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ANTIBACTERIAL ACTIVITIES OF Aspergillus fumigatus SSH01 CRUDE EXTRACTS

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By

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ANTIBACTERIAL ACTIVITIES OF *Aspergillus fumigatus* SSH01 CRUDE EXTRACTS

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Antimicrobial agents are mainly derived from natural products. The scientific evidence for the various antimicrobial activities from thermotroph, as referred here, thermotolerant *Aspergillus fumigatus* SSH01 is scarce and fragmented. Additionally, bacterial resistance to antibiotics is a public health problem that is increasing around the globe which later complicated illnesses. Therefore, the main objective of this study was to determine the antibacterial activities of fungal crude extracts and to investigate the modes of action of the extracts against susceptible pathogens. Methanol was used to extract metabolites from *A. fumigatus* SSH01 by submerged fermentation. The extract was investigated for its antimicrobial properties against pathogenic Gram-positive bacteria (methicillin-resistant *Staphylococcus aureus* S547, *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633 and *Listeria monocytogenes* L10) and pathogenic Gram-negative bacteria (*Escherichia coli* ATCC 8739, *Escherichia coli* 0157: H7 E187, *Salmonella enterica* serovar Thypimurium S836 and *Pseudomonas aeruginosa* ATCC 15442) as well as yeast, *Candida albicans* ATCC 10231 using disc diffusion method. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extract were determined by broth microdilution method. Time-killing kinetics, scanning electron microscopy (SEM), and detection of cellular leakage of proteins and 260_{nm} absorbing-compounds of susceptible Gram-positive pathogens were determined after exposure of bacterial cells to three concentrations (1/2, 1, and 2MIC) of 100% of methanolic crude extracts. The methanolic extract exhibited zone of inhibition ranged from 11.00 mm to 18.00 mm. The MIC values ranged from 0.097 mg/ml to 12.50 mg/ml whereas the MBC values ranged from 0.195 mg/ml to 25 mg/ml. The growth of all treated test microorganisms were inhibited at the concentration of twice MIC values in time-kill assay. Average log reduction of bactericidal effects (MIC and 2MIC) in viable cell count ranged from 7.75 Log_{10} to 5.52 Log_{10} CFU/ml and between 7.72 Log_{10} to 6.36 Log_{10} CFU/ml in MIC and 2MIC of the extracts, respectively. Detection of cellular leakage of proteins (595_{nm}) and 260_{nm} absorbing-compounds showed slightly increased to the time of exposure of cells to the extracts and suggesting its ability to damage the bacterial membrane. In addition, scanning electron micrograph (SEM) demonstrated disruption in morphology of the treated cells. Liquid chromatography/time-of-flight/mass spectrophotometry (LC/TOF-MS) was used to analyze the constituents of the extracts. Six metabolites were identified included L-tyrosine, kojic acid, fumagillin, fumigaclavine B, helvolic acid and citrinin. In MTT assay, SSH01 crude extract was found to be cytotoxic against HaCat with IC_{50} of 20 mg/ml and 0.78 mg/ml after 24 and 48 h of treatment. Further studies maybe required to explain the risk of toxicity (kojic acid and citrinin) of the crude extracts and its stability for the application of human use. The constituents of the extracts should also be isolated, identified, characterized perhaps improvise for better potential as antibacterial drugs.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

