ฤทธิ์ต้านเชื้อรา *CANDIDA ALBICANS* ของสารสกัดจากข่าเล็ก *ALPINIA OFFICINARUM* HANCE

นางคีตยา ฮูเวอร์์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาชีวเวชศาสตร์
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2556

ANTIFUNGAL ACTIVITY OF *ALPINIA OFFICINARUM* HANCE AGAINST *CANDIDA ALBICANS*

Keetaya Hoover

A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Master of Science in Biomedical Sciences

Suranaree University of Technology

Academic Year 2013

ANTIFUNGAL ACTIVITY OF *ALPINIA OFFICINARUM* HANCE AGAINST *CANDIDA ALBICAN*S

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

Thesis Examining Committee

_____
(Asst. Prof. Dr. Rungrudee Srisawat)
Chairperson

_____
(Assoc. Prof. Dr. Nuannoi Chudapongse)
Member (Thesis Advisor)

_____
(Dr. Nawarat Nantapong)
Member

_____
(Dr. Pongrit Krubphachaya)
Member

_____
(Prof. Dr. Sukit Limpijumnong)
Vice Rector for Academic Affairs and Innovation

_____
(Assoc. Prof. Dr. Prapun Manyum)
Dean of Institute of Science

คีตยา ฮูเวอร์ : ฤทธิ์ต้านเชื้อรา *CANDIDA ALBICANS* ของสารสกัดจากข่าเล็ก *ALPINIA OFFICINARUM* HANCE (ANTIFUNGAL ACTIVITY OF *ALPINIA OFFICINARUM* HANCE AGAINST *CANDIDA ALBICANS*) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.นวลน้อย จูฑะพงษ์, 81 หน้า.

ข่าเล็กถูกนำมาใช้เป็นยาแผนโบราณเพื่อรักษาโรคต่างๆ ในทวีปเอเชียมาเป็นเวลานาน สารสกัดจากข่าเล็กถูกพบว่ามีฤทธิ์ต้านเชื้อแบคทีเรีย แต่ยังไม่เคยมีรายงานถึงฤทธิ์ในการต้านเชื้อรา การศึกษานี้จึงมีวัตถุประสงค์เพื่อทดสอบฤทธิ์ต้านเชื้อรา *Candida albicans* ของสารสกัดจากเหง้าของข่าเล็ก เคยมีรายงานว่า galangin เป็นสารประกอบหลักกลุ่มฟลาโวนอยด์ที่พบในข่าเล็ก จากการวิเคราะห์ด้วยโครมาโทกราฟีสมรรถนะสูงในการศึกษานี้พบว่า สารสกัดข่าเล็กที่ใช้ 80% อะซิโตนสกัดมีปริมาณ galangin อยู่ 3.38 มิลลิกรัมต่อกรัม และจากการตรวจสอบด้วยวิธี disc diffusion พบว่าสารสกัดข่าเล็กมีฤทธิ์ต้านเชื้อรา *C. albicans* ถึงแม้ว่า galangin จะสามารถยับยั้งการเจริญของเชื้อราชนิดนี้ได้ แต่จากการทำ bioautography ทำให้ทราบว่า galangin ไม่ใช่สารออกฤทธิ์หลักในการต้านเชื้อราชนิดนี้ เนื่องจากปริมาณของ galangin ที่มีอยู่ในสารสกัดต่ำมาก ความเข้มข้นต่ำสุดของสารสกัดข่าเล็กที่สามารถยับยั้งการเจริญของเชื้อรา *C. albicans* (MIC) และความเข้มข้นต่ำสุดที่สามารถฆ่าเชื้อรา *C. albicans* (MFC) ได้ คือ 1.2 และ 2.0 มิลลิกรัมต่อมิลลิลิตร ตามลำดับ เมื่อสังเกตด้วยกล้องจุลทรรศน์แบบเฟสคอนทรัสต์และวัดการสร้างไบโอฟิล์มด้วยวิธี สเปกโทรสโกปี พบว่าสารสกัดสามารถยับยั้งการสร้างไบโอฟิล์มของเชื้อรา *C. albicans* ได้ ผู้วิจัยได้ทำการประเมินผลการทำลายเซลล์ของเชื้อราผ่านกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราด (SEM) เพื่อค้นหากลไกการออกฤทธิ์ในการต้านเชื้อรา *C. albicans* พบว่า สารสกัดทำให้เกิดการเปลี่ยนแปลงรูปร่างของเซลล์ โดยพบผิวเซลล์เกิดความขรุขระมากขึ้นเมื่อมีการเติมสารสกัดความเข้มข้นที่ MIC และเมื่อเลี้ยงเชื้อราด้วยด้วยอาหารเลี้ยงเชื้อที่มีสารสกัดข่าเล็กความเข้มข้นที่ MFC เซลล์จะเปลี่ยนแปลงรูปร่างจากวงรีไปสู่รูปร่างที่กลมมากกว่าเดิม จากผลการทดลองนี้ชี้แนะถึงความเป็นไปได้ที่สารสกัดข่าเล็กมีฤทธิ์ต้านเชื้อราโดยการไปทำลายผนังเซลล์ ส่งผลทำให้เซลล์เปลี่ยนเป็นสเฟียโรพลาส และตายในที่สุด อย่างไรก็ตามกลไกการออกฤทธิ์ที่นำเสนอนี้ยังมีความจำเป็นต้องศึกษาทดลองต่อไปเพื่อยืนยันว่าเกิดการทำลายผนังเซลล์จริง นอกจากนั้นสารออกฤทธิ์ที่รับผิดชอบต่อฤทธิ์ต้านเชื้อราของสารสกัดก็ยังจำเป็นต้องมีการศึกษาเพิ่มเติมต่อไป

สาขาวิชาเภสัชวิทยา ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2556 ลายมือชื่ออาจารย์ที่ปรึกษา_____

KEETAYA HOOVER : ANTIFUNGAL ACTIVITY OF *ALPINIA OFFICINARUM* HANCE AGAINST *CANDIDA ALBICANS*. THESIS ADVISOR : ASSOC. PROF. NUANNOI CHUDAPONGSE, Ph.D. 81 PP.

ALPINIA OFFICINARUM HANCE / *CANDIDA ALBICANS* / ANTIFUNGAL / MIC/ MFC

Alpinia officinarum Hance has long been used as traditional medicine for many ailments in Asia. The extract of *A. officinarum* has been shown to possess antibacterial activity. However, antifungal activity of this plant has not been reported. The purpose of this study was to determine an antifungal potential of *A. officinarum* on *Candida albicans*. Galangin was reported to be a major flavonoid compound in the rhizomes of *A. officinarum*. In the present study, a content of galangin in 80% acetone extract of *A. officinarum* was 3.38 mg/g, analyzed by a high performance liquid chromatography (HPLC). By a disc diffusion method, it was found that the *A. officinarum* extract possessed antifungal activity against *C. albicans*. Although galangin inhibited growth of this fungus, the results from bioautography revealed that galangin was not responsible for this activity of *A. officinarum* rhizomes extract due to its very low amount in the extract. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of the extract were 1.2 and 2.0 mg/ml, respectively. An inhibitory effect of the extract on *Candida* biofilm formation was revealed by using phase contrast microscopy and spectrometry. The assessment of cell damage was conducted through scanning electron microscope (SEM) observation to find the mechanism underlying this antimicrobial activity. SEM analysis showed that

the extract induced deformation of *C. albicans*. The cells treated with MIC of the extract had coarse surface and changed from oval to rounder shape when treated with MFC. These results suggested that the extract possess'd antifungal activity possibly by damaging cell wall, causing *C. albicans* to form spheroplast, and finally death. However, this postulated mechanism, which contributes to an explanation for its antifungal activity against *C. albicans*, needs more experiments to confirm the actual disruption of yeast cell wall. Moreover, the isolation and identification of the active ingredient(s) responsible for this action need further investigation.

School of Phamacology Student's Signature_____

Academic Year 2013 Advisor's Signature_____

ACKNOWLEDGEMENTS

During my graduate study, I have been fortunate enough to receive help and encouragement from several people. I would like to recognize and sincerely thank them all for making my studies at the Suranaree University of Technology a pleasant one and making a completion of this degree possible:

Special thanks are given to Assoc. Prof. Dr. Nuannoi Chudapongse, my advisor, for her consistent supervision, advice encouragement, valuable suggestion and support throughout my project, and to Dr. Nawarat Nantapong for her kind help in giving me some technical advices regarding the methodologies I have followed.

I would like to thank all my thesis committees for their suggestions and criticisms and all lecturers in School of Pharmacology, Institute of Science, Suranaree University of Technology for all knowledge and every suggestion.

Lastly, I have a great pleasure in thanking my family and all friends for their being with me and for their continuous encouragement.

Keetaya Hoover

CONTENTS

Page

ABSTRACT IN THAI I

ABSTRACT IN ENGLISH II

ACKNOWLEDGEMENTS IV

CONTENTS V

LIST OF TABLES IX

LIST OF SCHEME X

LIST OF FIGURES XI

LIST OF ABBREVIATIONS XIII

CHAPTER

I INTRODUCTION 1

1.1 Background/Problem 1

1.2 Research objectives 4

1.3 Research hypotheses 4

1.4 Scope and limitations of the study 4

II LITERATURE REVIEW 5

2.1 *Candida albicans* 5

2.1.1 Taxonomy of *C. albicans* 6

2.1.2 Structure of *C. albicans* 6

2.1.3 Morphology of *C. albicans* 6

CONTENTS (Continued)

Page

2.1.4 Biofilm formation of *C. albicans* 9

2.1.5 Epidemiology of *C. albicans* 10

2.1.6 Virulence of *C. albicans* 14

2.1.7 Treatment of *C. albicans* 16

2.2 Antifungal agents 18

2.2.1 Azole antifungals 18

2.2.2 Mechanism of resistance to azoles 20

2.2.3 Glucan synthesis inhibitors (Echinocadins) 24

2.2.4 Polyenes 24

2.2.5 Mechanism of resistance to polyenes 26

2.2.6 Allylamines 26

2.2.7 Mechanism of resistance to Allylamines 27

2.3 *Alpinia officinarum* Hance (Lesser galangal) 27

2.3.1 General background of *A. officinarum* 27

2.3.2 The phytochemistry of *A. officinarum* 29

2.3.3 The biological activity of *A. officinarum* 30

III MATERIALS AND METHODS 32

3.1 Chemical sources 32

3.2 Plant material 33

3.3 Extraction 34

CONTENTS (Continued)

Page

3.4 Antifungal susceptibility testing.....35
3.5 Determination of minimum inhibitory concentration 35
3.6 Determination of minimum fungicidal concentration.....37
3.7 Isolation of galangin from the extract by thin layer chromatography (TLC) 37
3.8 Quantitative analysis of galangin in the extract of *A. officinarum* by high-performance liquid chromatographic (HPLC) method 37
3.9 Determination of inhibition of growth by bioautography.....38
3.10 Scanning electron microscopy (SEM) 38
3.11 Biofilm assay.....39
3.12 Statistical analysis 40
IV RESULTS.....41
4.1 Antifungal activity of *A. officinarum* extract 41
4.2 Isolation and quantitative analysis of galangin in the extract of *A. officinarum*.....41
4.3 Bioautography detection in TLC.....42
4.4 Minimum inhibitory concentration (MIC) of the extract.....46
4.5 Minimum fungicidal concentration (MFC) of the extract.....46
4.6 Morphological changes observed by SEM.....46
4.7 Effect of the extract on biofilm formation.....48

CONTENTS (Continued)

Page

V DISCUSSION AND CONCLUSION.....51

REFERENCES 55

APPENDIX.....73

CURRICULUM VITAE.....81

LIST OF TABLES

Table **Page**

3.1 List of chemicals 32

4.1 Antifungal activity of *A. officinarum* rhizome extract against *C. albicans* 41

LIST OF SCHEME

Scheme **Page**

3.1 Extraction of the rhizomes of lesser galangal 34

LIST OF FIGURES

Figure **Page**

2.1 Schematic diagram of the cell wall of *C. albicans* 7

2.2 Morphology of *C.albicans* forms.....7

2.3 Pseudomembrane candidosis 12

2.4 Denture stomatitis 12

2.5 Esophageal candidiasis 13

2.6 Clinical phenotype in the patient with extensive cadidiasis of nails 13

2.7 Pathogenesis of invasive candidiasis 14

2.8 Structures of representative antifungal agents 17

2.9 Mechanism of action of antifungal drugs 19

2.10 Mechanism by which microbial cells might develop resistance.....23

2.11 The interaction between amphotericin B and cholesterol in a phospholipid bilayer 25

2.12 Aboveground and underground portion of *A. officinarum* 28

2.13 Lesser galangal (*Alpinia officinarum*) appearing in nature.....28

2.14 Chemical structures of compounds isolated from the rhizomes of *A. officinarum* 29

3.1 Growth profiles of *C. albicans* incubated at 30 °C.....36

4.1 HPLC chromatograms of *A. officinarum* extract and standard galangin 43

4.2 The scraped area from TLC 44

LIST OF FIGURES (Continued)

Figure **Page**

3.1 Growth profiles of *C. albicans* incubated at 30 °C……...…….36

4.1 HPLC chromatograms of *A. officinarum* extract and standard galangin …..….43

4.2 The scraped area from TLC……...…….44

4.3 HPLC chromatogram of the acetone extract from silica gel……..……44

4.4 Representative image of TLC plates of the *A. officinarum*.……...……45

4.5 TLC-direct bioautography of *A. officinarum* extract against *C. albicans*.……45

4.6 The inhibitory effect of lesser galangal on growth of *C. albicans* (n = 3).....…46

4.7 MFC of the extract on *C. albicans*…….....47

4.8 SEM micrographs of *C. albicans* treated with the extract of *A. officinarum*....48

4.9 Microscopic images on biofilm formation of *C. albicans* treated with *A. officinarum* extract. 49

4.10 The inhibitory effect of the extract on *C. albicans* biofilm by spectroscopic analysis. 50

LIST OF ABBREVIATIONS

°C	=	Degree Celsius
DMSO	=	Dimethylsulfoxide
g	=	Gram
HPLC	=	High performance liquid chromatography
h	=	Hour
kV	=	Kilovolt
L	=	Liter
µg	=	Microgram
µg/ml	=	Microgram per milliliter
µl	=	Microliter
µm	=	Micrometer
mg	=	Milligram
mg/ml	=	Milligram per milliliter
ml	=	Milliliter
MIC	=	Minimum inhibitory concentration
MFC	=	Minimum fungicidal concentration
min	=	Minute
nm	=	Nanometer
No.	=	Number
/	=	Per

LIST OF ABBREVIATIONS (Continued)

%	=	Percent
rpm	=	Round per minute
SDA	=	Sabouraud dextrose agar
SDB	=	Sabouraud dextrose broth
SEM	=	Scanning electron microscopy
TLC	=	Thin layer chromatography
UV	=	Ultraviolet radiation

CHAPTER I

INTRODUCTION

1.1 Background/Problem

Candida albicans resides in a diversity of organ surfaces of a healthy host, such as oral cavity, digestive tract, skin and vagina. As an opportunistic pathogen, it can cause life-threatening systemic infection in patients who are severely immunocompromised due to cancer chemotherapy, organ or bone marrow transplantation and human immunodeficiency virus (HIV) infection, especially in HIV-infected patients (Calderone and Fronzi, 2001). *C. albicans* is an organism that is most often associated with serious fungal diseases among those in immune-compromised patients (Fridkin and Jarvis, 1996). Moreover, candidal vaginitis is predominantly caused by strains of *C. albicans* (Sobel *et al*., 2001), and appears to be a common problem both in immunocompetent and healthy women. Despite advances in antifungal therapies, the number of problems remains to be solved for most antifungal drugs available, including resistance (Sojakova, Liptajova, Borovsky, and Subik, 2004) and toxicity (Laniado-Laborin and Cabrales-Vargas, 2009). These problems lead to the need of novel, safe, and effective antifungal compounds.

Biofilms are aggregates of unicellular micro-organisms forming multicellular structures that adhere to surfaces. Their formation occurs in response to a variety of cues, including high cell density, nutrient deprivation and physical environmental

stresses. Pathogenic bacteria and fungi can form biofilms on inert surfaces of implanted devices such as catheters, prosthetic heart valves and joint replacements. Because biofilms are generally more resistant to both host defence mechanisms and to antibiotics than planktonic unicellular microbes, they represent an ongoing source of infection for many patients (Li, Yan, and Xu, 2003), and therefore are increasingly recognized as important health problems for patients with microbial infections. Although the majority of these infections are caused by bacteria, fungal infections are becoming increasingly common, especially those caused by species in the genus *Candida*, including *C. albicans*. Since *C. albicans* forms biofilm on mucosal surfaces and on plastic surfaces of indwelling devices (Hasan, Xess, Wang, Jain, and Fries, 2009), it has been proposed that this fungus develops phenotypic adaptation within the biofilm matrix, resulting in drug-resistance.

Plants (fruits, vegetables, medicinal herbs, etc.) provide abundant resources of antimicrobial compounds, such as phenolic compounds, nitrogen containing compounds, vitamins, terpenoids and some other secondary metabolites. They are rich in valuable bioactivities, e.g., antioxidant, anti-inflammatory, antitumor, anti-mutagenic, anti-carcinogenic, antiviral and antibacterial activities. Flavonoids, a group of phenolic compounds in plants, have shown significant antimicrobial activities (Cushnie and Lamb, 2005).

Alpinia officinarum Hance (Zingiberaceae) known as lesser galangal, a pungent and aromatic plant, is used as spice ingredient for flavoring food throughout southeastern Asian countries (Ly, Shimoyamada, Kato, and Yamauchi, 2003). It has also been used in a traditional medicine for several purposes, such as relieving stomachache and pain, treating colds, invigorating the circulatory system, reducing

swelling and inflammation (An *et al*., 2008; Lee *et al*., 2009; Matsuda, Ando, Kato, Morikawa, and Yoshikawa, 2006). The crude extract of the rhizomes of *A. officinarum* as well as its bioactive components has been shown to possess antibacterial activity (Zhang, Dai, Liao, and Ding, 2010). However, antifungal activity of this plant has not been reported.

Several chemical compositions in the rhizomes of *A. officinarum* have been isolated and identified. They include monoterpenes, neolignans, diarylheptanoids, phenylpropanoids, and flavonoids (An, Zhang, Xu, Yang, and Zou, 2009; An et al. 2008; Ly, Yamauchi, and Kato, 2001; Shin, Kinoshita, Koyama, and Takahashi, 2002; Zhao, Qu, Fu, and Liang, 2010). The flavonol galangin (3,5,7-trihydroxyflavone) is a major constituent of *A. officinarum* (Cushnie and Lamb, 2006). Galangin possesses certain biological activities, such as anti-genotoxicity (Heo, Sohn, and Au, 2001) and antibacterial activity (Cushnie and Lamb, 2006). It is possible that the extract from rhizomes of *A. offcinarum* may have potential to possess antifungal activity against this type of fungus.

The aims of this study were to determine the inhibitory effect of the crude extracts from rhizomes of *A. officinarum* and its major flavonoid, galangin, against *C. albicans*. The crude extract of *Cassia spectabilis* has been reported to inhibit the biofilm formation and alter morphology of *C. albican* (Sangetha, Zuraini, Suryani, and Sasidharan, 2009). In the present study, cellular surface changes of *C. albicans* were examined by scanning electron microscope as well.

1.2 Research Objectives

1.2.1 To determine the inhibitory effect of galangin and the crude extract of rhizomes of *A. officinarum* against *C. albicans*.

1.2.2 To isolate and quantitate galangin from the crude extract of rhizomes of *A. officinarum*.

1.2.3 To determine whether crude extract from the rhizomes of *A. officinarum* changes cellular surface of *C. albicans*.

1.2.4 To determine whether the crude extract of rhizomes of *A. officinarum* prevents biofilm formation of *C. albicans*.

1.3 Research hypotheses

1.3.1 The crude extract of rhizomes of *A. officinarum* possesses anticandidal activity with galangin as bioactive substance.

1.3.2 The crude extract from rhizomes changes cellular surface of *C. albicans* and prevents biofilm formation of *C. albicans*.

1.4 Scope and limitations of the study

The obtained data is limited to *in vitro* study. Clinical trial is needed to confirm the beneficial use of the extract.

CHAPTER II

LITERATURE REVIEW

2.1 *Candida albicans*

The genus *Candida* belongs to yeasts. It is also the most common cause of opportunistic mycoses worldwide. It is a frequent colonizer of human skin and mucous membranes. *Candida* is a member of normal flora of skin, mouth, vagina, and bowel. In addition to being a colonizer and pathogen, it is found in the environment, particularly on leaves, flowers, water and soil. However, if the immune defenses of the host become compromised, they can cause severe systemic infections. These include especially the patients in risk factors include infection by the human immunodeficiency virus (HIV), anti-cancer therapy, organ transplantation, abdominal surgery, catheters, diabetes and the use of broad-spectrum antibiotics (Cruz *et al*., 2002; Morschhauser, 2002). The genus *Candida* includes around 154 species. Among these, six are most frequently isolated in human infections. While *Candida albicans* is the most abundant and significant species, *Candida tropicalis, Candida glabrata, Candida parapsilosis, Candida krusei* and *Candida lusitaniae* are also isolated as causative agents of *Candida* infections (Abi-Said *et al*., 1997). The most important of *Candida* spp. is *C. albicans* which is the most frequently isolated. It is also believed to be the most virulent in human (McCullough, Ross, and Reade, 1996).

