UNIVERSITI PUTRA MALAYSIA

IN VITRO STUDY OF ANTIFUNGAL EFFECT OF ALLICIN ON Aspergillus fumigatus ATCC 36607 GROWTH

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By

NURHAYATIE BINTI SAJALI

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March 2014

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Aspergillusfumigatus is a saprophytic fungus and it commonly affects immunocompromised hosts, leading to the development of invasive pulmonary aspergillosis. Mortality and morbidity rates due to aspergillosis are escalating over the past decades and the available commercial drugs come with adverse drug reactions. The fungal cell wall serves as an excellent antifungal drug target for development of new drugs due to its physiological features in providing mechanical strength, maintaining cell shape and integrity. Allicin, a pure compound extracted from garlic, has been proven to exhibit antimicrobial properties on a variety of microorganisms and the effect against *A. fumigatus*is of interest. The aims of this study were to examine the antifungal effect of allicin on *A. fumigatus*ATCC 36607, to assess the morphological alteration of the fungal cell surface and evaluate the expression pattern of cell wall related genes of *A. fumigatus*post treatment with allicin. The minimum inhibitory concentration (MIC) of allicin in *A. fumigatus*was determined by broth microdilution method according to the "Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi" (CLSI M38-A2) guidelines. The minimum fungicidal concentration (MFC) was determined by plating suspensions from optically clear wells from 96 well microtiter plate obtained in antifungal susceptibility testing onto Sabouraud dextrose agar (SDA). Changes in ultrastructure on the cell surface were observed through scanning electron microscopy (SEM) at 1× MIC (3.2 µg/ml), 2× MIC (6.4 µg/ml) and sub-MIC concentrations which were ¼× MIC (0.8 µg/ml) and ½× MIC (1.6 µg/ml) respectively. Additionally, *A. fumigatus*was incubated at predetermined time points within 24 h period at 3.2 µg/mlin time kill assay. For gene expression analysis, *A. fumigatus*was incubated at MIC (1× MIC) and sub-MIC concentrations (¼× MIC and ½× MIC). RNA was then extracted and converted to cDNA. Amplification efficiency for each primer set was evaluated from 5-fold serial dilutions of cDNA. Gene expression was evaluated using Pfaffl method and statistical analysis was performed

through Kruskal Wallis test. The present finding indicates that the MIC and MFC for allicin were both 3.2 µg/mL. Quantitative data in the form of optical density (OD) obtained indicated that $p<0.05$ at MIC value in comparison with untreated (growth) control. Complete suppression of hyphae formation at 3.2 µg/mL and reduced mycelial growth at 1.6 µg/mL and 0.8 µg/mL were observed through SEM. Alternatively, when *A. fumigatus*was incubated with 3.2 µg/mL allicin in the time kill assay, the inhibitory effect of allicin was observed after 12 h incubation. Down regulation pattern of *chsE, chsG, fksA, gelB, rhoA*and*rhoB*genes were observed at MIC and sub-MIC concentrations of allicin tested. With the exception for *rhoA* and *rhoB*, statistical analysis showed significant differences in the level of down regulation ($p<0.05$) at MIC and sub-MIC concentrations of allicin for *chsE, chsG, fksA*, and *gelB*indicating significant effect on the expression of the genes during exposure to allicin. On the contrary, *rhoD* showed an up regulation expression pattern which significantly differed at MIC and sub-MIC concentrations of allicin ($p<0.05$). As a whole, the present finding strongly implied that allicin exerts its antifungal activity against *A. fumigatus*by inhibiting the fungal cell proliferation, hindering transformation of the conidia into hyphae and down regulation of cell wall related genes.

Abstraktesis yang dikemukakankepadaSenatUniversiti Putra Malaysia sebagaimemenuhikeperluanuntukIjazahSarjanaSains.

KAJIAN *IN VITRO* BERKENAAN KESAN ALLICIN SEBAGAI ANTIKULAT TERHADAP PERTUMBUHAN*Aspergillusfumigatus*ATCC36607