AKTIVITI ANTIBAKTERIA DARIPADA EKSTRAK MENTAH *Aspergillus fumigatus* SSH01

Oleh

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Ejen antimikrob kebanyakannya diperolehi daripada produk semula jadi.Walau bagaimanapun, bukti saintifik untuk aktiviti antibakteria dari termotrof, *Aspergillus fumigatus* SSH01 yang mampu bertoleransi terhadap haba adalah terhad dan tidak cukup. Tambahan, rintangan bakteria terhadap antibiotik merupakan masalah kesihatan awam yang semakin meningkat di seluruh lapisan dunia seterusnya merumitkan lagi sesuatu penyakit. Oleh itu, objektif utama kajian ini adalah untuk menentukan aktiviti antibakteria daripada ekstrak mentah kulat dan menentukan mod tindakan ekstrak terhadap patogen. Metanol telah digunakan untuk mengekstrak metabolit daripada *A. fumigatus* SSH01 menggunakan penapaian kultur tenggelam. Sifat-sifat antimikrob ekstrak telah disiasat ke atas Gram-positif patogenik (rintang methisilin *Staphylococcus aureus* S547, *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633 dan *Listeria monocytogenes* L10) dan bakteria Gram-negatif patogenik (*Escherichia coli* ATCC 8739, *Escherichia coli* 0157: H7 E187, *Salmonella enterica* serovar Thypimurium S836 dan *Pseudomonas aeruginosa* ATCC 15442) serta yis, *Candida albicans* ATCC 10231 menggunakan kaedah sebaran cakera. Nilai kepekatan perencatan minimum (MIC) dan kepekatan bakterisidal minimum (MBC) ekstrak ditentukan dengan menggunakan kaedah mikro-pencairan. Kinetik masa-pembunuhan, imbasan mikroskop elektron (SEM), ukuran kebocoran sel terhadap protein dan penyerapan pada jarak gelombang 260_{nm} oleh patogen Gram-positif ditentukan selepas pendedahan sel-sel bakteria untuk tiga kepekatan (1/2, 1, dan 2MIC) daripada 100% ekstrak mentah metanol *A. fumigatus* SSH01. Ekstrak metanol mempamerkan zon perencatan diameter dari 11.00 mm hingga 18.00 mm dan terbatas kepada bakteria Gram-positif sahaja. Nilai MIC adalah di antara 0.097 mg/ml hingga 12.50 mg/ml manakala nilai MBC adalah antara 0.195 mg/ml hingga 25 mg/ml. Pertumbuhan semua mikroorganisma ujian direncat pada kepekatan nilai dua kali MIC dalam kinetik masa-pembunuhan. Purata pengurangan log bagi kesan bakterisidal (MIC dan 2MIC) sel yang hidup adalah dari Log_{10} 7.75 kepada Log_{10} 5.52 CFU/ml dan di antara Log_{10} 7.72 kepada Log_{10} 6.36 CFU/ml bagi MIC dan 2MIC daripada ekstrak. Analisis bagi ukuran kebocoran sel terhadap protein dan sebatian yang menyerap gelombang pada 260_{nm} menunjukkan sedikit peningkatan selepas pendedahan sel kepada ekstrak dan

disimpulkan bahasawanya ia mampu merosakkan membran bakteria. Di samping itu, imbasan elektron mikroskopi (SEM) menunjukkan perubahan morfologi terhadap sel-sel yang diuji. “Liquid chromatography/time-of-flight-mass spectrometry” (LC/TOF-MS) telah digunakan untuk menganalisis juzuk-juzuk ekstrak. Enam metabolit telah dikenal pasti termasuklah L-tirosin, asid kojik, fumagillin, fumigaclavine B, asid helvolik dan citrinin. Berdasarkan dari ujian MTT, ekstrak mentah SSH01 didapati memberi kesan sitotoksik terhadap HaCat dengan nilai IC_{50} 20 mg/ml dan 0.78 mg/ml selepas 24 dan 48 jam. Kajian lanjut mungkin diperlukan untuk menerangkan risiko ketoksikan (asid kojik dan sitrinin) daripada ekstrak mentah dan kestabilan bagi kegunaan manusia. Juzuk daripada ekstrak juga perlu diasingkan, dikenalpasti dan ditambah baik supaya berpotensi digunakan sebagai ubatan antibakteria.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
BLAST	Basic Local Alignment Search Tool
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Unit
cm	Centimeter
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
ESBL	Extended-spectrum Beta-Lactamase
g	Gram
GC-MS	Gas Chromatography-Mas Spectrometry
HIV	Human Immunodeficiency Virus
HPLC	High-Performance Liquid Chromatography
ITS	Internal transcribed spacer
L	Liter
lat.	Latitude
LC/TOF-MS	Liquid-Chromatography/Time-of-Flight Mass Spectrometry
Log	Logarithm
long.	Longitude
M	Molarity
MBC	Minimum Bactericidal Concentration
MDR	Multi-drug Resistance
mg	Milligram
MHA	Mueller-Hinton Agar
MHB	Mueller-Hinton Broth
MIC	Minimum Inhibitory Concentration
min	Minute
ml	Milliliter
mm	Millimeter
mRNA	messenger Ribonucleic Acid
MRSA	Methicillin-resistant *Staphylococcus aureus*
MTT	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium Bromide
NCBI	National Center of Biotechnology Information
ND	Not Determined
NI	No Inhibition
NIST	National Institute of Standards and Technology
nm	Nanometer
OD	Optical Density
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
pH	Exponential of Hydrogen Ion
rpm	Revolution per Minute
rRNA	Ribosomal Ribonucleic Acid
SD	Standard Deviation
SEM	Scanning Electron Microscopy