2.1.1 Taxonomy of *C. albicans*

C. albicans belongs to the Ascomycota class of fungi and is the most commonly studied species because it causes a variety of mycotic infections in humans (Siqueira and Sen, 2004)

Taxonomy

Kingdom: Fungi

Phylum: Ascomycota

Subphylum: Ascomycotina

Class: Ascomycetes

Order: Saccharomycetales

Family: saccharomycetaceae

Genus: *Candida*

Species: *albicans*

2.1.2 Structure of *C. albicans*

The cell wall of *C. albicans* is consisted of the polysaccharides mannan, glucan, and chitin (Calderone and Braun, 1991). The percent compositions of cell walls from yeast cells and germ tubes are relatively similar, although the amounts of glucans and chitin from *C. albicans* vary according to the growth form (Chattaway, Holmes, and Barlow, 1968). The relationships of these polymers to each other are presented in Figure 2.1.

2.1.3 Morphology of *C. albicans*

The fungal pathogen *C. albicans* can be found in three morphological states, which are shown in Figure 2.2 as yeasts, pseudohyphae and hyphae (Sudbery, Gow, and Berman, 2004).

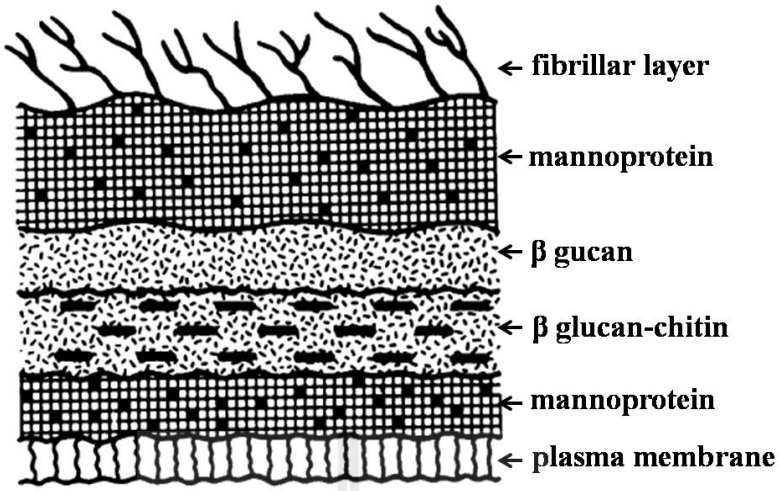


Figure 2.1 Schematic diagram of the cell wall of *C. albicans*. The layers are enriched in the indicated components. However, mannoprotein is found throughout the wall (Shepherd, 1987).

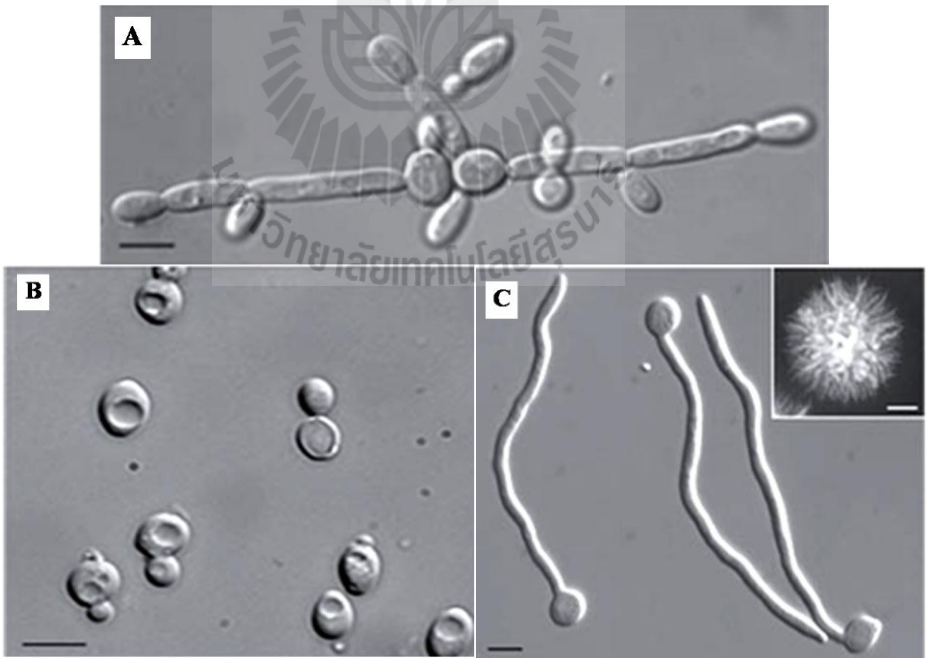


Figure 2.2 Morphology of (A) pseudohyphae, (B) yeasts and (C) hyphae forms (Sudbery, 2011).

Some mycologists prefer to consider the pseudohyphal and hyphal forms as one entity; therefore *C. albicans* is usually said to be dimorphic in nature as it has two distinct shapes. Yeast cells are unicellular and spherical or oval in shape, and normally form smooth, white dome-shaped colonies. Yeast multiply by a specific process of mitotic division known as budding, in which daughter cells exude from the mother cells. Several classes of fungi, including *C. albicans,* are featured with the ability to form spores i.e. blastospores and clamydospores (which are spherical, smooth surfaced and highly refractile (Nobile *et al*., 2003), thus more favorable for candidal survival). Pseudohyphae are considered modified yeasts which continued in polarized growth without separation from the mother cell at the end of each cell cycle (Sudbery, Gow, and Berman, 2004). Pseudohyphae are also characterized by unequal width of their cellular projections, being wider at the center than at the ends (Merson-Davies and Odds, 1989). Hyphae are microscopic tubes which contain compartmentalized cell units separated by septa, these units arise initially from blastospores or also from already existing hyphae (Webb, Thomas, Willcox, Harty, and Knox, 1998). When it takes up the hyphal form, it forms filamentous projection with parallel sided walls, so keeping the width of their compartments the same throughout the branched portion. Germ tube is a term applied to the projecting hyphae in the first cell cycle just before septation (Calderone *et al*., 2000). *C. albicans* is well known for its morphological plasticity, i.e. its ability to transform from one morphological pattern to another known as switching, which is thought to promote the pathologenicity of the organism (Whiteway and Oberholzer, 2004). There are other minor morphological changes which take place during switching. For example, opaque phase is a variety where the cell becomes oblong instead of the usual oval

form of the yeast. Cell signal transduction pathways and various transcriptional effects have been linked to the different array of morphological forms and switching of *C. albicans* (Liu, 2002).

2.1.4 Biofilm formation of *C. albicans*

Microorganisms can exist either in a floating planktonic state or attached to an external surface. A biofilm is an assembly of surface-coating microbial cells that is attached to the surface and enclosed in a matrix of polysaccharide material such as alginate, *Psl*- and *Pel*-encoded polysaccharide (Donlan, 2002; Ryder, Byrd, and Wozniak, 2007). In addition to its association with many hospital acquired nosocomial infections, biofilm formation is of clinical significance since it confers the associated microorganisms an ability to resist external threats, e.g. antimicrobial drugs (Chandra *et al*., 2001; Shinde, Raut, and Karuppayil, 2012). A biofilm formed of *C. albicans* renders it a hundred times more resistant to the antifungal fuconazole and 20-30 times more resistant to amphotericin B than planktonic cells (Ramage, Saville, Thomas, and Lopez-Ribot, 2005). Both yeast and hyphal forms of *C. albicans* can participate in biofilm formation. Due to biofilm formation, *C. albicans* yeast is considered as one of the most common microorganisms found in the bloodstream in hospitalized patients, in whom it originates from the biofilm composed of yeast cells embedded in a protective matrix of extracellular protein (Chaffin, Lopez-Ribot, Casanova, Gozalbo, and Martinez, 1998; Crump and Collignon, 2000; Soustre, Rodier, Imbert-Bouyer, Daniault, and Imbert, 2004). Biofilm of *C. albicans* has been noticed in dentures as well as other biomaterial, e.g. stents, shunts, endotracheal tubes and catheters (Andes *et al*., 2004; Bulad, Taylor, Verran, and McCord, 2004). In order to form biofilm *C. albicans* has to adhere first to a medical device, colonize it and

then establish a biofilm. Biofilm formation by *C. albicans* depends on many factors, e.g. nature of the device surface, whether host-derived conditioning film is present and liquid flow (Chandra *et al*., 2001; Kuhn, Chandra, Mukherjee, and Ghannoum, 2002). Colonization of different biomaterials by *C. albicans* has been accepted as an important major cause of medical device failure (Jones, McGovern, Adair, Woolfson, and Gorman, 2001). In the biofilm composed of *C. albicans*, a layer of yeast cells is located lowermost attached to the device. The yeast layer composed of filamentous cells in the hyphal form surrounded by extensive exoplasmic matrix (Baillie and Douglas, 1999).

2.1.5 Epidemiology of *C. albicans*

It is believed that 90% of healthy individuals carry *C. albicans* if the sensitive enough tests were developed. The intraoral commensally living of this organism appears at least 50% of the population. However, it has been shown that *Candida* spp. may heaven more than one species at the same time in hospitalized and immunocompromised patients (Odds, 1987).

Gastrointestinal (GI) tract is the major environment of the commensal *Candida* spp. It has been shown that, in sufficient high numbers of *Candida*, *C. albicans* can spread from human gut which causes fungaemia and funguria (Krause, Matheis, and Wulf, 1969) and also believed that the gut is the source of the most forms of *Candida* infection (Odds, 1987).

From the previous study, it was reported that the prevalence and density of *C. albicans* are greater in edentulous patients, who wear denture, than in normal subjects. The dentures have been suggested to help the growth of *C. albicans*. Although the appearance of *C. albicans* was greater in these patients than in healthy

subjects, there was no significant difference in the concentration of *C. albicans* between these groups (Borromeo, McCullough, and Reade, 1992).

Candidiasis encompasses infections that range from superficial, such as oral thrush and vaginitis, to systemic and potentially life-threatening diseases. *Candida* infections of the latter category are also referred to as candidemia or invasive candidiasis, and are usually confined to severely immunocompromised persons, such as cancer, transplant and AIDS patients, as well as nontrauma emergency surgery patients (Kourkoumpetis *et al*., 2010). Candidiasis is devided into four types, which are mucosal candidiasis, cutaneous candidiasis, onychomycosis, and invasive candidiasis. Mucosal candidiasis includes oral candidasis, which is shown as pseudomembranous candidosis in Figure 2.3 and denture stomatitis in Figure 2.4 (Tarcin, 2011). Esophageal candidiasis is also included in mucosal candidiasis as shown in Figure 2.5 (Choi *et al*., 2013). Cutaneous candidiasis includes candidal intertrigo, diaper cadidiasis and congenital cutaneous candidiasis. Onychomycosis (nail candidiasis) involving the yeasts *C. albicans* was shown in Figure 2.6 (Nolting, Brautigam, and Weidinger, 1994). Invasive candidiasis is infections affecting an ever-increasing number of hospitalized patients. Invasive candidiasis causes considerable morbidity and mortality in patients with medical comorbidities who possess risk factors for these infections such as broad-spectrum antibacterial therapy, intense myelosuppression and cytotoxic therapies, recent gastrointestinal surgery and the presence of central venous access devices. A variety of *Candida* species may produce invasive cadidiasis, but *C. albicans* continues to be the most common inciting pathogen (Bow *et al*., 2010). Pathogenesis of invasive candidiasis was shown in Figure 2.7.

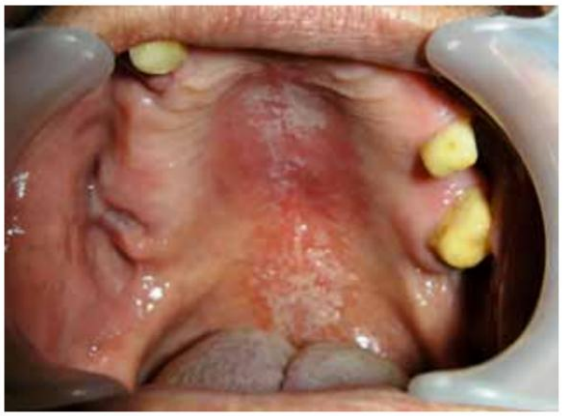


Figure 2.3 Pseudomembranous candidosis in a patient using steroid inhaler for management of asthma (Tarcin, 2011).

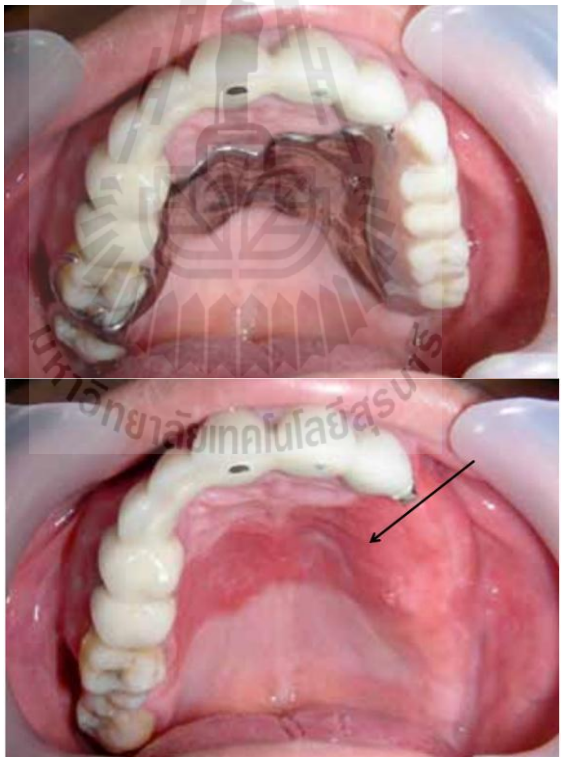


Figure 2.4 Denture stomatitis, showing localized erythema of tissues covered by dentures (Tarcin, 2011).

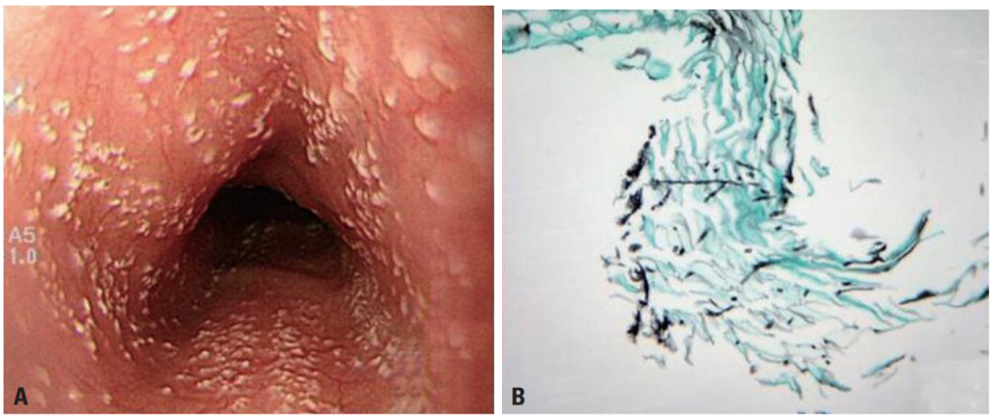


Figure 2.5 Esophageal candidiasis. (A) Endoscopic finding; multiple whitish plaques were identified. (B) Histopathologic finding; esophageal mucosa containing candida spores and pseudohyphae were noted (Grocott methenamine silver stain, ×400) (Choi *et al*., 2013).

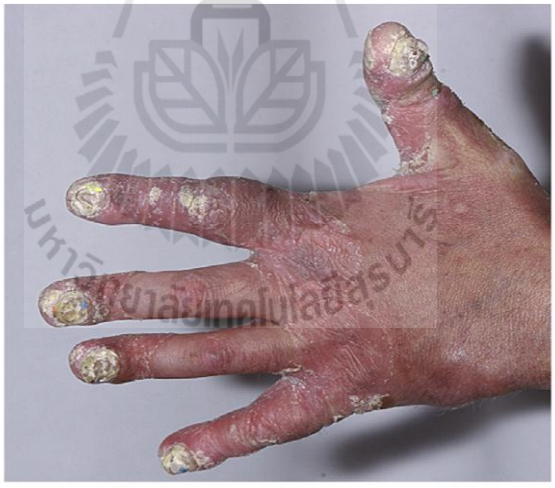


Figure 2.6 Clinical phenotype of the patient with extensive cadidiasis of nails (Uzel *et al*., 2013).

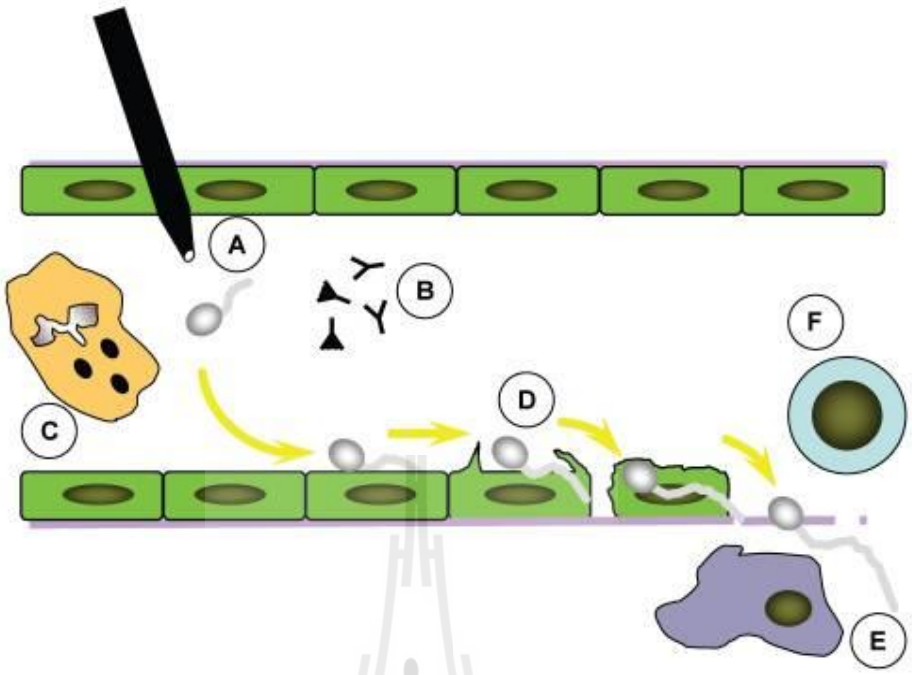


Figure 2.7 Pathogenesis of invasive candidiasis. Blastospores of *Candida* gain access to the endovascular compartment via gut translocation or endovascular catheter colonization (A). Candida blastospores germinate within the bloodstream and interact with soluble serum components (B) and circulating neutrophils (C). Organisms then adhere to and penetrate vascular endothelial cells (D) to gain access to the deep organs, where they interact with dendritic cells and monocyte macrophages (E) that activate adaptive immune response (F) (Bow *et al*., 2010).

2.1.6 Virulence of *C. albicans*

Among human fungal pathogen, the yeast *C. albicans* is the major opportunistic fungal pathogen of humans. *C. albicans* lives commensally in humans such as at the gut, oral cavity and vaginal mucosa. This microorganism causes diseases when given the opportunity. On mucosal surfaces, limitation of nutrient and competition surrounded by bacteria and fungi provide selective pressures that result in

the elimination of less adapted microorganism. From this competition among mucosal bacteria and fungi, the host site (tissue) has specific pressures that a pathogen, such as *C. albicans,* must adapt its growth to a range of physiological extremes such as pH (Calderone and Fonzi, 2001).

C. albicans expresses several virulence factors include host recognition biomolecules (adhesins), morphogenesis (the reversible transition between unicellular yeast and filamentous, growth from), secreted aspartyl proteinases (SAPs) and phospholipases and probably phenotypic switching (Calderone and Fonzi, 2001).Successful colonization and infection of host tissues by the pathogenic *Candida* species depend on the ability of these organisms to adhere to mucosal surfaces. A hierarchy exists among *Candida* spp. such that the more commonly presenting pathogens, such as *C. albicans,* adhere to host cells *in vitro* to a greater extent than the relatively nonpathogenic species. This is a clear correlation between adhesion and virulence. This correlation has been shown in a comparative study of the adherence of different *Candida* spp. to human epithelial cells (Asakura *et al*., 1991).