Oleh

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*Aspergillusfumigatus*ialahsejeniskulatsaprofit yang seringmenjangkitipesakit yang kurangdayatahanimunseterusnyamenyebabkanjangkitanpenyakit yang dikenalisebagaiaspergillosispeparuinvasif.Sejakbeberapadekad yang lalu, kadarkematiandanmorbiditisemakinmeningkatsertapenggunaanubat yang telahdikomersilkanmemberikesanburukterhadappesakit. Di sampingitu, dindingselmerupakansalahsatusasaranantikulatterbaikdisebabkanolehciri-cirifisiologinya yangmemberikankekuatanmekanikaldanmengekalkanbentuk sel. Ekstraktulendaripadabawangputih yang dikenalisebagaiallicintelahterbuktimerencatkanpertumbuhankebanyakanmicroorganis madankesanterhadap*A. fumigatus*merupakansesuatu yang pentinguntukdikajidenganlebihmendalam. Objektifkajianiniadalahuntukmengkajikesanallicinsebagaiantikulatterhadap*A. fumigatus*ATCC 36607, perubahanmorfologipadapermukaanselkulatdanmenilaiekspresi gen yang terlibatdalampembentukandindingselselepas 48 jam pengeramandenganallicin. Kepekatanperencatan minimum (MIC) untukallicinterhadap*A. fumigatus*ditentukanmelaluikaedahkaldupencairanmikroberdasarkangarispanduandari padaujiansensitivitiantikulatuntukkulatberfilamen (CLSI M38-A2).Kepekatanmembunuhkulat minimum (MFC) pula ditentukanmelaluikaedahpenyebaran medium yang tidakditumbuhikulat yang diperolehidaripada plat-mikrotiter 96-perigimelaluiujiansensitivitiantikulatdandisebarkanpadapermukaan agar Sabouraud dextrose (SDA).Perubahanultrastrukturpadapermukaanselkulatpadakepekatan1× MIC (3.2 µg/ml), 2× MIC (6.4 µg/ml) dankepekatan sub-MIC iaitu ¼× MIC (0.8 µg/ml) dan ½× MIC (1.6 µg/ml) diperhatikan di bawahimbasanmikroskopelektron (SEM) setelahdiproses. Di sampingitu, *A. fumigatus*jugadieramkanpadaselangmasa yang telahditetapkanselama 24 jam pada3.2 µg/ml melaluikaedah “time kill assay”.

Bagianalisisekspresi gen, *A. fumigatus*dieramkanpadakepekatan 1× MIC dan sub-MIC iaitu ¼× MIC dan ½× MIC. Kemudian, RNA diekstraksebelumditukarkankepadacDNA.Keberkesananamplifikasiuntuksetiap set primer ditentukandenganmemplotgarisan linear berdasarkan 5 siripencairancDNA. Ekspresi gen dinilaidenganmenggunakankaedahPfaffldananalisisstatistik "Kruskal Wallis". Kajianinimenunjukkankedua-dua MIC dan MFC bagiallicinialah 3.2 µg/mL.Data kuantitatifmenunjukkanp<0.05 padanilai MIC apabiladibandingkandengansampelkawalan.Perencatanpertumbuhanhifatelahdilaporkanpadakepekatan 3.2 µg/mL manakalapadakepekatan 1.6 µg/mL dan 0.8 µg/mL kepadatanpertumbuhanjaringanhifasemakinberkurang.Selaindaripadaitu, apabila*A. fumigatus*dieramkanpadakepekatan3.2 µg/mL mengikutkaedah "time kill assay", perencatanpertumbuhandiperhatikanselepaspengeramanselama 12 jam. Penurunanekspresi gen padakepekatan MIC dan sub-MIC allicindilihatpada*chsE*, *chsG*, *fksA*, *gelB*, *rhoA*dan*rhoB* gen. Analisisstatistikmenunjukkanperbezaanpadatahappenurunanekspresi gen (p<0.05) padakepekatan MIC dan sub-MIC allicinuntuk*chsE*, *chsG*, *fksA*, and *gelB*di manakeadaaninimenunjukkanperbezaankesanpendedahanpelbagaikepekatanallicinterhadap gen dinding sel. Walaupunbegitu, dua gen iaitu*rhoA*dan*rhoB*tidakmenunjukkankesanperbezaanselepaspendedahanpadakepekatan MIC dan sub-MIC (p>0.05). Sebaliknya, *rhoD*menunjukkankenaikanpadaekspresi gen di manaterdapatperbezaanpadakepekatan MIC dan sub- MIC allicin (p<0.05).Kesimpulannya, kajianinimenujukkankesanantikulatolehekstrakallicinterhadap*A. fumigatus*di manaekstrakiniberupayamerencatkanpembahagianselkulat, mengelakkanpercambahan conidia kepadahifadanpenurunanekspresisesetengah gen dinding sel.