sp.	Species
tRNA	transfer Ribonucleic Acid
UV	Ultraviolet
VRE	Vancomycin-resistant Enterococci
VRSA	Vancomycin-resistant *Staphylococcus aureus*
%	Percentage
µ	Micro
°C	Degree Celsius
α	Alpha
β	Beta
(v/v)	Volume per volume

CHAPTER 1

INTRODUCTION

For thousand years, natural products have played such crucial role worldwide in treating and preventing human diseases (Chin et al., 2006). The drugs production from the natural product is mainly produced by microbes. Of 23000 active compounds discovered from microorganisms (antimicrobial, antiviral, cytotoxic and immunosuppressive compounds), 42% are isolated from fungi while 32% by filamentous bacteria, the actinomycetes (Demain, 2014). In addition, 20% of antibiotics found contributed by fungi, 10-15% by non-filamentous bacteria while over half of it are from actinomycetes and these compounds are semi-synthetic derivatives from novel. Penicillin, cephalosporins, tetracycline, aminoglycosides, macrolides, ansamycins, polyenes and glycopeptides are examples of antimicrobial agents produced by chemistry and bioconversion (Demain, 2014).

However, the cost of healthcare including morbidity and mortality has increased due to the emergence of antibiotic-resistant (Deutscher and Friendman, 2010). Malaysia, the 67th largest country by total land area is facing the same issues like elsewhere in Africa, America, Europe, and Eastern Mediterranean Region (WHO, 2015). In 2005, Malaysia faced prevalence of multi-drug resistance (MDR) of nosocomial infections caused by *Pseudomonas aeruginosa* strains with 6.9% higher than Japan (2.8%) in Asia-Pacific (Tsuji et al., 2005; Raja and Singh, 2007; Giske et al., 2008). A study that was carried out by Pathmanathan et al. (2009), showed that 25 out of 97 clinical *Pseudomonas aeruginosa* isolated from Hospital Kuala Lumpur were resistant towards at least one of antimicrobial agents used (cephalosporin, carbapenem, aminoglycoside and quinolone).

To date, it is reported that majority of *Enterobacteriaceae* and *P. aeruginosa* exhibited multiple resistance to ampicillin, augmentin, cefuroxime and ceftriaxone (Kor et al., 2013). Gould et al. (2014) stated that emergence of extended-spectrum beta-lactamase (ESBL) Gram-positive bacteria, as well as multidrug-resistant *Pseudomonas* has been unchanging for years. In addition, the incidence of drug resistance in *Acinetobacter* against nearly all major antibiotics is increasing.

Fungi provide such priceless secondary metabolites values in potential biomedical applications (Gao et al., 2012). It produces a great variety of unique natural products ranging from peptides to alkaloids to terpenes and polyketides (Keller et al., 2005). Penicillin and cephalosporins are the best-known metabolites from fungi that serve as antimicrobial agents in pharmacology (Gloer, 2007). The production of fungal metabolites is renewable, modifiable and desirable. It can be produced in large scale (Gloer, 2007), improved and modified structurally by the genetic mutation, and the production can be increased by optimization of medium and culture conditions (Masurekar, 2005; Aly et al., 2011). Nonetheless, data on natural products from thermotolerant for biological activities are still fragmented. Its useful thermostable enzymes probably the major reason why it is often to be reported as prior candidates in industries.

Taken together, the research in the field of natural products isolated from thermotolerant fungal isolates perhaps could serve the humans demand to overcome the emerging and known diseases. Thus, this study was performed to investigate the antimicrobial activity of fungal crude extracts. The objectives of the study were:

1. To screen for antimicrobial properties of methanolic fungal crude extracts
2. To determine the modes of action of fungal crude extracts against susceptible test pathogens
3. To identify the fungal isolate and its metabolites as well as to perform the cytotoxicity of the crude extract

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