The extracellular proteinase activities of *C. albicans* is associated with virulence. Secretory acid proteinase can be shown in patients infected with *C. albicans*. The enzymes produced by these yeasts are carboxyl proteinases capable of degrading secretory immunoglobulin A (IgA) which is the major immunoglobulin in mucous membrane. Some of these proteinases have keratinolytic or collagenolytic activity (Douglas, 1988). The previous investigation suggested that the secretion of proteinase is an important factor allowing invasion of the choriollantoic membrane (Shimizu, Kondoh, and Tanaka, 1987).

2.1.7 Treatment of *C. albicans*

C. albicans is widely treated with the antifungal agent fluconazole that inhibits the biosynthesis of ergosterol. This azole group, include ketoconazole, fluconazole, and itraconazole, explain a major advance in systemic antifungal therapy. From these azole drugs, fluconazole has the most attractive pharmacologic profile which is the capacity to produce high concentrations of active drug in cerebrospinal fluid and urine. Ketoconazole is less well tolerated than either fluconazole or itraconazole and associated with more important toxic effects, including hepatitis and inhibition of steroid hormone synthesis. However, ketoconazole is less expensive than fluconazole and itraconazole and it is also important consideration for patients receiving long-term therapy. Ketoconazole and itraconazole are effective in patients with the chronic, indolent forms of the endemic mycoses, including blastomycosis, histoplasmosis, and coccidioidomycosis. Itraconazole is also effective in patients with sporotrichosis and it is the most effective of the azoles for the treatment of aspergillosis. Fluconazole is useful in the common forms of fungal meningitis--namely, coccidioidal and cryptococcal meningitis and it is also effective for selected patients with serious candida syndromes such as candidemia (Como and Dismukes, 1994). However, the emergence of azole-resistant *C. albicans* strains is a problem after long-term treatment of recurrent oropharyngeal candidiasis (OPC) in acquired immunodeficiency syndrome (AIDS) patients (Morschhauser, 2002).

Different classes of antifungals are now available to manage any type of candidal infection. Azoles, fluconazole in particular (Charlier *et al.*, 2006), have become the main stay of therapy over the past few years. This include topical and systemic agents. Posaconazole is the most recent addition to this group of antifungals.

Polyenes include amphotericin B, liposomal amphotericin B formulations, and topical nystatin. Allylamins include terbinafine, which is formulated in a topical preparation and an oral tablet. The newest group of antifungals is echinocadins, including caspofungin, micafungin, and anidulafugin. These drugs have shown excellent clinical efficacy, a low incidence of adverse events, a good safety profile, and ease of use (Kauffman, 2006; Sable, Strohmaier, and Chodakewitz, 2008).

In addition to azoles, there are another three major groups of antifungal agents for clinical use. They are derivatives of echinocadins, polyenes and allylamine of which mechanisms will be discussed in the next section. The structures of representative antifungal agents, including amphotericin B, fluconazol, terbinafine, voriconazol, and echinocandin B, were shown in Figure 2.8.

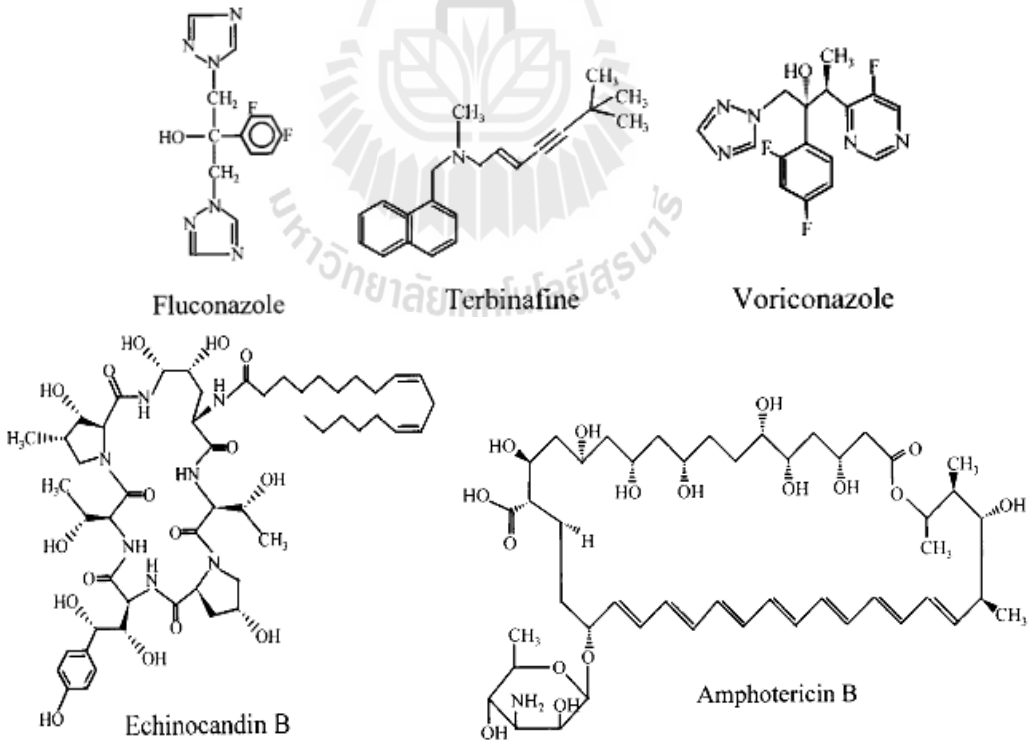


Figure 2.8 Structures of representative antifungal agents (Ghannoum and Rice, 1999).

2.2 Antifungal agents

2.2.1 Azole antifungals

These agents are synthetic compounds that include two groups, imidazole and triazoles. Imidazole agents include miconazole, ketoconazole, and clotrimazole. Triazole agents, which are the most commonly used azoles, include fluconazole, itraconazole, econazole, terconazole, butonazole, and tioconazole. Newer triazoles are active against fluconazole-resistant strains of *Candida* such as voriconazole, posaconazole, and ravuconazole. Voriconazole and posaconazole have shown high efficacy against candidiasis in recent clinical trials (Kullberg *et al*., 2005; Ostrosky-Zeichner, Oude Lashof, Kullberg, and Rex, 2003; Skiest *et al*., 2007). The primary mechanism of action is inhibition of lanosterol 14-alpha-demethylase, an enzyme required for ergosterol biosynthetic pathway as showed in Figure 2.9 (Hitchcock, Dickinson, Brown, Evans, and Adams, 1990; Lupetti, Danesi, Campa, Del Tacca, and Kelly, 2002). Ergosterol, the primary sterol in the fungal membrane, serves as a bioregulator of membrane fluidity in fungal cells (Ellis, 2002; Espinel-Ingroff and Shadomy, 1989).

Inhibition of 14α-demethylase leads to depletion of ergosterol and accumulation of sterol precursors, including 14α-demethylated sterol (lanosterol, 4,14-dimethylzymosterol, and 24-methylenedihydrolanosterol). These result in an alteration of the structure and function of plasma membrane. The recent triazole derivatives, such as fluconazole, itraconazole, and voriconazole, have their own antifungal activity at least in part to inhibition of cytochrome P-450-dependent 14α-sterol demethylase (Sanati, Belanger, Fratti, and Ghannoum, 1997).

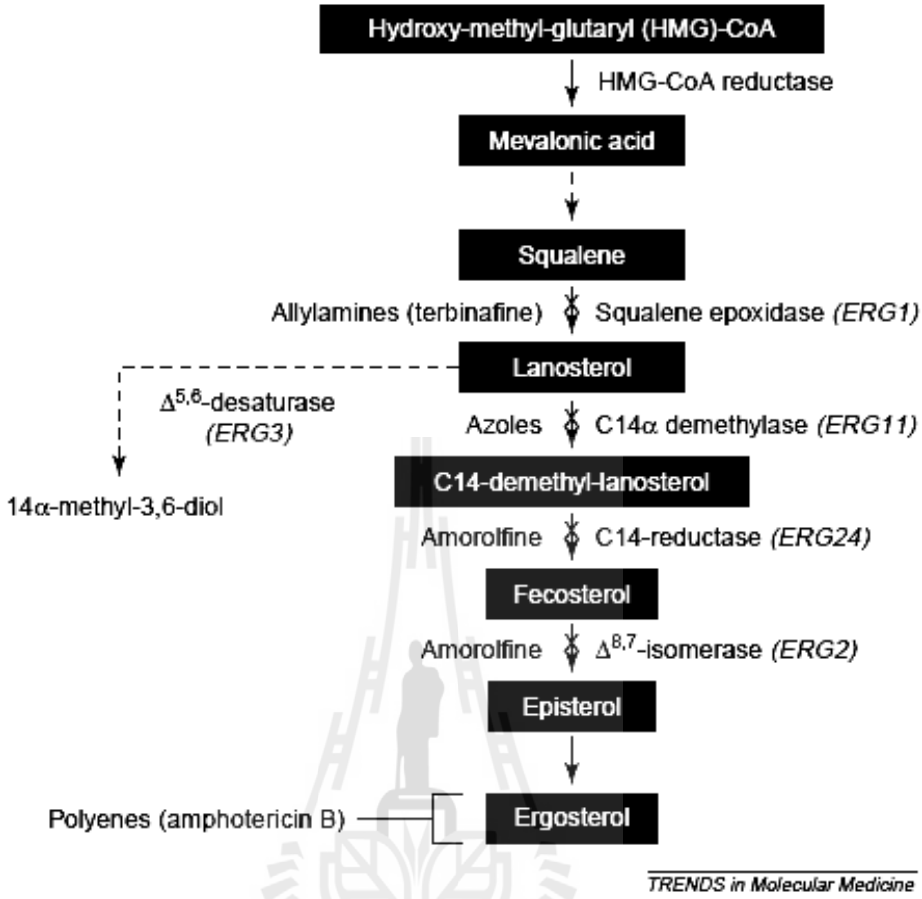


Figure 2.9 Mechanism of action of antifungal drugs affecting the ergosterol biosynthetic pathway. Steps at which various antifungal agents exert their inhibitory activities are shown (Lupetti *et al*., 2002).

Although more recent azole antifungals are 14α-demethylase inhibitors, there exists a heterogeneity of action among these antifungals (Beggs, 1983; Sud and Feingold, 1981). The imidazole derivatives, such as miconazole, econazole, and ketoconazole, have a complex mode of action, inhibiting several membrane-bound enzymes as well as membrane lipid biosynthesis (Sheehan, Hitchcock, and Sibley, 1999). In *Candida krusei*, voriconazole significantly inhibited ergosterol synthesis.

The *C. krusei* cells treated with voriconazole accumulated the following biosynthetic intermediates: squalene, 4,14-dimethylzymosterol, and 24-methylenedihydrolanosterol. Accumulation of these methylated sterols is consistent with the premise that this agent functions by inhibiting fungal P-450-dependent 14α-demethylase (Sanati *et al*., 1997).

Azole activity may possess organism-dependent fungicidal activities (Manavathu, Cutright, and Chandrasekar, 1998). In addition to inhibiting the 14α-demethylase in *Cryptococcus neoformans*, fluconazole and itraconazole affect the reduction of obtusifolione to obtusifoliol, which results in the accumulation of methylated sterol precursors (Ghannoum *et al*., 1994; Van den Bossche *et al*., 1993). Mammalian cholesterol synthesis is also blocked by azoles at the stage of 14α-demethylation. However, the dose required to affect the same degree of inhibition is much higher than that required for fungi (Hitchcock *et al*., 1990; Van den Bossche, Willemsens, Cools, Lauwers, and Le Jeune, 1978). The clinical effects of inhibition of human sterol biosynthesis are most prominently seen with ketoconazole (Ghannoum and Rice, 1999). Moreover, the accumulation and importation of fluconazole was tested in the pathogenic fungus, such as *C. albicans*. These antifungal agents import via facilitated diffusion through a transporter rather than passive diffusion. The other fungi, including *C. neoformans*, *Saccharomyces cereviciae*, and *C. krusei*, have been observed on import of fluconazole by facilitated diffusion (Mansfield *et al*., 2010).

2.2.2 Mechanisms of resistance to azoles

Several lines of evidence implicate a modification in the quantity or quality of 14α-demethylase in the expression of resistance to azole antifungal agents. A recent study examined the biochemical mechanisms for resistance to fluconazole by

comparing sterol composition, fluconazole accumulation, and inhibition of 14α-demethylase by fluconazole in two clinical *C. krusei* strains and a susceptible isolated *C. albicans*. The sterol content of *C. krusei* and *C. albicans* were detected (ergosterol was the major sterol in both species) and also performed on cell extracts indicated that the fluconazole concentration required to inhibit the synthesis of ergosterol approximately 24 to 46-fold higher in *C. krusei* than in *C. albicans*, advising that affinity of the target enzyme is different in the two species (Orozco *et al*., 1998). A comparison of fluconazole accumulation between *C. albicans* and *C. krusei* showed that fluconazole accumulation in the first 60 min was not different from all studied strains. However, the analysis after 90 minutes of the incubation displayed that *C. krusei* accumulated 60% less fluconazole accumulation than did *C. albicans*, suggesting active efflux in the fluconazole resistance expressed by these *C. krusei* strains.

Other studies have showed an alteration of 14α-demethylase activity in resistance to azoles. Reduced susceptibility of *C. albicans* B41628 to miconazole, ketonazole, itranazole, and fluconazole was attributed to differences in the microsomal cytochrome P-450 enzyme (Smith *et al*., 1986; Sokol-Anderson, Brajtburg, and Medoff, 1986). In addition, the enzyme had a low binding affinity for azole antifungal (Van den Bossche, Willemsens, Cools, Marichal, and Lauwers, 1983).

Overexpression of 14α-demethylase has been involved as a mechanism of resistance to azole antifungals. The characterization of an azole-resistant *C. glabrata* had been isolated and showed that its ergosterol content was increased when compared with pretreatment isolate. This increase in ergosterol synthesis suggested

that both lower fluconazole uptake and increased P-450-dependent ergosterol synthesis are involved in the mechanism of fluconazole resistance, suggesting an overexpression of the enzyme (Van den Bossche, Marichal, Odds, Le Jeune, and Coene, 1992). It has been suggested that the increased P-450 levels may be responsible for the cross-resistance to these triazoles.

Considerable evidence has now been accumulated to suggest that active efflux is an important mechanism of resistance to azole antifungals. A group of researchers have reported that fungi possess at least two efflux systems: (1) proteins belonging to the major facilitator super family (MFS) and (2) ATP-binding cassette (ABC) superfamily of proteins. The MFS drug efflux proteins are associated with the transport of structurally diverse compounds and accounted for a range of resistance to toxic compounds in microorganisms (Jenkinson, 1996)

Alteration in membrane composition had been studied in an interaction between sterols and phospholipids in the cytoplasmic membrane. Using cerulenin as a lipid modulator showed that altered phospholipids and fatty acid profiles affected *C. albicans* cell permeability and rendered the cells more resistant to miconazole (Mago and Khuller, 1989). A decrease in the amount of drug taken up by the fungal cell was suggested to be a result from changes in the sterol and/or the phospholipids composition of the fungal cell membrane.

It has been shown that an azole-resistant and polyene-resistant *C. albicans* mutant had a larger lipid content and lower polar-lipid-to-neutral-lipid ratio than did strains susceptible to azoles. However, the most significant change in the lipid of the resistant strain was in the membrane sterol pattern, where ergosterol was replaced by methylated sterols, such as methylfecosterol (Hitchcock, Barrett-Bee, and

Russell, 1987). The summary of mechanisms by which microbial cells might develop resistance as showed in Figure 2.10.

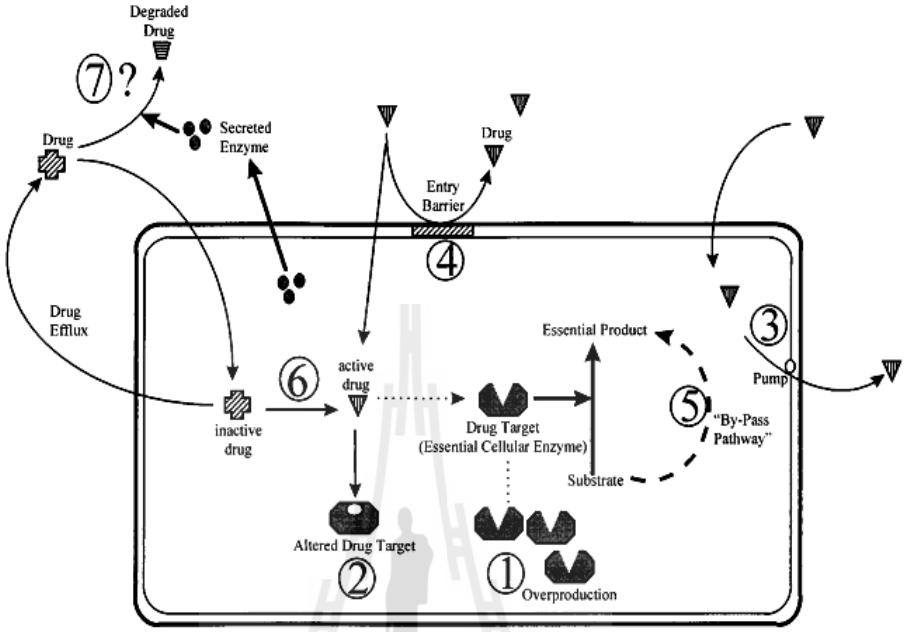


Figure 2.10 Mechanisms by which microbial cells might develop resistance (Ghannoum and Rice, 1999).

Figure 2.10 demonstrates the postulated mechanisms of resistance to antifungal. First, the target enzyme is overproduced, so that the drug does not inhibit the biochemical reaction completely. Secondly, the drug target is altered so that the drug cannot bind to the target. Thirdly, the drug is pumped out by an efflux pump. Fourthly, the entry of the drugs is prevented at the cell membrane/cell wall level. The fifth mechanism is that the cell has a bypass pathway that compensates for the loss-of-function inhibition due to the drug activity. Some fungal enzymes that convert an inactive drug to its active form are inhibited. The last is that the cell secretes some enzymes to the extracellular medium, which degrade the drug (Ghannoum and Rice, 1999).

2.2.3 Glucan synthesis inhibitors (Echinocadins)

Echinocadins inhibit the synthesis of (1,3) β-D-glucan, an essential component of the fungal cell wall. This component is not found in mammalian cell wall. These agents have expanded with the approvals of caspofungin, micafungin, and anidulafungin. Indication are evolving but have been approved for complicated forms of invasive candidiasis, candidemia, disease refractory to other systemic antifungals, and intolerance to amphotericin B. They are board spectrum and fungicidal against most *Candida* species, except *C. parapsilosis* and *C. guilliermondii*.

2.2.4 Polyenes

These are board-spectrum fungicidal agents. Mechanism of action is by insertion into fungal cytoplasmic membrane, causing increases in permeability. Membrane channel activity is increased at lower dose, and pores are formed at higher concentration. The polyene class of antifungals includes amphotericin B, nystatin, and natamycin. All organisms susceptible to polyenes, e.g. yeasts, algae, andprotozoa, contain sterols in their outer membrane, while resistant organisms do not susceptible to polyenes (Norman, Demel, de Kruyff, Geurts van Kessel, and van Deenen, 1972). For larger polyenes, such as amphotericin B, it has been proposed that the interaction of the antifungal with membrane sterol results in the production of aqueous pores consisting of an annulus of eight amphotericin B molecules linked hydrophobically to the membrane sterols (de Kruijff and Demel, 1974; Holz, 1974) as showed in Figure 2.11. This configuration gives rise to a pore in which the polyene hydroxyl residues face inward, leading to altered permeability, leakage of vital cytoplasmic components, and death of the organism. Moreover, amphothericin B in the concentration of 1.25 to

10 µg/ml killed both *Aspergillus fumigates* and *C. albicans* (Manavathu, Cutright, and Chandrasekar, 1998).