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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 **Master of Science**. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
CFU/ml	Colony forming unit per milliliter
CHS	Chitin synthase
ITS	Internal Transcribed Spacer
MFC	Minimum fungicidal concentration
MIC	Minimum inhibitory concentration
MOPS	3-[N-morpholino] propanesulfonic acid
NCBI	National Center for Biotechnology Information
NRT	Non reverse transcriptase control
NTC	Non template control
PDA	Potato Dextrose Agar
RPMI	Roswell Park Memorial Institute
SDA	Sabouraud Dextrose Agar
SEM	Standard error of mean
VPSEM	Variable pressure scanning electron microscope
C_q	Cycle threshold
Δ	Mutant

CHAPTER 1

INTRODUCTION

1.1 Introduction

Invasive fungal infection (IFI) has gained much attention since the past two to three decades. The most common IFI caused by *Aspergillus* genus is termed as invasive pulmonary aspergillosis (IPA). IPA was first described as opportunistic infection in 1953 (Rankin, 1953). The incidence of IPA has been escalating over the past two decades due to extensive use of immunosuppressive agents and chemotherapy, affecting majority of hematologic malignancy patients (Chamilos et al., 2006). In addition, IPA is a fatal infection associated with serious morbidity and mortality (Walsh et al., 2008).

Aspergillus fumigatus is the most common species that causes aspergillosis followed by other non-*fumigatus* species such as *A. flavus* and *A. terreus* (Warnock, 2007; Lass-Flörl et al., 2005). *A. fumigatus* is a saprophytic mold, found abundantly in decaying organic matter and soil. It produces plentiful miniature airborne spores or conidia that can endure extensive range of environmental conditions. Inhalation of these minute conidia by healthy individual is not detrimental as it will be eradicated by defense mechanisms in respiratory system. However, the conidia could persist and subsequently germinate into invasive hyphae leading to development of IPA in immunocompromised patients (Balloy & Chignard, 2009).

The success of *A. fumigatus* species as a human fungal pathogen is attributed to its various putative virulence factors such as production of conidia, hyphae, proteolytic enzymes activities, and even some toxins (such as gliotoxin) (Tomee & Kauffman, 2000). Additionally, the cell wall of *Aspergillus*, like most fungi, provides shape, mechanical strength and shields the fungi from adverse environmental conditions. It acts as communication layer between the internal and external environmental states. Disruption of cell wall affects the fungal cell's physiology which makes it susceptible to cell lysis. Hence, the fungal cell wall is an excellent antifungal drug target given its essential physiological role in fungal survival (Bowman and Free, 2006).

Garlic or scientifically known as *Allium sativum* has been in use since many years ago as a traditional remedy to cure various kinds of ailments. However, it was not until 1944 when two scientists discovered allicin, one of the active compounds from garlic which possesses antibacterial potential (Cavallito and Bailey, 1944). Apart from inhibiting bacteria growth, allicin has been studied extensively for other biotherapeutic applications. Allicin demonstrated various antimicrobial effects including antifungal property (Khodavandi et al., 2011a), antischistosomal activity (Lima et al., 2011) as well as antimalarial activity (Coppi et al., 2006).

In the recent past, amphotericin B deoxycholate was regarded as the "gold standard" for IFI therapy despite its toxicities and other potential side effects. Lipid preparation of amphotericin B has been regarded as the new "gold standard" in place of amphotericin B deoxycholate and this phenomenon has led to major shift in polyene therapy (Ostrosky-Zeichner et al., 2003). Nonetheless, several reasons have impeded the use of this formulation as an option for aspergillosis therapy. Scarcity of published safety data to guide clinicians and higher cost are two common causes that withhold the usage of the drug (Kleinberg, 2006).

Alternatively, other classes of commercial drug such as echinocandins have been developed. Although adverse effect of this drug to humans is mild compared to amphotericin B deoxycholate, its usage has been hampered due to compensatory mechanism which compensates for the loss of β-(1,3)-glucan and reduces the effectiveness of echinocandins in fungal cell wall and mutation in sequences of amino acids in some clinical isolates. This mechanism implied that there is an adaptive response by the fungi in ensuring their survival following the administration of this drug (Munro, 2010). However, echinocandin resistant in *Aspergillus* spp has only been observed in laboratory strains (Gardiner et al., 2005).

Despite current advances in the development of therapeutic antifungal agents to treat aspergillosis, exploring natural products to serve as an antifungal agent that exhibits fewer side effects, toxicities and possesses prophylactic potential against IPA is warranted. To date, there are few studies done on the potential antifungal effects of allicin on *A. fumigatus*. The mechanism of action in which the compound exerts its inhibitory or suppressive effects on *A. fumigatus* growth has also yet to be elucidated.

1.2 Objectives

The general objective of this study was to investigate the antifungal effect of allicin on *Aspergillus fumigatus* ATCC 36607.

In addition, the specific objectives of this study are listed below:

1. To determine the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of allicin against *A. fumigatus* ATCC 36607.
2. To examine the ultrastructural changes of *A. fumigatus* ATCC 36607 treated with allicin.
3. To determine the gene expression pattern of selected cell wall associated genes of *A. fumigatus* ATCC 36607 upon exposure to allicin.

1.3 Hypothesis

A. fumigatus growth will be inhibited by allicin.

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