

Aflatoxins are produced by several species of *Aspergillus* and are not produced by other fungi (Frisvad *et al.,* 2007). Chemically, there are four major aflatoxins, B1, B2, G1 and G2. OTA is a nephrocarcinogenic and teratogenic mycotoxin that has been detected in several food products (Joosten *et al.*, 2001; Nguyen *et al.,* 2007; Kumar *et al.,* 2008). Therefore, there is a need for screening their toxin production abilities. Several methods were described as techniques suitable for detection of mycotoxins produced by *Aspergillus* in culture media (Davis *et al.,* 1966, 1987; Saito and Machida, 1999; Fente *et al*., 2001; Kumar *et al*., 2007) but the efficacy of these techniques has not been proven for all *Aspergillus* species. Another simpler technique described by Filtenborg *et al.* (1983) for detecting aflatoxins and OTA in pure culture is the agar plug sampling TLC method. TLC, also known as flat bed chromatography is one of the most widely used separation techniques in mycotoxin analysis. The aflatoxins are well suited for analysis by TLC since most of the compounds fluoresce strongly under long-wave UV light. Besides TLC many analytical and immunological methods are available for estimation of aflatoxin such as HPLC.

Many chemicals, including acetic, formic and propionic acids, fungicides and natural oils have been evaluated for the prevention of *Aspergillus* but have often been unsuccessful when applied to grain (Chelkowski, 1991). In recent years, public pressure to reduce the use of synthetic fungicides in agriculture has increased (Abad *et al*., 2007).

The hypothesis of this study is that plant extracts have potential for suppression of growth and toxin production by *Aspergillus* contaminants in milled rice. The main objective of this study was to identify *Aspergillus* contaminants in milled rice and their toxin inhibition by ehnomedicinal plant extracts in Malaysia.

The specific objectives of this study were as follows:

(1) To isolate and identify *Aspergillus* species from rice samples in Peninsular Malaysia based on morphological characteristics and molecular methods.

(2) To screen *Aspergillus* isolates with ability of toxin production using cultural and chromatography techniques.

(3) To screen plants species in Malaysia with previous ethnobotanical records for antifungal and antitoxic properties against *Aspergillus* using Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of extracts.

(4) To isolate major fractions exhibiting the most antifungal activity potential and identify the chemical constituents.

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LIST OF PUBLICATION

Articles:

1. Yazdani, D., Zainal Abidin, M. A., Tan, Y. H. and Kamaruzaman, S. (2010). Evaluation of the detection techniques of toxigenic *Aspergillus* isolates. *African Journal of Biotechnology*, Vol. 9(45), pp. 7654-7659
2. Yazdani, D., Zainal Abidin, M.A.;Tan, Y.H. and Kamaruzaman, S.(2011) Molecular identification of A*spergillus* and *Eurotium* species isolated from rice and their toxin- producing ability. *Microbiology, Vol. 80(5),* pp. *720-727.*
3. Yazdani, D., Zainal Abidin, M.A.; Tan, Y.H. Jaganath, I.B. (2011). A review on bioactive compounds isolated from plants against plant pathogenic fungi. Submitted to *Journal of Medicinal Plants Research.*
4. Yazdani, D., Zainal Abidin, M.A.;Tan, Y.H. and Kamaruzaman, S.(2011) Isolation and identification of *Eurotium* species from contaminated rice by morphology and DNA sequencing .Submitted to Brazilian Journal of Microbiology
5. Yazdani, D., Zainal Abidin, M.A.; Tan, Y.H., Kamaruzaman, S and Jaganath, I.B.(2011) Phytochemicals from ethnomedicinal plants used in Malaysia against *Aspergillus flavus* involved in toxin production. Under revision in *Natural Product Research.*
6. Yazdani, D., Zainal Abidin, M.A.; Tan, Y.H., Kamaruzaman, S and Jaganath, I.B.(2011) Inhibition of aflatoxin biosynthesis in *Aspergillus flavus* by *Piper betle* phytochemicals. Under editing by co- authors for submission in *Natural Product communication.*

Proceedings:

1. Yazdani, D., Zainal Abidin, M.A.;Tan, Y.H. and Kamaruzaman,S.(2009). A rapid identification technique for aflatoxin producing *Aspergillus* strains using Thin Layer Chromatography (TLC). *International Congress of Malaysian Society for Microbiology,* 1-4 December 2009, Penang, Malaysia.
2. Yazdani, D., Zainal Abidin, M.A., Tan, Y.H. and Kamaruzaman, S. (2010). Toxin producing ability of *Aspergillus* and *Eurotium* species isolated from rice. *Biomalaysia 2010 Conference & Exhibition*, 1-3 November, Kuala Lumpur Convention Center, Malaysia

3. Yazdani, D., Zainal Abidin, M.A., Tan, Y.H. and Kamaruzaman, S. (2010). Aflatoxin- producing potential of *Aspergillus* and *Eurotium* isolated from rice. *ISSAAS International Congress*, 14-18 November, Inna Grand Bali Beach Hotel, 14-18 November 2010, Bali, Indonesia