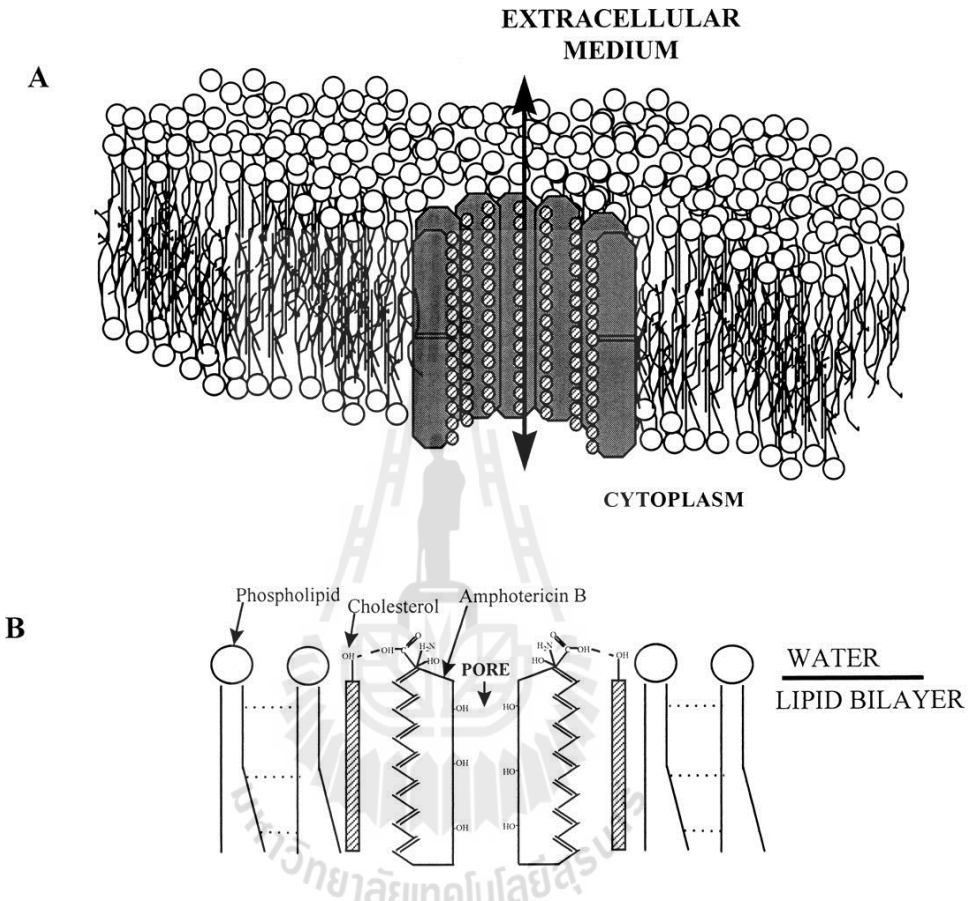


Figure 2.11 The interaction between amphotericin B and cholesterol in a phospholipid bilayer. (A) The conducting pore is formed by the end-to-end union of two wells or half pores. (B) Molecular orientation in an amphotericin B-cholesterol pore. The dotted lines between the hydrocarbon chains of phospholipids represent short-range van der Waals forces. The dashed lines represent hydrogen bonds formed between amphotericin B and cholesterol molecules (Ghannoum and Rice, 1999).

2.2.5 Mechanism of resistance to polyenes

Most of the mechanisms of resistance to polyenes in fungal species have come from studies using mutants generated by (1) growing cells in the presence of increasing concentrations of antifungal agents, (2) exposing the cells to a gradient concentration (Athar and Winner, 1971), or (3) creating mutants by one-step mutation with mutagenic agents (Hamilton-Miller, 1972). It is postulated that the resistant cells with altered sterol content bind smaller amounts of polyene than do susceptible cells. This decreased binding of polyenes in *C. albicans* mutants could be attributed to (1) a decrease in the total ergosterol content of the cell, without concomitant changes in sterol composition; (2) replacement of some or all of the polyene-binding sterols by ones which bind polyene less well, e.g., substitution of ergosterol, cholesterol, or stigmasterol by a 3-hydroxy or 3-oxosterol; or (3) reorientation, or masking, of existing ergosterol, so that binding with polyenes is sterically or thermodynamically less favored (Michaelis and Berkower, 1995).

2.2.6 Allylamines

Allylamines, such as terbinafine and naftifine, have been developed as a new class of ergosterol biosynthetic inhibitors that are functionally as well as chemically distinct from the other major classes of ergosterol-inhibiting antifungal agents. Terbinafine is highly effective against dermatophytes *in vivo* and *in vitro*. Terbinafine has good activity against at least some azole-resistant *C. albicans* strains (Ghannoum and Rice, 1999). Allylamines act by inhibiting early steps of ergosterol biosynthesis, suggesting that allylamine inhibition of sterol synthesis occurs at the point of squalene epoxidation, a reaction catalyzed by squalene epoxidase, causing fungal-cell death (Birnbaum, 1990).

2.2.7 Mechanism of resistance to allylamines

Although clinical failure has been observed in patients treated with terbinafine, allylamine resistance in association with clinical use of terbinafine and naftifine has not been found in human pathogenic fungi. However, with the increased use of this agent, resistance may be expected, since it was reported that a *C. glabrata* strain that became resistant to fluconazole expressed cross-resistance to terbinafine (Van den Bossche *et al*., 1992).

2.3 *Alpinia officinarum* Hance (Lesser galangal)

2.3.1 General background of *A. officinarum*

Alpinia officinarum Hance, known as lesser galangal, is of Zingiberaceae family and *Alpinia* genus. It is a pungent and aromatic rhizome, cultivated in several countries in Asia such as China, Vietnam and Thailand. It is used as a spice ingredient for flavoring food throughout southeast Asian countries (Ly, Shimoyamada, Kato, and Yamauchi, 2003; Ly, Yamauchi, Shimoyamada, and Kato, 2002). The dried rhizome of *A. officinarum* has been used as a popular traditional herbal medicine for several purposes, such as relieving stomachache, treating colds, strengthening the circulatory system, and reducing swelling (An *et al*., 2008; Seddeag, Madawe, Badwi, and Bakhiet, 2010).

Lesser galangal has a pungent and spicy taste. It grows to a height of about 5 feet. The leaves are long and rather narrow blades. The flowers are grown in terminal spike. The petals are white, with deep-red veining distinguishing the lippetal. The most commonly used part is its rhizome. The branch pieces of rhizome are from 1.5 to 3 inches in length with a reddish-brown color (Grieve, 1995-2013). Figure 2.12

and 2.13 are the morphological features of rhizome, leaves and flowers of *A. officinarum*.

Figure 2.12 Aboveground and underground portion of *Alpinia officinarum* (Grieve, 1995-2013).

Figure 2.13 Lesser galangal (*Alpinia officinarum*) appearing in nature.

2.3.2 The chemistry of *A. officinarum*

In the chemical studies of the rhizomes, the isolation and structure determination of monoterpenes, diarylheptanoids, flavonoids, phenylpropanoids, and neolignans have been reported. The constituents isolated from *A. officinarum* rhizomes consist of a diarylheptanoid [7-(4''-hydroxy-3''-methoxyphenyl)-1-phenylhept-4-en-3-one (2)], flavonols [kaempferide (5), galangin (6)], 5-Hydroxy-1,7-diphenyl-3-heptanone (1), 5-hydroxy-7-(4''-hydroxy-3-methoxyphenyl)-1-phenyl-3-heptanone (3), and 3,5-dihydroxy-1,7-diphenylheptane (4) (Matsuda, Nakashima, Oda, Nakamura, and Yoshikawa, 2009). Their structures were presented in Figure 2.15.

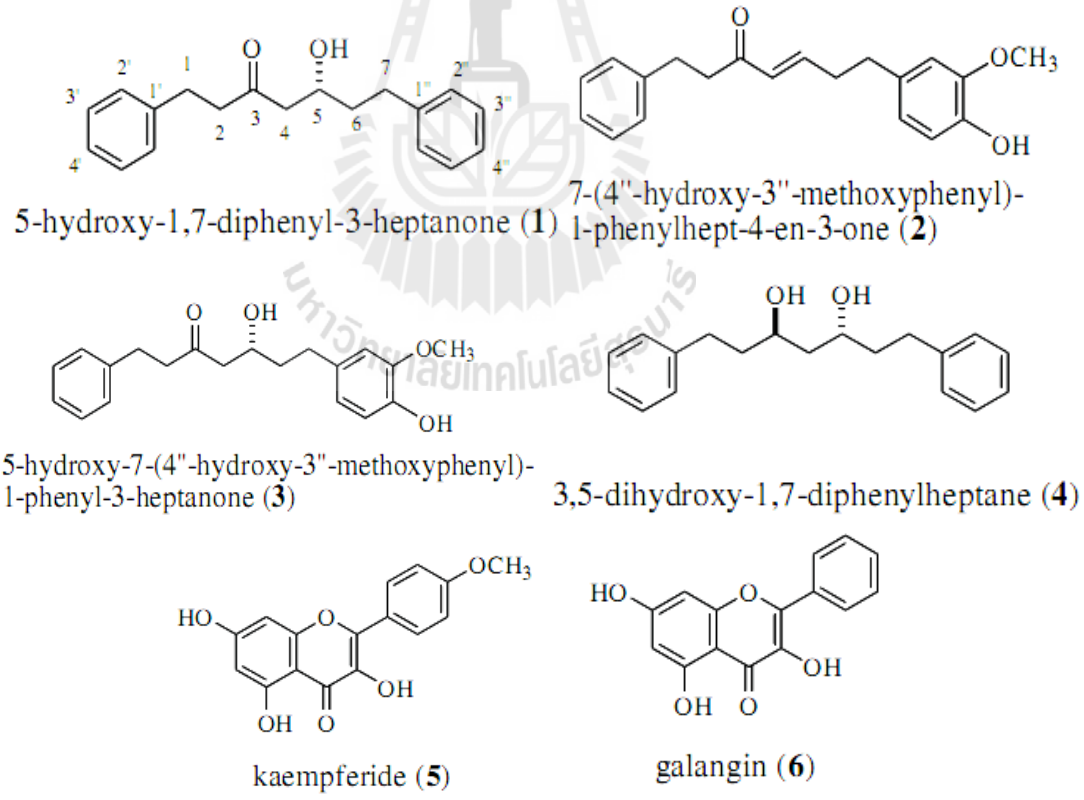


Figure 2.14 Chemical structures of compounds isolated from the rhizomes of *A. officinarum* (Matsuda *et al*., 2009).

2.3.3 The biological activity of *A. officinarum*

A. officinarum has been shown to possess many biological activities. It showed an activity against 5alpha-reductase (Kim *et al*., 2003) and also showed moderate cytotoxicity against several human tumor cell lines, including HepG2, MCF-7 and SF-268 (An *et al*., 2008). The rhizomes of *A. officinarum* posses anti-inflammatory, anti-nociceptive, and anti-psychiatric activities (Lee *et al*., 2009). The crude extract of the rhizomes of *A. officinarum* as well as its bioactive components have been shown to possess antioxidant and antibacterial activities (Srividya, Dhanabal, Misra, and Suja, 2010; Zhang, Dai, Liao, and Ding, 2010).

The rhizomes of *A. officinarum* have been shown anti-inflammatory effect by inhibiting nitric oxide (NO) production in lipopolysaccharide (LPS)-activated macrophages (Lee *et al*., 2009; Matsuda, Ando, Kato, Morikawa, and Yoshikawa, 2006). Nitric oxide (NO), involved in inflammation, is produced and released by induction of inducible nitric oxide synthase (iNOS), resulting in cytotoxicity, and tissue and DNA damage (Raso, Meli, Di Carlo, Pacilio, and Di Carlo, 2001). Moreover, this plant showed anti-rheumatic and analgesic activities by suppressing the swelling volume and inhibiting the flexion scores in complete Freund's adjuvant which stimulated arthritis (Lee *et al*., 2009).

Some glycosides and anti-oxidative compounds from *A. officinarum* rhizomes have been separated and identified (Ly *et al*., 2003; Ly *et al*., 2002)**.** The rhizomes of *A. officinarum* were found to have potent antibacterial, antioxidant (Ly, Shimoyamada, Kato, and Yamauchi, 2004; Srividya *et al*., 2010) and anti-genotoxic activities (Heo, Sohn, and Au, 2001). This plant contains several potent enzyme inhibitors. For example, it has been shown that they block pancreatic lipase (Shin,

Han, and Kim, 2003), 5α-reductase (Kim *et al*., 2003) and enzymes that involved in arachidonate inflammatory pathway, prostaglandin synthetase and 5-lipoxygenase (Kiuchi, Iwakami, Shibuya, Hanaoka, and Sankawa, 1992). In addition, diarylheptanoids from *A. officinarum* has been reported to correlate to the inhibition of nitric oxide (NO) production in lipopolysaccharide-activated macrophages (Matsuda *et al*., 2006). The acetone extract from rhizomes of *A. officinarum* was shown to inhibit melanogenesis in theophylline-stimulated murine B16 melanoma (Matsuda *et al*., 2009). Several compounds isolated from *A. officinarum* have shown the property of combating nausea (Shin, Kinoshita, Koyama, and Takahashi, 2002).

Galangin, a member of flavonol class of flavonoids, is present in high concentrations in *A. officinarum* (Heo, Sohn, and Au, 2001). Galangin, isolated from *A. officinarum* rhizomes, is a topic of interest because it has been shown to possess many pharmacological activities. It showed an inhibitory effect on acetylcholinesterase (AChE) activity (Guo *et al*., 2010). Because AChE inhibitors are widely used for Alzheimer's disease treatment, it is suggested that the flavonoid galangin from rhizomes of *A. officinarum* may has potential for development of anti-Alzheimer agents. Galangin may be a useful chemopreventive compound due to its *in vivo* potent anticlastogenic effects (Heo, Jae, Jung, and Au, 1996). Galangin also possess anti-microbial activities. Antibacterial activity of galangin against 4-quinolone resistant strains of *Staphylococcus aureus* have been demonstrated (Cushnie and Lamb, 2006). Moreover, the combination of galangin with gentamycin showed synergism against Methicillin-Resistant *S. aureus* (Lee *et al*., 2008). However, antifungal, especially anticandidal, activity of this plant has not been reported.

CHAPTER III

MATERIALS AND METHODS

3.1 Chemical sources

All chemicals were purchased from various suppliers (Carlo Erba, Himedia, Merck, and Sigma-Aldrich) as listed in Table 3.1.

Table 3.1 List of used chemicals.

Chemicals	Supplier
Acetone	Carlo Erba, Italy
D-Glucose	Carlo Erba, Italy
Ethanol	Carlo Erba, Italy
Ethyl acetate	Carlo Erba, Italy
Galangin	Sigma-Aldrich, USA
Ketoconazole	Sigma-Aldrich, USA
Methanol	Carlo Erba, Italy
Peptone	Himedia, India

Table 3.1 List of used chemicals (Continued).

Analytical grade chemicals	Supplier
Acetone	Carlo Erba, Italy
Hexane	Carlo Erba, Italy
HPLC grade chemicals	**Supplier**
Methanol	Sigma-Aldrich, USA
Phosphoric acid	Sigma-Aldrich, USA

Silica gel 60 F_{254} precoated TLC aluminum sheet (20 × 20 cm^2 in size with layer thickness of 0.2 mm) was purchased from Merck, USA. Standard galangin was bought from Sigma-Aldrich, USA.

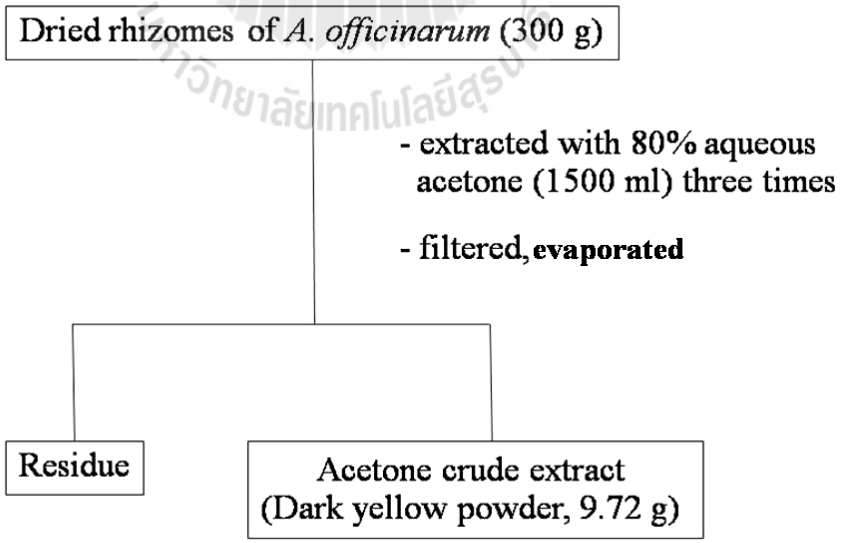
3.2 Plant material

A. officinarum was bought from local markets in Nakhon Ratchasima province, Thailand. The rhizomes of lesser galangal were separated from the stem, washed thoroughly, and oven dried at 50 °C for 3 days. The sample was then grounded to powder and kept in -20 °C until used. The dried rhizomes of *A. officinarum* (300 g) were extracted with 1500 ml of 80% aqueous acetone three times at room temperature. The extracted solutions were filtered through filter paper (Whatman No.1). The filtrate was concentrated by evaporation under reduced pressure to obtain

a semisolid material, which was then lyophilized by freeze dryer. The extract (yield: 3.24%) was kept at -20 °C until used.

3.3 Extraction

The rhizomes of lesser galangal were separated from the stem, washed thoroughly, and oven dried at 50 °C for 3 days. The sample was then grounded to powder and kept in -20 °C until used. The dried rhizomes of *A. officinarum* (300 g) were extracted with 1500 ml of 80% aqueous acetone three times at room temperature. The extracted solutions were filtered through filter paper (Whatman No.1). The filtrate was concentrated by evaporation under reduced pressure to obtain a semisolid material, which was then lyophilized by freeze dryer. The extract (yield: 3.24%) was kept at -20 °C until used. The extraction is shown in Scheme 3.1.



Scheme 3.1 Extraction of the rhizomes of lesser galangal.

3.4 Antifungal susceptibility testing

Antifungal activity of lesser galangal against *C. albicans* was evaluated using agar disc diffusion method. One hundred microliters of fresh culture suspension (1×10^6 cells/ml) of *C. albicans* was spread on SDA plates. Sterile filter paper discs (5 mm diameter) were impregnated with 10 µl of *A. officinarum* extract (250 µg/disc) or galangin (50 µg/disc). After dried, the discs were placed on the surface of the inoculated SDA plates. The plates were incubated at 30 °C for 24 h. Clear inhibition zone around the discs indicated the presence of antifungal activity. All the tests were performed in triplicate with ketoconazole (15 µg/disc) as positive control and 10 µl of 5% dimethyl sulfoxide (DMSO) (v/v) as vehicle control.

3.5 Determination of minimum inhibitory concentration

The starter of *C. albicans* cultures was prepared by inoculation of the stock culture in fresh Sabouraud dextrose broth (SDB) and incubated for 24 h at 30 °C on a rotary shaker (200 rpm). The cultures were transferred to fresh SDB in dilution 1:100 and further incubated for 12 h due to the growth profiles of *C. albicans* are shown in Figure 3.1.

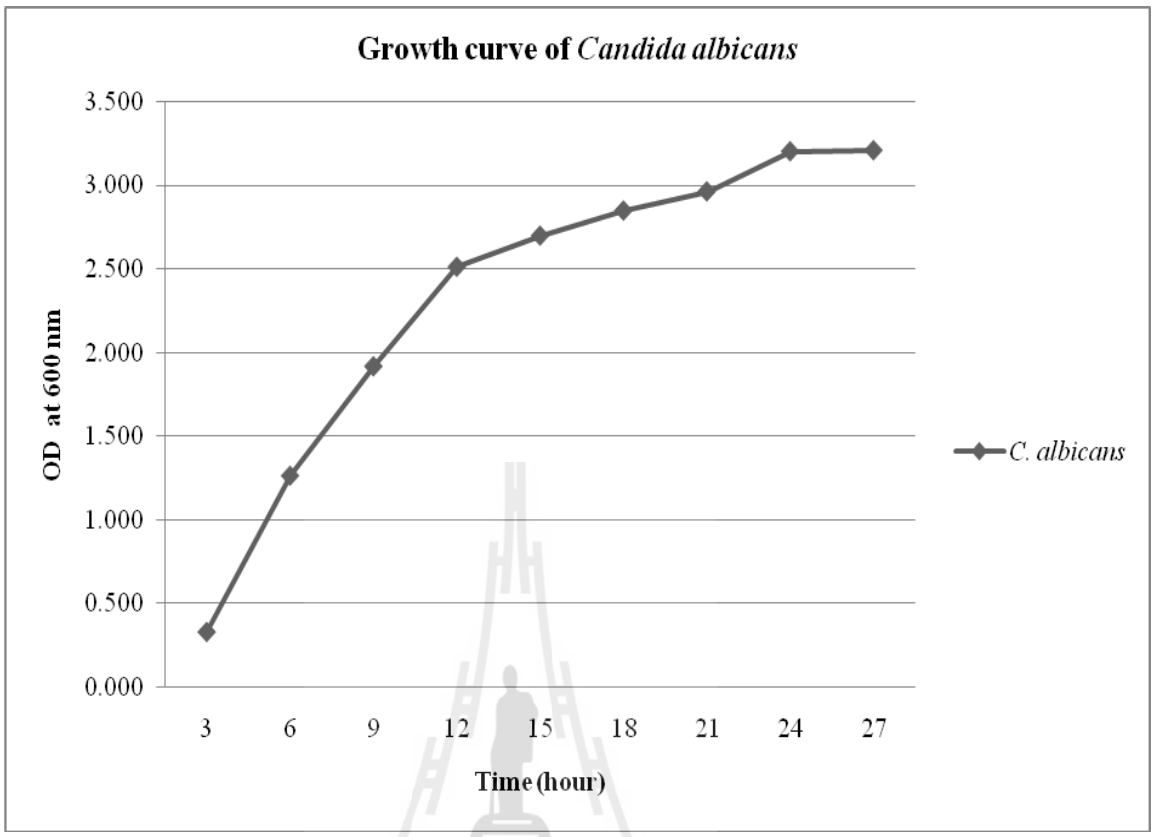


Figure 3.1 Growth profiles of *C. albicans* incubated at 30°C.

After 12 hours of incubation, the concentration of 1×10^6 cells/ml, standardized spectrophotometrically to an absorbance of 2.50 at 600 nm, was used for testing minimum inhibitory concentration (MIC).

The concentration of crude extract at 50 mg/ml was used as stock solution in DMSO. Three milliliters of SDB containing various concentrations of the extract and 30 µl of *C. albicans* (10^6 cells/ml) were mixed in test tube and incubated at 30 °C. The growth of *C. albicans* was spectrophotometrically monitored. The MIC is defined as the lowest concentration that inhibits the growth of *C. albicans* at the end of 24 h of incubation.

3.6 Determination of minimum fungicidal concentration

Based on the result from MIC method, cells from the tubes showing no growth were subcultured on SDA plates and incubated at 30 °C for 24 hours. The MFC is defined as the lowest concentration that causes total inhibition of visible growth of *C. albicans* at the end of 24 hours of incubation. All the tests were done in three replicates.

3.7 Isolation of galangin from the extract by thin layer chromatography (TLC)

Two hundred and fifty micrograms of the crude extract were applied to silica gel 60 F_{254} for fingerprint compared to standard galangin (0.5 μg). The plates were developed in mobile phase of hexane and ethyl acetate (7:3) and visualized under UV light at 254 nm.

3.8 Quantitative analysis of galangin in the extract of *A. officinarum* by high-performance liquid chromatographic (HPLC) method

The amount of galangin in the acetone extract from rhizomes of *A. officinarum* was examined by high performance liquid chromatographic (HPLC). The HPLC system was equipped with the Waters 600 HPLC system. Separation was performed on a reverse phase column (Novapack ODS, 5 μm, 4.6×250 mm) provided with C18 guard column (C18 Waters, MA, USA) and methanol-water-phosphoric acid (60:38:2) were used as the mobile phase. The flow rate was constantly kept at 0.8

ml/min and peaks were identified by using UV absorbance at 254 nm. The temperature of the column during analysis was maintained at 40 °C and the volume of 10 µl of the sample was injected each time.

3.9 Determination of inhibition of growth by bioautography

For bioautographic analysis, developed TLC plates were carefully dried for complete removal of solvent, overlaid with agar containing an aliquot of *C. albicans* culture (10^6 cells/ml) and incubated at 30 ºC for 24 h. The areas of inhibition were compared with the retention factor (R_f) of the galangin. The clear zones indicated the presence of compounds in the extract that inhibited the growth of *C. albicans*.

3.10 Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) was performed on *C. albicans* cells. *C. albicans* cells at the concentration of 1×10^6 cells/ml were added into 3 ml of SDB and incubated at 30 ºC. After incubated for 24 h, *C. albicans* cells were treated with the crude extract of *A. officinarum* at the concentration of 0.8, 1.2, 1.6 and 2.0 mg/ml and then incubated for further 36 h. The DMSO-treated culture was used as vehicle control. Cell pellets were fixed with 2.5% glutaraldehyde in carcodylate buffer overnight and washed three times in 0.1 M carcodylate buffer solution (pH 7.2). Then, the cells were post fixed with 1% osmium tetroxide (OsO_4) in 0.1 M carcodylate buffer solution (pH 7.2) for 1 h and washed again with 0.1 M carcodylate buffer solution (pH 7.2) for 15 minutes three times. Then each suspension was serially dehydrated with 30%, 50%, 75%, 90% and 100% ethanol for 15 minutes each. Then the cells were freeze dried in critical point dryer. The dried cells were coated with

gold for SEM study. The analytical condition for SEM was as the following: L = SE1, WD = 17 mm, and EHT = 10.0 kV.

3.11 Biofilm assay

Many types of materials have been used for growing *Candida* biofilms in vitro, including polystyrene (Kuhn, Chandra, Mukherjee, and Ghannoum, 2002; Tarcin, 2011), polyvinyl chloride (Hawser and Douglas, 1994), silicone elastomer and polymethylmethacrylate (Chandra *et al.*, 2001). Although polystyrene is not a material used in indwelling medical devices, it has been used widely for *in vitro* diagnostics and shown to be an excellent material for promoting adherence of cells. In addition, a standardized method for biofilm formation based on polystyrene 96-well plates has been established (Li, Yan, and Xu, 2003). But in this study polystyrene 24-well plates has been used.

3.11.1 Direct microscopic analysis

The inhibitory effect of the extract on biofilm formation was examined by microscopic analysis. *C. albicans* was cultured in SDB at 30 °C for 24 h. The *C. albicans* starter (30 μl) was pipette into each well of 24-well plates in the presence of 3 ml SDB and incubated at 37 °C for 9 h (adhesion period). Supernatant including planktonic cells was then discarded and wells were gently washed twice with PBS to get rid of any non-adherent cells. For inhibition of biofilm growth, the crude extract was then added to each well in the final concentrations of 0.8, 1.2, 1.6 and 2.0 mg/ml. The plates were covered, wrapped with parafilm to prevent evaporation and incubated at 37 °C for further 48 h. After the incubation period, planktonic cells were discarded through three rounds of washing with 2 ml sterile PBS buffer. DMSO-treated cells

were used as vehicle control. The inhibitory effect on biofilm formation was observed under phase contrast microscope.

3.11.2 Spectroscopic examination

Biofilm formation was prepared using the same method described in section 3.11.1. After treated with the extract for 48 h, the medium and non-adherent cells were removed from wells and washed three times with sterile phosphate buffer solution (PBS) and dried in the room temperature for 15 minutes. Then 1% crystal violet was added to stain the biofilms in the wells. After that the unadsorbed stain were removed by washing with PBS 3 times. The wells were destained by 2 ml of ethanol and the destained solution was transferred to a new plate. Optical density of cells was determined for each suspension at 590 nm (Benchmark Plus Microplate spectrophotometer, Bio-Rad, USA).

3.12 Statistical analysis

Data are expressed as mean ± S.D. Comparison among different groups was performed by analysis of variance (ANOVA) followed by Student-Newman-Keuls test. *P*- values less than 0.05 were set as the level of significance.

CHAPTER IV

RESULTS

4.1 Antifungal activity of *A. officinarum* extract

By disc diffusion method, 250 μg of the extract and 15 μg of ketoconazole (positive control) produced 11.0 and 23.0 mm diameter of inhibition zone (Table 4.1), respectively, whereas 100 μg of galangin produced only 8 mm diameter of clear zone. These results indicated that both galangin and the extract possessed antifungal effect against *C. albicans* with lower potency than ketoconazole.

Table 4.1 Antifungal activity of *A. officinarum* rhizome extract against *C. albicans*.

Microorganism	Diameters of inhibition zone (nm)		Positive control
	The extract (250 μg)	Galangin (100 μg)	Ketoconazole (15 μg)
C. albicans	11.0 ± 1.0	8.0 ± 1.0	23.0 ± 1.0

4.2 Isolation and quantitative analysis of galangin in the extract of *A. officinarum*

To further investigate whether galangin was accountable for anticandidal activity, the content of galangin in the extract was determined. At first, 80% acetone crude extract was subjected to HPLC analysis, however it was found that the crude extract of *A. officinarum* contained relatively low concentration of galangin which

could not be detected by HPLC as shown in Figure 4.1A. Figure 4.1B represented a typical HPLC chromatogram of galangin standard (retention time: 23.4 min). In order to increase its concentration in the sample, galangin was isolated from the crude extract by TLC. Figure 4.2 showed that galangin was isolated from the crude extract co-migrating with galangin standard. The scraped area was eluted with acetone and then the acetone extract from the scraped silica gel was reanalyzed by HPLC (Figure 4.3) and calculated for its concentration. The result showed that the content of galangin in the extract was 3.8 mg/g crude extract.

4.3 Bioautography detection in TLC

Bioautography, a microbiological screening method, is commonly used for the detection of antimicrobial activity. In this study, bioautography was conducted to investigate whether galangin was the active ingredient responsible for anticandidal activity of *A. officinarum* extract. First, 250 μg of *A. officinarum* extract was separated by solvent system (hexane, ethyl acetate (7:3) on TLC (Figure 4.4). From bioautography analysis, it was found that 10 μg of galangin which is about 10 times more than the equivalent dose of galangin in the extract (0.85 μg/250 μg) showed no effect on candidal growth. The clear zone on bioautograph of the extract appeared on the spotting position as shown in Figure 4.5.

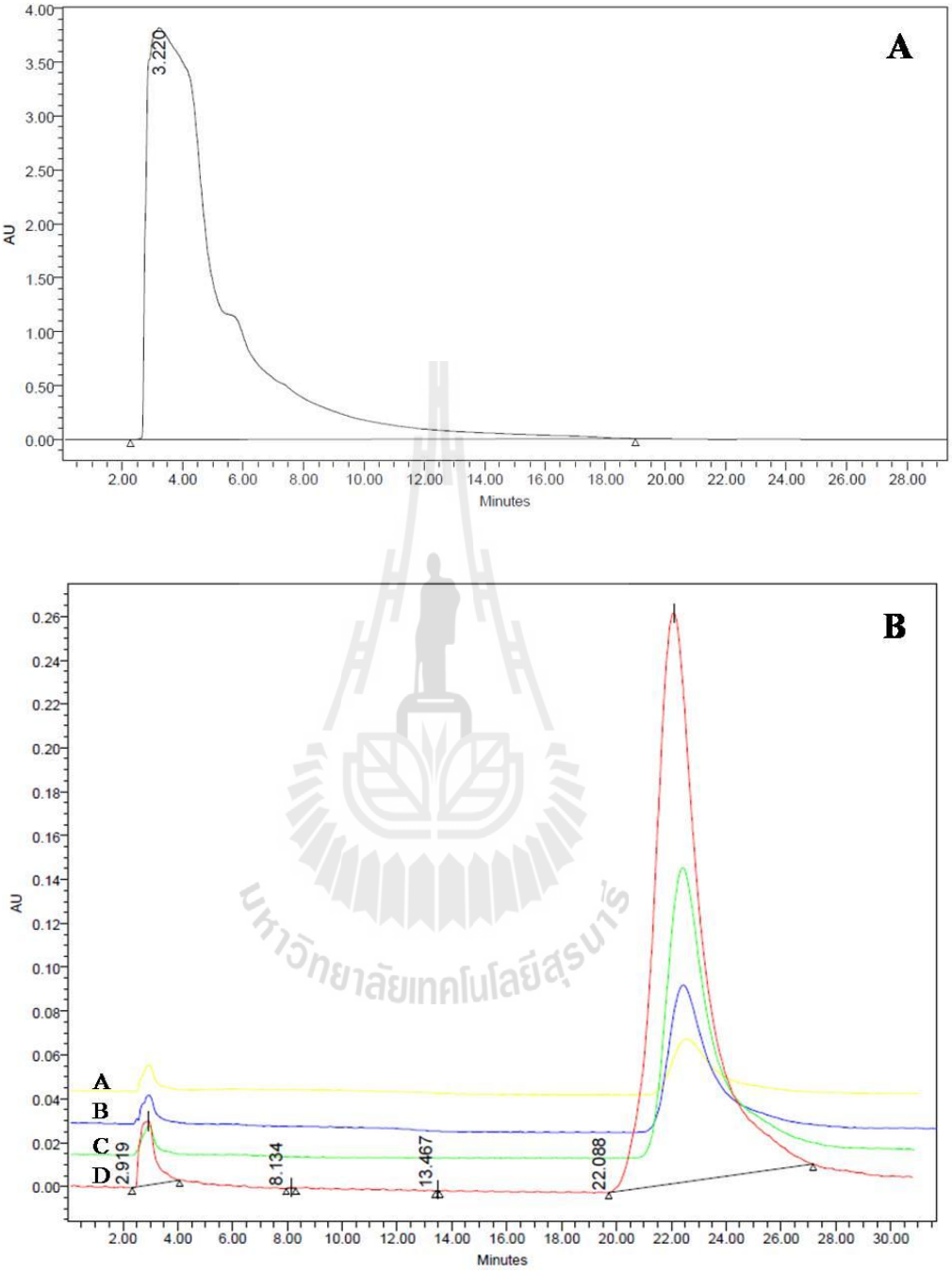


Figure 4.1 HPLC chromatograms of *A. officinarum* extract (A) and standard galangin (B). Panel A showed no detectable peak at the retention time of 23.4 min. In panel B, A-D are standard galangin at the concentration of 0.01, 0.025, 0.05 and 0.1 mg/ml, respectively.

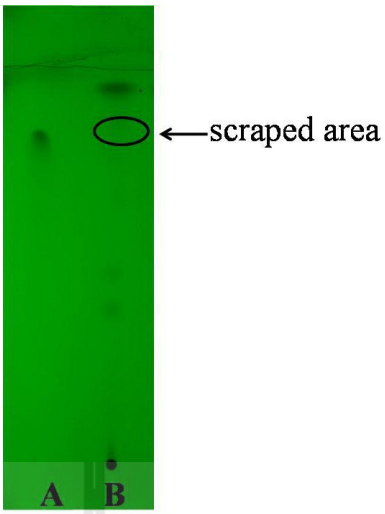


Figure 4.2 The scraped area from TLC. Lane A is the galangin standard (10 μg) whereas lane B is the extract of *A. officinarum* (250 μg).

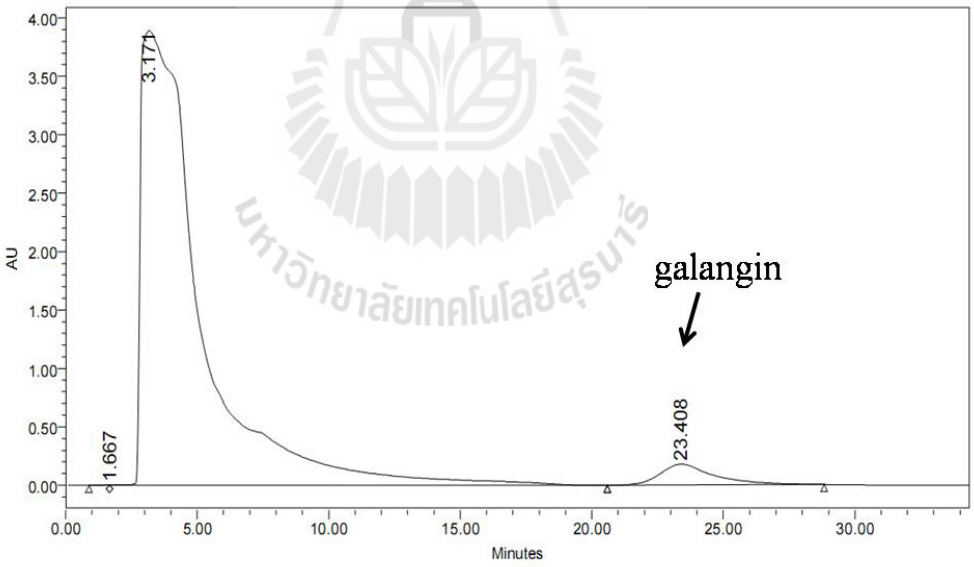


Figure 4.3 HPLC chromatogram of the acetone extract from silica gel. Galangin was detected at the retention time of 23.4 min and the calculated concentration was 3.38 mg/g extract.

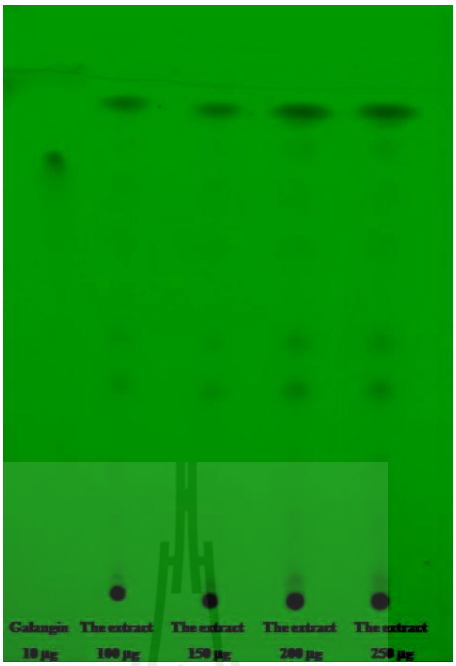


Figure 4.4 Representative image of TLC plates of the *A. officinarum* extract. The observation was done at 254 nm along with the standard galangin.

Figure 4.5 TLC-direct bioautograph of *A. officinarum* extract against *C. albicans*.

4.4 Minimum inhibitory concentration (MIC) of the extract

The MIC is defined as the lowest concentration that inhibits the growth of *C. albicans* at the end of 24 h of incubation. The inhibitory effect of lesser galangal on *C. albicans* is shown in Figure 4.6. It was found that the extract exhibited antifungal activity against *C. albicans* with MIC at 1.2 mg/ml.

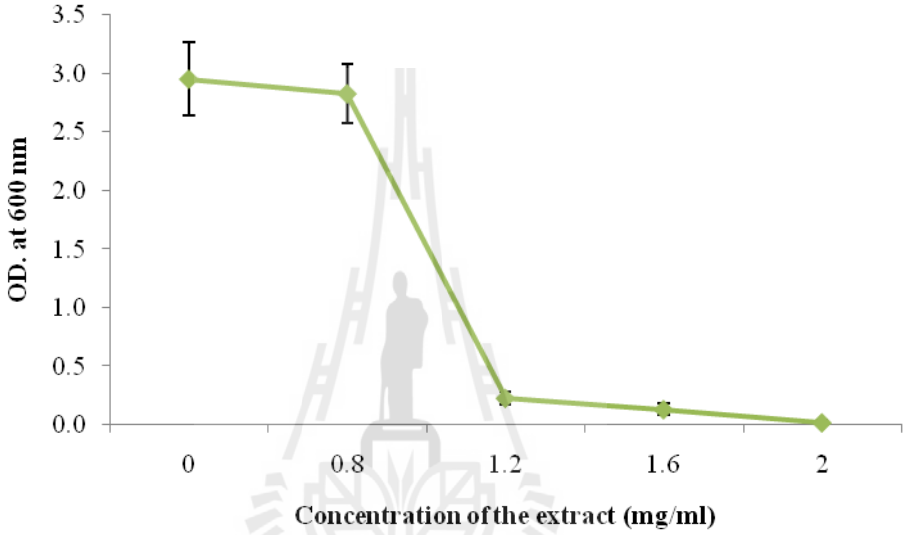


Figure 4.6 The inhibitory effect of lesser galangal on growth of *C. albicans* (n = 3).

4.5 Minimum fungicidal concentration (MFC) of the extract

The MFC is defined as the lowest concentration that causes total inhibition of visible growth of *C. albicans* at the end of 24 h of incubation. It was found that the MFC value of the extract was 2.0 mg/ml.

4.6 Morphological changes observed by SEM

In order to investigate a mechanism underlie the antifungal activity of the extract, scanning electron microscopy (SEM) was conducted. SEM analysis showed that the extract induced deformation of *C. albicans* in dose-response manner. Figure

4.8 showed that cell changed from smooth (Figure 4.8A: untreated cells) to coarse surface when treated with low doses as shown in Figure 4.8C, 4.8D and 4.8E, and from oval to rounder shape when treated with higher dose (MFC) as shown in Figure 4.8F.

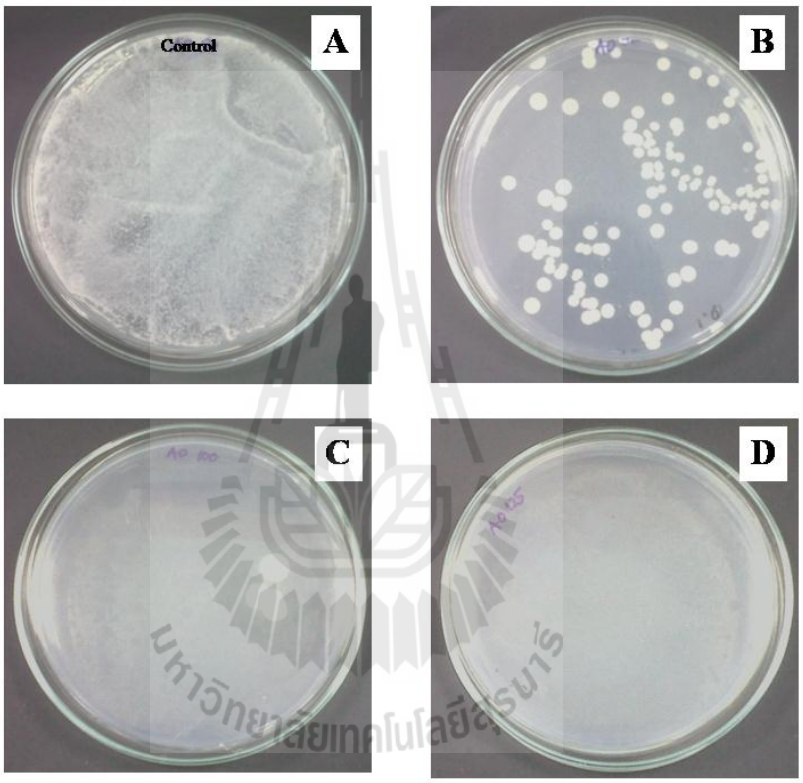


Figure 4.7 MFC of the extract on *C. albicans*. A: untreated *C. albicans*, B-D: cells treated with 1.2, 1.6 and 2.0 mg/ml extract, respectively. There was no fungal growth on the plate treated with the extract at the concentration of 2.0 mg/ml (panel D).

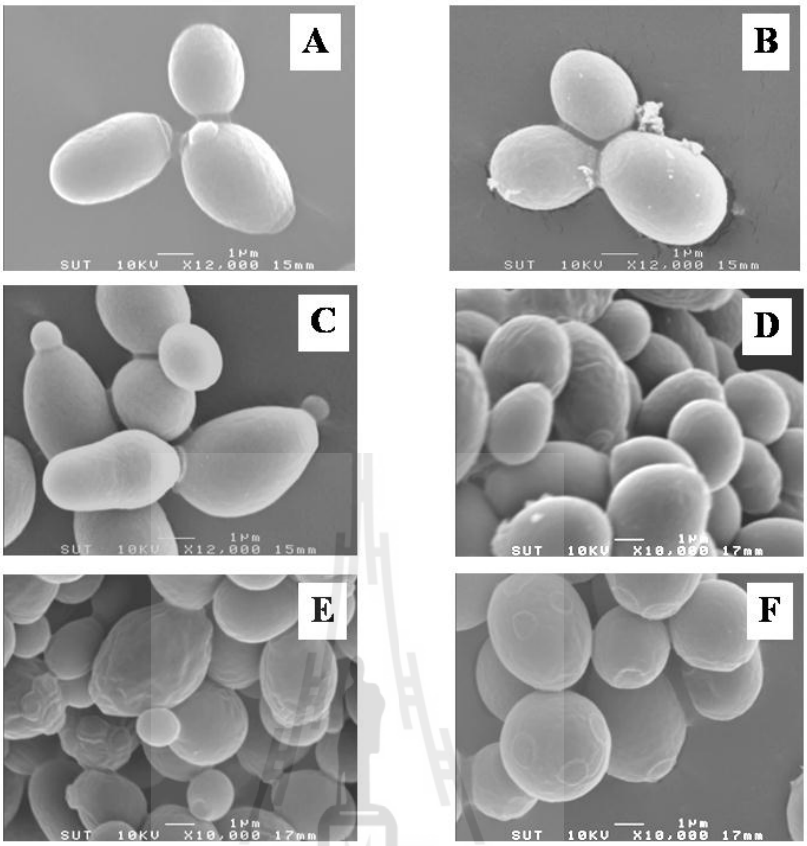


Figure 4.8 SEM micrographs of *C. albicans* treated with the extract of *A. officinarum*. Cells were treated as described in Materials and Methods. Coarse surfaces of the damaged cells were seen in the cells treated with the extract at 0.8 (C) and 1.2 mg/ml (D), and more clearly seen in higher concentration (E: 1.6 mg/ml). The cell treated with MFC at 2.0 mg/ml (F) of *A. officinarum* extract changed from oval to rounder shape. 5% DMSO (B) did not affect morphology of the candidal cells compared to control (A).

4.7 Effect of the extract on biofilm formation

Crystal violet staining of surface-attached cells by microtiter plate assay is a convenient method for quantitative detection of *C. albicans* biofilm formation (Kuhn, Chandra, Mukherjee, and Ghannoum, 2002). In this study the effect of the extract on

biofilm formation was investigated by direct microscopic analysis and spectroscopic determination at 590 nm. Figure 4.9 showed phase contrast microscopic images of *C. albicans* treated with various concentrationa of *A. officinarum* extract. It was found that *C. albicans* formed vast empty regions on plate surface with clusters of cells when treated with the extract at the concentration of 0.8 (C), 1.2 (D), 1.6 (E) and 2.0 (F) mg/ml compared to control (A) and DMSO-treated cells (B). In accordance, the result from spectroscopic measurement as shown in Figure 4.10 revealed that *A. officinarum* extract had inhibitory effect on biofilm formation in dose-dependent fashion.

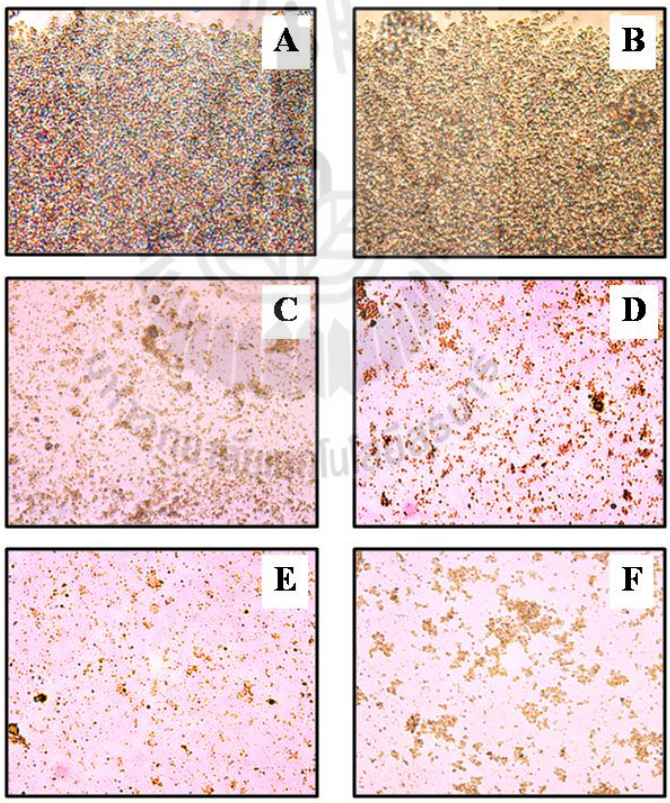


Figure 4.9 Microscopic images on biofilm formation of *C. albicans* treated with *A. officinarum* extract. Cells treated with the extract at the concentration of 0.8 (C), 1.2 (D), 1.6 (E) and 2.0 mg/ml (F) formed less biofilm than sample (A) and DMSO-treated cells (B).

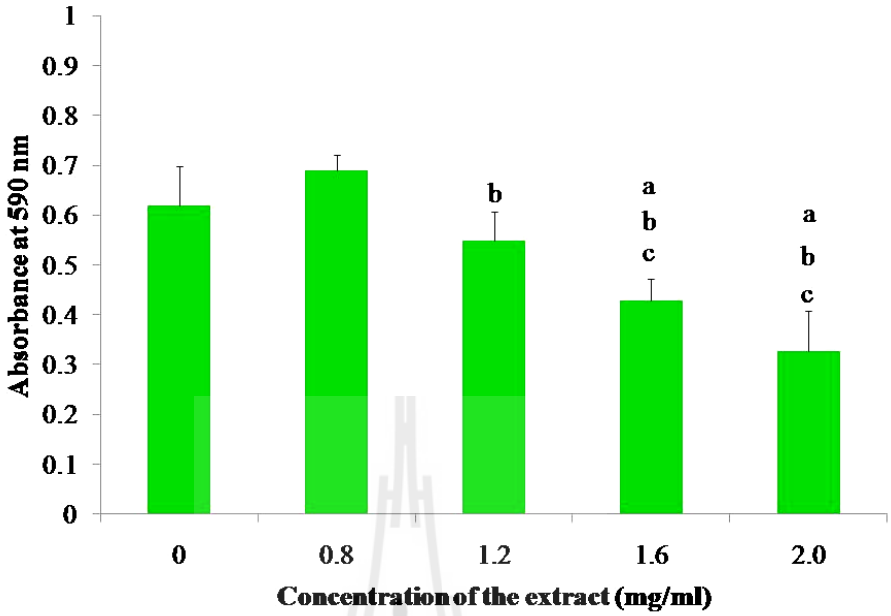


Figure 4.10 The inhibitory effect of the extract on *C. albicans* biofilm by spectroscopic analysis. [a, b,] and [c] statistically significant difference from control, 0.8 and 1.2 mg/ml, respectively ($p < 0.05$).

CHAPTER V

DISCUSSION AND CONCLUSION

The various therapeutic effects of lesser galangal such as anti-emetic, anti-inflammatory, anti-nociceptive, anti-psychiatric, anticancer and antioxidant activities, have been previously reported (An *et al*., 2008; Lee *et al*., 2009; Shin, Kinoshita, Koyama, and Takahashi, 2002; Zhang, Dai, Liao, and Ding, 2010). The antibacterial activities of *A. officinarum* have also been documented by several groups of researchers. For example, Huang and coworkers (Huang, Wu, Tian, Ma, and Wu, 2008) showed that 40% ethanolic extract of the rhizomes of *A. officinarum* possessed antibacterial activity against *Staphylococcus aureus*, hemolytic streptococcus and *S. pneumonia*. Moreover, a group of investigators from India found that galangal extracts by three different methods showed moderate to potent antimicrobial activity against *Bacillus cereus, S. aureas, Pseudomonas auroginosa,* and *Escherichia coli* (Srividya, Dhanabal, Misra, and Suja, 2010). Active compounds of *A. officinarum* rhizomes which are responsible for antimicrobial activities have been identified. Diarylheptanoids have been isolated and shown that they strongly inhibit growth of *Helicobactor pylori* (Zhang *et al*., 2010). The flavonol galangin has been reported to be a major component of *A. officinarum* (Cushnie and Lamb, 2006) as well as several other medicinal natural products including *Helichrysum aureonitens* and propolis (Patel, Patel, Gadewar, and Tahilyani, 2012). Data from several studies suggested that

galangin be responsible for the antibacterial activity of natural products from plants (Cushnie and Lamb, 2006).

In the present study, antifungal activity of *A. officinarum* rhizome extract against *C. albicans* was evaluated. Contrary to the previous study reported by Srividya and colleagues (Srividya *et al*., 2010), it was found that 80% acetone extract of *A. officinarum* rhizomes had anticandidal activity. An explanation could be the different methods used in extraction. From the previous report, the hydro-alcoholic extract by hot and cold maceration and the methanol extract by percolation did not show such antifungal activity.

As mentioned earlier that galangin has been proposed to be responsible for antibacterial activities of several natural products, it was skeptical whether it was also responsible for anticandidal activity found in this study. By disc diffusion method, 250 μg of the extract and 15 μg of ketoconazole (positive control) produced 11.0 and 23.0 mm diameter of clear zone, respectively, whereas 100 μg of galangin produced only 8.0 mm diameter of clear zone. These results indicated that both galangin and the extract possessed antifungal effect against *C. albicans*, but lower potency than ketoconazole.

Because galangin was reported to be a major compound in *A. officinarum* and antimicrobial agent, an investigation whether galangin was accountable for anticandidal activity was conducted. In this study, the content of galangin in the extract was determined. The crude extract of *A. officinarum* by 80% acetone contained relatively low concentration of galangin. After increasing its concentration in the sample by TLC, the content of galangin in the extract appear to 3.8 mg/g crude extract which is in the range previously reported (2.63-11.6 mg/g) by Tao and

coworker (Tao, Wang, Zhu, Lu, and Wei, 2006). Bioautography was conducted to investigate whether galangin was the active ingredient. It was found that 10 μg of galangin which is about 10 times more than the equivalent dose of galangin in the extract (0.85 μg/250 μg) showed no effect on candidal growth. The clear zone on bioautography appeared on the spotting position, not galangin. This result indicated that galangin was not responsible for antifungal activity of the extract of *A. officinarum* rhizomes found in this study. According to this result, galangin was excluded from further experiments. Macro-diffusion method was performed to further determine MIC and MFC of the extract. It was found that the extract exhibited antifungal activity against *C. albicans* with MIC and MFC at 1.2 and 2.0 mg/ml respectively.

Application of SEM analysis of fungal cells has been recommended as useful tool for providing lifelike three-dimensional visualization at high resolution. For fungi, SEM is commonly used to document morphology and spatial relationships (Kaminskyj and Dahms, 2008; Tyagi and Malik, 2010). In this study, SEM analysis of the *Candida* cells revealed changes in morphology after treatment with the crude extract. The *Candida* cells treated with the extract at MIC had coarse surface (Figure 4.8D), and changed from oval to rounder shape (Figure 4.8F) when treated with MFC. These results suggested that the extract possess antifungal activity possibly by damaging cell wall, causing *C. albicans* to form spheroplast and finally death. However, this postulated mechanism needs more experiments to confirm the actual damage of yeast cell wall.

A number of previous studies have shown that *Candida* biofilms exhibit increased resistance to antifungals. Surface roughness has been shown to influence the

adhesion of microorganisms to medical devices such as catheters and dental implants (Dohnt, Sauer, Müller, Atallah, Weidemann, Gronemeyer, Rasch, Tielen, and Krull 2011; Douglas, 2002; Estivill, Arias, Torres-Lana, Carrillo-Muñoz, and Arévalo, 2011; Tarcin, 2011). Although none of many hypotheses has not succeeded to clearly explain the phenomenon of increased resistance caused by *Candida* biofilms, any agents which can inhibit its biofilm formation would be clinically useful. An inhibitory effect on *Candida* biofilm formation was investigated using phase contrast microscopy and spectrometry. It appeared that both MIC and MFC of the crude extract prevented biofilm formation of *C. albicans*.

In conclusion, the result from the present study showed that 80% acetone extract of *A. officinarum* rhizomes or lesser galangal possessed antifungal activity against *C. albicans* with MIC and MFC of 1.2 and 2.0 mg/ml respectively. It also has inhibitory effect on biofilm formation. The extract obtained from this study contained galangin, which was earlier reported to be a major component, at the amount of 3.8 mg/g extract. Although galangin exhibited antifungal activity against *C. albicans*, it was not the major active ingredient for this activity because its content in the extract is too low. For mechanism underlying the antifungal activity, the results suggested that the extract may cause cell wall damage, resulting in cell death. However, this postulated mechanism needs more experiments to confirm the actual disruption of yeast cell wall. Moreover, the active ingredient(s) responsible for this action need further investigation.

REFERENCES

REFERENCES

Abi-Said, D., Anaissie, E., Uzun, O., Raad, I., Pinzcowski, H., and Vartivarian, S. (1997). The epidemiology of hematogenous candidiasis caused by different *Candida* species. **Clinical Infectious Diseases**. 24(6): 1122-1128.

An, N., Zhang, H. W., Xu, L. Z., Yang, S. L., and Zou, Z. M. (2009). New diarylheptanoids from the rhizome of *Alpinia officinarum* Hance. **Food Chemistry**. 119(2): 513-517.

An, N., Zou, Z. M., Tian, Z., Luo, X. Z., Yang, S. L., and Xu, L. Z. (2008). Diarylheptanoids from the rhizomes of *Alpinia officinarum* and their anticancer activity. **Fitoterapia**. 79(1): 27-31.

Andes, D., Nett, J., Oschel, P., Albrecht, R., Marchillo, K., and Pitula, A. (2004). Development and characterization of an *in vivo* central venous catheter *Candida albicans* biofilm model. **Infection and Immunity**. 72(10): 6023-6031.

Asakura, K., Iwaguchi, S., Homma, M., Sukai, T., Higashide, K., and Tanaka, K. (1991). Electrophoretic karyotypes of clinically isolated yeasts of *Candida albicans* and *C. glabrata*. **Journal of General Microbiology**. 137(11): 2531-2538.

Athar, M. A., and Winner, H. I. (1971). The development of resistance by *Candida* species to polyene antibiotics *in vitro*. **Journal of Medical Microbiology**. 4(4): 505-517.

Baillie, G. S., and Douglas, L. J. (1999). Role of dimorphism in the development of *Candida albicans* biofilms. **Journal of Medical Microbiology**. 48(7): 671-679.

Beggs, W. H. (1983). Comparison of miconazole- and ketoconazole-induced release of K^+ from *Candida* species. **Journal of Antimicrobial Chemotherapy**. 11(4): 381-383.

Birnbaum, J. E. (1990). Pharmacology of the allylamines. **Journal of the American Academy of Dermatology**. 23(4): 782-785.

Borromeo, G. L., McCullough, M. J., and Reade, P. C. (1992). Quantitation and morphotyping of *Candida albicans* from healthy mouths and from mouths affected by erythematous candidosis. **Journal of Medical and Veterinary Mycology**. 30(6): 477-480.

Bow, E. J., Evans, G., Fuller, J., Laverdiere, M., Rotstein, C., Rennie, R., .Vinh, D. C. (2010). Canadian clinical practice guidelines for invasive candidiasis in adults. **The Canadian Journal of Infectious Diseases and Medical Microbiology**. 21(4): e122-150.

Bulad, K., Taylor, R. L., Verran, J., and McCord, J. F. (2004). Colonization and penetration of denture soft lining materials by *Candida albicans*. **Dental Materials**. 20(2): 167-175.

Calderone, R., Suzuki, S., Cannon, R., Cho, T., Boyd, D., Calera, J., and Watanabe, T. (2000). *Candida albicans*: adherence, signaling and virulence. **Medical Mycology**. 38(1): 125-137.

Calderone, R. A., and Braun, P. C. (1991). Adherence and receptor relationships of *Candida albicans*. **Microbiological Reviews**. 55(1): 1-20.

Calderone, R. A., and Fonzi, W. A. (2001). Virulence factors of *Candida albicans*. **Trends in Microbiology.** 9(7): 327-335.

Chaffin, W. L., Lopez-Ribot, J. L., Casanova, M., Gozalbo, D., and Martinez, J. P. (1998). Cell wall and secreted proteins of *Candida albicans*: identification, function, and expression. **Microbiology and Molecular Biology Reviews**. 62(1): 130-180.

Chandra, J., Kuhn, D. M., Mukherjee, P. K., Hoyer, L. L., McCormick, T., and Ghannoum, M. A. (2001). Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. **Journal of Bacteriology**. 183(18): 5385-5394.

Charlier, C., Hart, E., Lefort, A., Ribaud, P., Dromer, F., Denning, D. W., and Lortholary, O. (2006). Fluconazole for the management of invasive candidiasis: where do we stand after 15 years?. **Journal of Antimicrobial Chemotherapy**. 57: 384-410.

Chattaway, F. W., Holmes, M. R., and Barlow, A. J. (1968). Cell wall composition of the mycelial and blastospore forms of *Candida albicans*. **Journal of General Microbiology**. 51(3): 367-376.

Choi, J. H., Lee, C. G., Lim, Y. J., Kang, H. W., Lim, C. Y., and Choi, J. S. (2013). Prevalence and risk factors of esophageal candidiasis in healthy individuals: a single center experience in Korea. **Yonsei Medical Journal**. 54(1): 160-165.

Como, J. A., and Dismukes, W. E. (1994). Oral azole drugs as systemic antifungal therapy. **The New England Journal of Medicine**. 330(4): 263-272.

Crump, J. A., and Collignon, P. J. (2000). Intravascular catheter-associated infections. **European Journal of Clinical Microbiology and Infectious Diseases**. 19(1): 1-8.

Cruz, M. C., Goldstein, A. L., Blankenship, J. R., Del Poeta, M., Davis, D., Cardenas, M. E., and Heitman, J. (2002). Calcineurin is essential for survival during membrane stress in *Candida albicans*. **EMBO Journal**. 21(4): 546-559.

Cushnie, T. P., and Lamb, A. J. (2006). Assessment of the antibacterial activity of galangin against 4-quinolone resistant strains of *Staphylococcus aureus.* **Phytomedicine**. 13(3): 187-191.

Cushnie, T. P. T., and Lamb, A. J. (2005). Antimicrobial activity of flavonoids. **International Journal of Antimicrobial Agents**. 26(5): 343-356.

de Kruijff, B., and Demel, R. A. (1974). Polyene antibiotic-sterol interactions in membranes of *Acholeplasma laidlawii* cells and lecithin liposomes. 3. Molecular structure of the polyene antibiotic-cholesterol complexes. **Biochimica et Biophysica Acta**. 339(1): 57-70.

Donlan, R. M. (2002). Biofilms: microbial life on surfaces. **Emerging Infectious Diseases**. 8(9): 881-890.

Dohnt, K., Sauer, M., Muller, M., Atallah, K., Weidemann, M., Gronemeyer, P., and Krull, R. (2011). An *in vitro* urinary tract catheter system to investigate biofilm development in catheter-associated urinary tract infections. **Journal of Microbiological Methods**. 87(3): 302-308.

Douglas, L. J. (2002). Medical importance of biofilms in *Candida* infections. **Revista Iberoamericana de Micologia**. 19(3): 139-143.

Douglas, L. J. (1988). *Candida* proteinases and candidosis. **Critical Reviews in Biotechnology**. 8(2): 121-129.

Ellis, D. (2002). Amphotericin B: spectrum and resistance. **Journal of Antimicrobial Chemotherapy**. 49(1): 7-10.

Espinel-Ingroff, A., and Shadomy, S. (1989). *In vitro* and *in vivo* evaluation of antifungal agents. **European Journal of Clinical Microbiology and Infectious Diseases**. 8(4): 352-361.

Estivill, D., Arias, A., Torres-Lana, A., Carrillo-Muñoz, A. J., and Arevalo, M. P. (2011). Biofilm formation by five species of *Candida* on three clinical materials. **Journal of Microbiological Methods**. 86(2): 238-242.

Fridkin, S. K., and Jarvis, W. R. (1996). Epidemiology of nosocomial fungal infections. **Clinical Microbiology Reviews**. 9(4): 499-511.

Ghannoum, M. A., and Rice, L. B. (1999). Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. **Clinical Microbiology Reviews**. 12(4): 501-517.

Ghannoum, M. A., Spellberg, B. J., Ibrahim, A. S., Ritchie, J. A., Currie, B., Spitzer, E. D., and Casadevall, A. (1994). Sterol composition of *Cryptococcus neoformans* in the presence and absence of fluconazole. **Antimicrobial Agents and Chemotherapy**. 38(9): 2029-2033.

Grieve, M. (1995-2013). Galangal: Botanical of galangal. (online). Available: http://www.botanical.com/botanical/mgmh/g/galang01.html.

Guo, A. J. Y., Xie, H. Q., Choi, R. C. Y., Zheng, K. Y. Z., Bi, C. W. C., Xu, S. L., and Tsim, K. W. K. (2010). Galangin, a flavonol derived from Rhizoma

Alpinia officinarum, inhibits acetylcholinesterase activity *in vitro*. **Chemico-Biological Interactions**. 187(1-3): 246-248.

Hasan, F., Xess, I., Wang, X., Jain, N., and Fries, B. C. (2009). Biofilm formation in clinical *Candida* isolates and its association with virulence. **Microbes and Infection**. 11(8-9): 753-761.

Hamilton-Miller, J. M. (1972). Physiological properties of mutagen-induced variants of *Candida albicans* resistant to polyene antibiotics. **Journal of Medical Microbiology**. 5(4): 425-440.

Heo, M. Y., Jae, L. H., Jung, S. S., and Au, W. W. (1996). Anticlastogenic effects of galangin against mitomycin C-induced micronuclei in reticulocytes of mice. **Mutation Research**. 360(1): 37-41.

Heo, M. Y., Sohn, S. J., and Au, W. W. (2001). Anti-genotoxicity of galangin as a cancer chemopreventive agent candidate. **Mutation Research**. 488(2), 135-150.

Hitchcock, C. A., Barrett-Bee, K. J., and Russell, N. J. (1987). The lipid composition and permeability to azole of an azole- and polyene-resistant mutant of *Candida albicans*. **Journal of Medical and Veterinary Mycology**. 25(1): 29-37.

Hitchcock, C. A., Dickinson, K., Brown, S. B., Evans, E. G., and Adams, D. J. (1990). Interaction of azole antifungal antibiotics with cytochrome P-450-dependent 14 alpha-sterol demethylase purified from *Candida albicans*. **Biochemical Journal**. 266(2): 475-480.

Holz, R. W. (1974). The effect of the polyene antibiotics nystatin and amphotericin B on thin lipid membranes. **Annals of the New York Academy of Sciences**. 235(1): 469-479.

Huang, H., Wu, D., Tian, W. X., Ma, X. F., and Wu, X. D. (2008). Antimicrobial effect by extracts of rhizome of *Alpinia officinarum* Hance may relate to its inhibition of β-ketoacyl-ACP reductase. **Journal of Enzyme Inhibition and Medicinal Chemistry**. 23(3): 362-368.

Jenkinson, H. F. (1996). Ins and outs of antimicrobial resistance: era of the drug pumps. **Journal of Dental Research**. 75(2): 736-742.

Jones, D. S., McGovern, J. G., Adair, C. G., Woolfson, A. D., and Gorman, S. P. (2001). Conditioning film and environmental effects on the adherence of *Candida* spp. to silicone and poly (vinylchloride) biomaterials. **Journal of Materials Science. Materials in Medicine**. 12(5): 399-405.

Kaminskyj, S. G. W., and Dahms, T. E. S. (2008). High spatial resolution surface imaging and analysis of fungal cells using SEM and AFM. **Micron**. 39(4): 349-361.

Kauffman, C. A. (2006). Clinical efficacy of new antifungal agents. **Current Opinion in Microbiology**. 9(5): 483-488.

Kim, Y. U., Son, H. K., Song, H. K., Ahn, M. J., Lee, S. S., and Lee, S. K. (2003). Inhibition of 5alpha-reductase activity by diarylheptanoids from *Alpinia officinarum*. **Planta Medica**. 69(1): 72-74.

Kiuchi, F., Iwakami, S., Shibuya, M., Hanaoka, F., and Sankawa, U. (1992). Inhibition of prostaglandin and leukotriene biosynthesis by gingerols and

diarylheptanoids. **Chemical and Pharmaceutical Bulletin (Tokyo)**. 40(2): 387-391.

Kourkoumpetis, T., Manolakaki, D., Velmahos, G., Chang, Y., Alam, H. B., De Moya, M. M., and Mylonakis, E. (2010). *Candida* infection and colonization among non-trauma emergency surgery patients. **Virulence**. 1(5): 359-366.

Krause, W., Matheis, H., and Wulf, K. (1969). Fungaemia and funguria after oral administration of *Candida albicans*. **Lancet**. 1(7595): 598-599.

Kuhn, D. M., Chandra, J., Mukherjee, P. K., and Ghannoum, M. A. (2002). Comparison of biofilms formed by *Candida albicans* and *Candida parapsilosis* on bioprosthetic surfaces. **Infection and Immunity**. 70(2): 878-888.

Kullberg, B. J., Sobel, J. D., Ruhnke, M., Pappas, P. G., Viscoli, C., Rex, J. H., and Hodges, M. R. (2005). Voriconazole versus a regimen of amphotericin B followed by fluconazole for candidaemia in non-neutropenic patients: a randomised non-inferiority trial. **Lancet**. 366(9495): 1435-1442.

Laniado-Laborin, R., and Cabrales-Vargas, M. N. (2009). Amphotericin B: side effects and toxicity. **Revista Iberoamericana de Micologia**. 26(4): 223-227.

Lee, J., Kim, K. A., Jeong, S., Lee, S., Park, H. J., Kim, N. J., and Lim, S. (2009). Anti-inflammatory, anti-nociceptive, and anti-psychiatric effects by the rhizomes of *Alpinia officinarum* on complete Freund's adjuvant-induced arthritis in rats. **Journal of Ethnopharmacology**. 126(2): 258-264.

Lee, Y. S., Kang, O. H., Choi, J. G., Oh, Y. C., Chae, H. S., Kim, J., and Kwon, D. Y. (2008). Synergistic effects of the combination of galangin with gentamicin

against methicillin-resistant *Staphylococcus aureus.* **The Journal of Microbiology**. 46(3): 283-288.

Li, X., Yan, Z., and Xu, J. (2003). Quantitative variation of biofilms among strains in natural populations of *Candida albicans*. **The Journal of Microbiology**. 149(2): 353-362.

Liu, H. (2002). Co-regulation of pathogenesis with dimorphism and phenotypic switching in *Candida albicans*, a commensal and a pathogen. **International Journal of Medical Microbiology**. 292(5–6): 299-311.

Lupetti, A., Danesi, R., Campa, M., Del Tacca, M., and Kelly, S. (2002). Molecular basis of resistance to azole antifungals. **Trends in Molecular Medicine**. 8(2): 76-81.

Ly, T. N., Shimoyamada, M., Kato, K., and Yamauchi, R. (2003). Isolation and characterization of some antioxidative compounds from the rhizomes of smaller galanga (*Alpinia officinarum* Hance). **Journal of Agricultural and Food Chemistry**. 51(17): 4924-4929.

Ly, T. N., Shimoyamada, M., Kato, K., and Yamauchi, R. (2004). Antioxidative compounds isolated from the rhizomes of smaller galanga (*Alpinia officinarum* Hance). **Biofactors**. 21(1-4): 305-308.

Ly, T. N., Yamauchi, R., and Kato, K. (2001). Volatile components of the essential oils in galanga (*Alpinia officinarum* Hance) from Vietnam. **Food Science and Technology Research**. 7(4): 303-306.

Ly, T. N., Yamauchi, R., Shimoyamada, M., and Kato, K. (2002). Isolation and structural elucidation of some glycosides from the rhizomes of smaller galanga

(*Alpinia officinarum* Hance). **Journal of Agricultural and Food Chemistry**. 50(17): 4919-4924.

Mago, N., and Khuller, G. K. (1989). Influence of lipid composition on the sensitivity of *Candida albicans* to antifungal agents. **Indian Journal of Biochemistry and Biophysics**. 26(1): 30-33.

Manavathu, E. K., Cutright, J. L., and Chandrasekar, P. H. (1998). Organism-dependent fungicidal activities of azoles. **Antimicrobial Agents and Chemotherapy**. 42(11): 3018-3021.

Mansfield, B. E., Oltean, H. N., Oliver, B. G., Hoot, S. J., Leyde, S. E., Hedstrom, L., and White, T. C. (2010). Azole drugs are imported by facilitated diffusion in *Candida albicans* and other pathogenic fungi. **PLoS Pathogens**. 6(9): e1001126.

Matsuda, H., Ando, S., Kato, T., Morikawa, T., and Yoshikawa, M. (2006). Inhibitors from the rhizomes of *Alpinia officinarum* on production of nitric oxide in lipopolysaccharide-activated macrophages and the structural requirements of diarylheptanoids for the activity. **Bioorganic and Medicinal Chemistry**. 14(1): 138-142.

Matsuda, H., Nakashima, S., Oda, Y., Nakamura, S., and Yoshikawa, M. (2009). Melanogenesis inhibitors from the rhizomes of *Alpinia officinarum* in B16 melanoma cells. **Bioorganic and Medicinal Chemistry**. 17(16): 6048-6053.

McCullough, M. J., Ross, B. C., and Reade, P. C. (1996). *Candida albicans*: a review of its history, taxonomy, epidemiology, virulence attributes, and methods of strain differentiation. **International Journal of Oral and Maxillofacial Surgery**. 25(2): 136-144.

Merson-Davies, L. A., and Odds, F. C. (1989). A morphology index for characterization of cell shape in *Candida albicans*. **Journal of General Microbiology**. 135(11): 3143-3152.

Michaelis, S., and Berkower, C. (1995). Sequence comparison of yeast ATP-binding cassette proteins. **Cold Spring Harbor Symposia on Quantitative Biology**. 60: 291-307.

Morschhauser, J. (2002). The genetic basis of fluconazole resistance development in *Candida albicans*. **Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease**. 1587(2-3): 240-248.

Nobile, C. J., Bruno, V. M., Richard, M. L., Davis, D. A., and Mitchell, A. P. (2003). Genetic control of chlamydospore formation in *Candida albicans*. **Microbiology**. 149(12): 3629-3637.

Nolting, S., Brautigam, M., and Weidinger, G. (1994). Terbinafine in onychomycosis with involvement by non-dermatophytic fungi. **The British Journal of Dermatology**. 130(43): 16-21.

Norman, A. W., Demel, R. A., de Kruyff, B., Geurts van Kessel, W. S., and van Deenen, L. L. (1972). Studies on the biological properties of polyene antibiotics: comparison of other polyenes with filipin in their ability to interact specifically with sterol. **Biochimica et Biophysica Acta**. 290(1): 1-14.

Odds, F. C. (1987). Candida infections: an overview. **Critical Reviews in Microbiology**. 15(1): 1-5.

Orozco, A. S., Higginbotham, L. M., Hitchcock, C. A., Parkinson, T., Falconer, D., Ibrahim, A. S., and Filler, S. G. (1998). Mechanism of fluconazole resistance

in *Candida krusei*. **Antimicrobial Agents and Chemotherapy**. 42(10): 2645-2649.

Ostrosky-Zeichner, L., Oude Lashof, A. M. L., Kullberg, B. J., and Rex, J. H. (2003). Voriconazole salvage treatment of invasive candidiasis. **European Journal of Clinical Microbiology and Infectious Diseases**. 22(11): 651-655.

Patel, D. K., Patel, K., Gadewar, M., and Tahilyani, V. (2012). Pharmacological and bioanalytical aspects of galangin-a concise report. **Asian Pacific Journal of Tropical Biomedicine**. 2(1): S449-S455.

Ramage, G., Saville, S. P., Thomas, D. P., and Lopez-Ribot, J. L. (2005). *Candida* biofilms: an update. **Eukaryot Cell**. 4(4): 633-638.

Raso, G. M., Meli, R., Di Carlo, G., Pacilio, M., and Di Carlo, R. (2001). Inhibition of inducible nitric oxide synthase and cyclooxygenase-2 expression by flavonoids in macrophage J774A.1. **Life Sciences**. 68(8): 921-931.

Ryder, C., Byrd, M., and Wozniak, D. J. (2007). Role of polysaccharides in *Pseudomonas aeruginosa* biofilm development. **Current Opinion in Microbiology**. 10(6): 644-648.

Sable, C. A., Strohmaier, K. M., and Chodakewitz, J. A. (2008). Advances in antifungal therapy. **The Annual Review of Medicine**. 59: 361-379.

Sanati, H., Belanger, P., Fratti, R., and Ghannoum, M. (1997). A new triazole, voriconazole (UK-109,496), blocks sterol biosynthesis in *Candida albicans* and *Candida krusei*. **Antimicrobial Agents and Chemotherapy**. 41(11): 2492-2496.

Sangetha, S., Zuraini, Z., Suryani, S., and Sasidharan, S. (2009). *In situ* TEM and SEM studies on the antimicrobial activity and prevention of *Candida albicans* biofilm by *Cassia spectabilis* extract. **Microscopy**. 40(4): 439-443.

Seddeag, M., Madawe, G., Badwi, S. M. A. E., and Bakhiet, A. O. (2010). The effect of dietary *Alpinia officinarum* (Hance) supplementation in Bovns-type chicks. **International Journal of Poultry Science**. 9(5): 499-502.

Sheehan, D. J., Hitchcock, C. A., and Sibley, C. M. (1999). Current and emerging azole antifungal agents. **Clinical Microbiology Reviews**. 12(1): 40-79.

Shepherd, M. G. (1987). Cell envelope of *Candida albicans*. **Critical Reviews in Microbiology**. 15(1): 7-25.

Shimizu, K., Kondoh, Y., and Tanaka, K. (1987). Proteinase production and pathogenicity of *Candida albicans*. I. Invasion into chorioallantoic membrane by *C. albicans* strains of different proteinase activity. **Microbiology and Immunology**. 31(11): 1045-1060.

Shin, D., Kinoshita, K., Koyama, K., and Takahashi, K. (2002). Antiemetic Principles of *Alpinia officinarum*. **Journal of Natural Products**. 65(9): 1315-1318.

Shin, J. E., Han, M. J., and Kim, D. H. (2003). 3-Methylethergalangin isolated from *Alpinia officinarum* inhibits pancreatic lipase. **Biological and Pharmaceutical Bulletin**. 26(6): 854-857.

Shinde, R., Raut, J., and Karuppayil, M. (2012). Biofilm formation by *Candida albicans* on various prosthetic materials and its fluconazole sensitivity: a kinetic study. **Mycoscience**. 53(3): 220-226.

Siqueira, J. F., and Sen, B. H. (2004). Fungi in endodontic infections. **Oral Surgery, Oral medicine, Oral pathology, Oral radiology, and Endodontics**. 97(5): 632-641.

Skiest, D. J., Vazquez, J. A., Anstead, G. M., Graybill, J. R., Reynes, J., Ward, D., and Isaacs, R. (2007). Posaconazole for the treatment of azole-refractory oropharyngeal and esophageal candidiasis in subjects with HIV infection. **Clinical Infectious Diseases**. 44(4): 607-614.

Smith, K. J., Warnock, D. W., Kennedy, C. T. C., Johnson, E. M., Hopwood, V., van Cutsem, J., and Vanden Bossche, H. (1986). Azole resistance in *Candida albicans*. **Medical Mycology**. 24(2): 133-144.

Sobel, J. D., Kapernick, P. S., Zervos, M., Reed, B. D., Hooton, T., Soper, D., and Wittes, H. (2001). Treatment of complicated *Candida vaginitis*: Comparison of single and sequential doses of fluconazole. **American Journal of Obstetrics and Gynecology**. 185(2): 363-369.

Sojakova, M., Liptajova, D., Borovsky, M., and Subik, J. (2004). Fluconazole and itraconazole susceptibility of vaginal yeast isolates from Slovakia. **Mycopathologia**. 157: 163-169.

Sokol-Anderson, M. L., Brajtburg, J., and Medoff, G. (1986). Sensitivity of *Candida albicans* to amphotericin B administered as single or fractionated doses. **Antimicrobial Agents and Chemotherapy**. 29(4): 701-702.

Soustre, J., Rodier, M. H., Imbert-Bouyer, S., Daniault, G., and Imbert, C. (2004). Caspofungin modulates in vitro adherence of *Candida albicans* to plastic coated with extracellular matrix proteins. **Journal of Antimicrobial Chemotherapy**. 53(3): 522-525.

Srividya, A. R., Dhanabal, S. P., Misra, V. K., and Suja, G. (2010). Antioxidant and antimicrobial activity of *Alpinia officinarum*. **Indian Journal of Pharmaceutical Sciences**. 72(1): 145-148.

Sud, I. J., and Feingold, D. S. (1981). Mechanisms of action of the antimycotic imidazoles. **Journal of Investigative Dermatology**. 76(6): 438-441.

Sudbery, P., Gow, N., and Berman, J. (2004). The distinct morphogenic states of *Candida albicans*. **Trends in Microbiology**. 12(7): 317-324.

Sudbery, P. E. (2011). Growth of *Candida albicans* hyphae. **Natures Reviews Microbiology**. 9(10): 737-748.

Tao, L., Wang, Z. T., Zhu, E. Y., Lu, Y. H., and Wei, D. Z. (2006). HPLC analysis of bioactive flavonoids from the rhizome of *Alpinia officinarum*. **South African Journal of Botany**. 72(1): 163-166.

Tarcin, B. G. (2011). Oral candidosis: aetiology, clinical manifestations, diagnosis and management. **Musbed.** 1(2): 140-148.

Tyagi, A. K., and Malik, A. (2010). *In situ* SEM, TEM and AFM studies of the antimicrobial activity of lemon grass oil in liquid and vapour phase against *Candida albicans*. **Micron**. 41(7): 797-805.

Uzel, G., Sampaio, E. P., Lawrence, M. G., Hsu, A. P., Hackett, M., Dorsey, M. J., and Holland, S. M. (2013). Dominant gain-of-function STAT1 mutations in FOXP3 wild-type immune dysregulation–polyendocrinopathy–enteropathy–X-linked–like syndrome. **Journal of Allergy and Clinical Immunology**. 131(6): 1611-1623.

Van den Bossche, H., Marichal, P., Le Jeune, L., Coene, M. C., Gorrens, J., and Cools, W. (1993). Effects of itraconazole on cytochrome P-450-dependent

sterol 14 alpha-demethylation and reduction of 3-ketosteroids in *Cryptococcus neoformans*. **Antimicrobial Agents and Chemotherapy**. 37(10): 2101-2105.

Van den Bossche, H., Marichal, P., Odds, F. C., Le Jeune, L., and Coene, M. C. (1992). Characterization of an azole-resistant *Candida glabrata* isolate. **Antimicrobial Agents and Chemotherapy**. 36(12): 2602-2610.

Van den Bossche, H., Willemsens, G., Cools, W., Lauwers, W. F. J., and Le Jeune, L. (1978). Biochemical effects of miconazole on fungi. II. Inhibition of ergosterol biosynthesis in *Candida albicans*. **Chemico-Biological Interactions**. 21(1): 59-78.

Van den Bossche, H., Willemsens, G., Cools, W., Marichal, P., and Lauwers, W. (1983). Hypothesis on the molecular basis of the antifungal activity of N-substituted imidazoles and triazoles. **Biochemical Society Transactions**. 11(6): 665-667.

Webb, B. C., Thomas, C. J., Willcox, M. D. P., Harty, D. W. S., and Knox, K. W. (1998). Candida-associated denture stomatitis. Aetiology and management: A review: Part1. Factors influencing distribution of candida species in the oral cavity. **Australian Dental Journal**. 43(1): 45-50.

Whiteway, M., and Oberholzer, U. (2004). *Candida* morphogenesis and host-pathogen interactions. **Current Opinion in Microbiology**. 7(4): 350-357.

Zhang, B. B., Dai, Y., Liao, Z.-X., and Ding, L.-S. (2010). Three new antibacterial active diarylheptanoids from *Alpinia officinarum*. **Fitoterapia**. 81(7): 948-952.

Zhao, L., Qu, W., Fu, J. Q., and Liang, J. Y. (2010). A New Diarylheptanoid from the Rhizomes of *Alpinia officinarum*. **Chinese Journal of Natural Medicines**. 8(4): 241-243.

APPENDIX

APPENDIX

LIST OF PUBLICATIONS

Poster presentation

Klahan, K., Nantapong, N., Chudapongse, N. (2011). **Antifungal activity of the extract of *Alpinia officinarum* Hance rhizomes on *Candida albicans*.** The 59th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (GA 2011), 4-9 September 2011, Antalya, Turkey.

Proceeding

Hoover, K., Nantapong, N., Chudapongse, N. (2012). **Antifungal activity of *Alpinia officinarum* Hance against *Candida albicans*.** The 38th Congress on Science and Technology of Thailand (STT 38), 17-19 October 2012, Chiang Mai, Thailand.

ANTIFUNGAL ACTIVITY OF *Alpinia officinarum* Hance AGAINST *Candida albicans*

Keetaya Hoover, Nawarat Nantapong, Nuannoi Chudapongse*
School of Biology, Institution of Science, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand
*e-mail: nuannoi@sut.ac.th

Abstract: *Alpinia officinarum* Hance has been used as traditional medicine in Asia. The extract of *A. officinarum* has been shown to possess antibacterial activity. However, antifungal activity of this plant has not been reported. The purpose of this study was to determine an antifungal potential of *A. officinarum* on *Candida albicans*. Galangin was reported to be a major flavonoid compound in the rhizomes of *A. officinarum*. In the present study, content of galangin in 80% acetone extract of *A. officinarum* was 3.38 mg/g, analyzed by high performance liquid chromatography (HPLC). It was found that the *A. officinarum* extract, but not galangin, exhibited antifungal activity against *C. albicans*. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) value of the *A. officinarum* extract were 1.2 mg/ml and 2.0 mg/ml, respectively. The assessment of cell damage was conducted through scanning electron microscope (SEM) observation. SEM analysis showed that the extract induced deformation of *C. albicans*. The cells had coarse surface when treated with MIC and changed from oval to rounder shape when treated with MFC. The result suggested that the extract damage cell wall, causing *C. albicans* to form spheroplast and finally death. However, this postulated mechanism which contributes to an explanation for its antifungal activity against *C. albicans* needs more experiments to confirm the damage of yeast cell wall.

Introduction: *Candida albicans* is an organism which resides in many human organ surfaces, such as oral cavity, digestive tract, skin and vagina.[1] It is an opportunistic pathogen which can cause life-threatening systemic infection in severely immunocompromised patients.[2] Candidal vaginitis is predominantly caused by strains of *C. albicans*, and appears to be a common problem both in immunocompetent and healthy women.[3] Despite advances in antifungal therapies, the number of problems remains to be solved for most antifungal drugs available, including resistance and toxicity.[4,5] These problems lead to the need of novel, safe, and effective antifungal compounds. *Alpinia officinarum* Hance, known as lesser galangal, is a pungent and aromatic plant which is used as spice for flavoring food throughout Asian countries. This plant has also been used as traditional medicine for several purposes such as relieving stomachache, treating colds, invigorating the circulatory system, and reducing inflammation. The crude extract of *A. officinarum* rhizomes as well as its bioactive components has been shown to possess antibacterial activity. However, antifungal activity of this plant has not been reported. The aim of this study was to determine an antifungal potential of *A. officinarum* and its component, galangin. We found that lesser galangal extract, but not galangin, exhibited antifungal activity against *C. albicans*. The mechanism underlie was also investigated.

Methodology:

Microorganism

C. albicans ATCC 90028 strain used in this study was obtained from Thailand Institute of Scientific and Technology Research, Thailand. The yeast was cultured on Sabouraud dextrose agar (SDA) at 30 °C for 24 h and the stock culture was maintained on SDA at 4 °C.

Plant collection and preparation

A. officinarum was bought from local markets in Nakhon Ratchasima province, Thailand. The rhizomes of lesser galangal were separated from the stem, washed thoroughly, and oven dried at 50 °C for 3 days. The sample was then grounded to powder and kept in -20 °C until used. The dried rhizomes of *A. officinarum* (300 g) were extracted with 1500 ml of 80% aqueous acetone three times at room temperature.[6] The extracted solutions were filtered through filter paper (Whatman No.1). The filtrate was concentrated by evaporation under reduced pressure to obtain a semisolid material, which was then lyophilized by freeze dryer. The extract (yield: 3.24%) was kept at -20 °C until used.

Antifungal susceptibility

Antifungal activity of lesser galangal against *C. albicans* was evaluated using agar disc diffusion method. One hundred microliters of fresh culture suspension (1×10^6 cells/ml) of *C. albicans* was spread on SDA plates. Sterile filter paper discs (5 mm diameter) was impregnated with 10 µl of *A. officinarum* extract (250 µg/disc) or galangin (50 µg/disc). After dried, the discs were placed on the surface of the inoculated SDA plates. The plates were incubated at 30°C for 24 h. Clear inhibition zone around the discs indicated the presence of antifungal activity. All the tests were performed in triplicate with ketoconazole (15 µg/disc) as positive control; 5% dimethyl sulfoxide (DMSO) (v/v) as negative control.

Minimum inhibitory concentration (MIC) determination

The starter of *C. albicans* cultures were prepared by inoculation of the stock culture in fresh Sabouraud dextrose broth (SDB) and incubated for 24 h at 30 °C on a rotary shaker (200 rpm). The cultures were transferred to fresh SDB in dilution 1:100 and further incubated. The concentration of 1×10^6 cells/ml, standardized spectrophotometrically to an absorbance of 2.50 at 600 nm, was used for testing minimum inhibitory concentration (MIC).

The concentration of crude extract solution at 50 mg/ml was used as stock solution in DMSO. Three milliliters of SDB containing varied crude extract solution and 30 µl of *C. albicans* (10^6 cells/ml) were added in test tube. The growth of *C. albicans* was monitored visually. The MIC is defined as the lowest concentration that inhibits the growth of *C. albicans* at the end of 24 h of incubation.

Minimum fungicidal concentration (MFC) determination

Base on the result from MIC method, cells from the tubes showing no growth were subcultured on SDA plates and incubated at 30 °C for 24 h. The MFC is defined as the lowest concentration that causes total inhibition of visible growth of *C. albicans* at the end of 24 h of incubation. All the tests were done in three replicates.

Isolation of galangin from the extract by thin layer chromatography (TLC)

Two hundred and fifty micrograms of the crude extract were applied to silica gel 60 F_{254} for fingerprint compared to standard galangin (0.5 µg). The plates were developed in mobile phases of hexane and ethyl acetate (7:3). The plates were visualized under UV light at 254 nm.

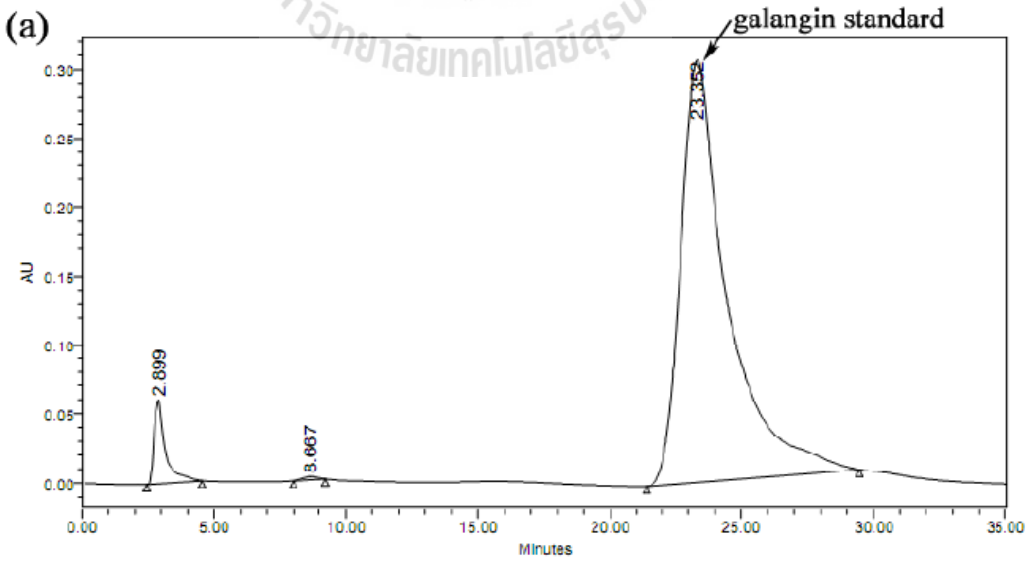
Quantitative analysis of galangin in the extract of *A. officinarum*

The amount of galangin in the acetone extract from rhizomes of *A. officinarum* was examined by high performance liquid chromatographic (HPLC). The HPLC system was equipped with the Waters 600 HPLC system. Separation was performed on a reverse phase column (Novapack ODS, 5 µm, 4.6×250 mm) provided with C18 guard column (C18 Waters, MA, USA) and methanol-water-phosphoric acid (60:38:2) were used as the mobile phase.[7] The flow rate was constantly kept at 0.8 ml/min and peaks were identified by using UV absorbance at 254 nm. The temperature of the column during analysis was maintained at 40 °C and the volume of 10 µl of the sample was injected each time.

Scanning electron microscopy (SEM)

Scanning electron microscope (SEM) observations was performed on *C. albicans* cells. The stock solution (50 mg/ml) of the crude extract were added into 3 ml of SDB containing 30 µl of the cells suspension at concentration of 1×10^6 cells/ml to yield solution containing the concentrations at 0.8, 1.2, 1.6 and 2.0 mg/ml and incubated for 36 h. The DMSO-treated culture was used as control. The collected cell pellets were fixed with 2.5% glutaraldehyde in carcodylate buffer overnight and washed three times in 0.1 M carcodylate buffer solution (pH 7.2). Then, the cells were post fixed with 1% Osmium tetroxide (OsO_4) in 0.1 M carcodylate buffer solution (pH 7.2) for 1 h. The cells were washed again with 0.1 M carcodylate buffer solution (pH 7.2) for 15 minutes three times. Now each suspension was serially dehydrated with 30%, 50%, 75%, 90% and 100% ethanol for 15 minutes twice. Then the cells were freeze dried in critical point dryer. The dried cells were coated with gold for SEM study.[8] The analytical condition for SEM was as the following: L = SE1, WD = 17mm, and EHT = 10.0 kV.

Results, Discussion and Conclusion: The extract of *A. officinarum* as well as its bioactive compounds have been shown to posses antibacterial activity,[9] however, antifungal activity of this plant has never been documented. Galangin was reported to be a major flavonoid compound in the rhizomes of *A. officinarum*.[10] In this study, the crude extract of *A. officinarum* contained relatively low concentration of galangin which could not be detected by HPLC. The retention time of galangin standard in the system used in this study was 23.4 min (Figure 1a). In order to increase its concentration, galangin was isolated from the crude extract by TLC (Figure 1b). The extract of galangin from collecting scraped silica gel was subjected into HPLC and calculated for its concentration. The content of galangin in the extract was 3.38 mg/g which is in the range previously reported (2.63-11.6 mg/g) by Tao and coworker.[7]



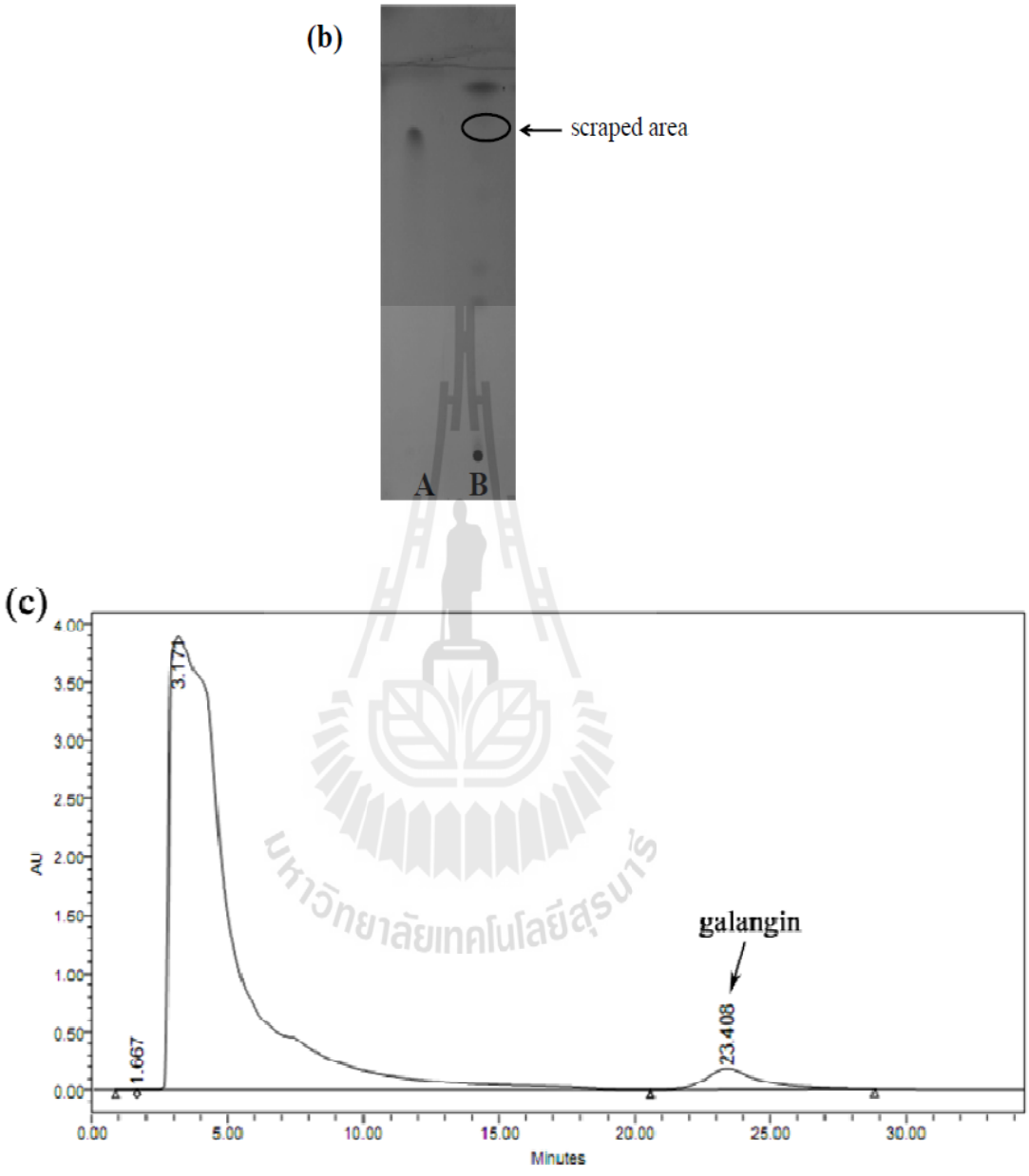


Figure 1. HPLC and TLC of the *A. officinarum* extract. Figure 1a is the chromatogram of galangin standard (retention time: 23.4 min). Figure 1b shows the scraped area (Lane B) that co-migrating with galangin standard (Lane A) in TLC. After extracted from silica gel and concentrated, galangin in the extract was analyzed by HPLC and the chromatogram is shown in Figure 1c.

We further investigated antifungal activities of *A. officinarum* extract by agar disc diffusion method. The result showed that *C. albicans* was susceptible to the extract, but not to galangin. The diameter of inhibition zone of the *A. officinarum* extract (250 µg) was about 11 mm while that of ketoconazole, a fungistatic agent, was about 23 mm. The MIC and MFC value of the extract were 1.2 mg/ml and 2.0 mg/ml, respectively.

In order to investigate a mechanism underlie the antifungal activity of the extract, scanning electron microscopy (SEM) was conducted. SEM analysis showed that the extract induced deformation of *C. albicans* in dose-response manner. The cells had coarse surface when treated with low doses as shown in Figure 2c, 2d and 2e, and changed from oval to rounder shape when treated with higher dose (MFC) as shown in Figure 2f. These results suggested that the extract possess antifungal activity by damaging cell wall, causing *C. albicans* to form spheroplast and finally death. However, this postulated mechanism needs more experiments to confirm the actual damage of yeast cell wall. Moreover, the active ingredient(s) need further investigation.

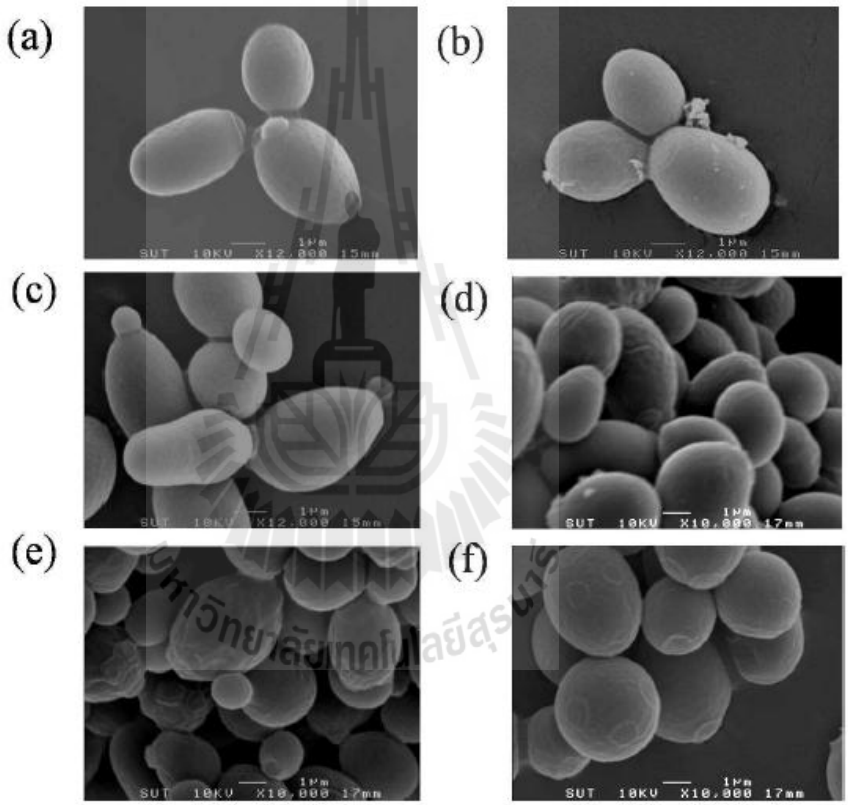


Figure 2. SEM micrographs of *C. albicans* treated with the extract of *A. officinarum*. Cells were incubated with the extract for 36 h. Figures 2a and 2b are control and DMSO-treated cells. Figures 2c, 2d, 2e and 2f are cells treated with *A. officinarum* extract at the concentration of 0.8, 1.2, 1.6 and 2.0 mg/ml, respectively. Coarse surfaces of the damaged cells were seen in the cells treated with MIC (1.2 mg/ml) of the extract and more clearly seen in higher concentration. Furthermore, the cell treated with MFC (2.0 mg/ml) of *A. officinarum* extract changed from oval to rounder shape (Figure 2f).

References:

1. Calderone RA, Fonzi WA. Trends Microbiol 2001;9:327-335.
2. Fridkin SK, Jarvis WR. Clin Microbiol Rev 1996;9:499-511.
3. Sobel JD, Kapernick PS, Zervos M, Reed BD, Hooton T, Soper D, Nyirjesy P, Heine MW, Willems J, Panzer H, Wittes, H. Am J Obstet Gynecol 2001;185:363-369.
4. Sojakova M, Liptajova D, Borovsky M, Subik J. Mycopathol 2004;157:163-169.
5. Fanos V, Cataldi L. J Chromatogr 2000;12:463-470.
6. Matsuda H, Ando S, Kato T, Morikawa T, Yoshikawa M. Bioorg Med Chem 2006;14:138-142.
7. Tao L, Wang ZT, Zhu EY, Lu YH, Wei DZ. Afr J Bot 2006;72:163-166.
8. Sangetha S, Zuraini Z, Suryani S, Sasidharan S. Micron 2009;40:439-443.
9. Cushnie TP, Lamb AJ. Phytomedicine 2006;13:187-191.
10. Mutsuda H, Nakashima S, Oda Y, Nakamura S, Yoshikawa M. Bioorg Med Chem 2009;17:6048-6053.

Keywords: *Alpinia officinarum*, *Candida albicans*, MIC, HPLC, SEM

CURRICULUM VITAE

Name Mrs. Keetaya Hoover

Date of Birth 8 March 1985

Place of Birth Nakhon Ratchasima, Thailand

Education Bachelor's degree in Medical Technology from the Faculty of Associated Medical Science, Khon Kaen University in 2007.

Publications

Proceeding

Keetaya Hoover, Nawarat Nantapong, Nuannoi Chudapongse (2012). Antifungal activity of *Alpinia officinarum* Hance against *Candida albicans*. **Proceeding of the 38th Congress on Science and Technology of Thailand.** 17-19 October 2012, Chiang Mai, Thailand

Abstract

Keetaya Klahan, Nawarat Nantapong, Nuannoi Chudapongse (2011). Antifungal activity of the extract of *Alpinia officinarum* Hance rhizomes on *Candida albicans*. **Planta Medica**. 77: PM27.