ROLES OF *RDS2* AND *PDR5* GENES IN CONFERRING RESISTANCE TO *XYLARIA* EXTRACTS IN *SACCHAROMYCES CEREVISIAE*

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE (BIOCHEMICAL TECHNOLOGY)
SCHOOL OF BIORESOURCES AND TECHNOLOGY
KING MONGKUT'S UNIVERSITY OF TECHNOLOGY THONBURI
2012

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Thesis Title	Roles of *RDS2* and *PDR5* Genes in Conferring Resistance to *Xylaria* Extracts in *Saccharomyces cerevisiae*
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Program	Master of Science
Field of Study	Biochemical Technology
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ABSTRACT

In *Saccharomyces cerevisiae,* the expression of drug resistant genes is regulated by a group of proteins called zinc cluster or zinc binuclear cluster proteins. The zinc cluster Pdr1 and Pdr3 are the master transcriptional regulators that control expression of various drug transporter genes, including *PDR5, SNQ2,* and *YOR1*. Recently, the phenotypic analysis performed with different drugs showed that another zinc cluster protein called Rds2 is also associated with antifungal ketoconazole drug sensitivity. Moreover, Rds2 is found at the promoter of *PDR5* during the glycerol shift. However, the role of Rds2 in drug resistance/sensitivity remains unclear. In this work, the goal is to characterize the function of Rds2 regulator in mediating drug resistance/sensitivity and to find new natural products with antifungal properties. The antifungal activities of four *Xylaria* species were investigated through the clear zone of inhibition assay using the *S. cerevisiae* wild-type and the deletion Δ*rds2* and Δ*pdr5* strains. All *Xylaria* extracts including *Xylaria cubensis, Xylaria globosa, Xylaria obovata,* and *Xylaria* sp. showed some clear zones of inhibition which were increased when tested in combination with ketoconazole. The antifungal activities of four *Xylaria* extracts were further confirmed by minimal inhibitory/fungicidal concentration (MIC and MFC) assays. The *Xylaria* sp. extract produced the lowest MIC_{50} values of 0.10, 0.03, and 0.02 mg/ml for the wild-type and deletion Δ*rds2* and Δ*pdr5* strains, respectively. The Δ*pdr5* strain showed greatest sensitivity to all extracts. The involvement of Rds2 regulator on expression of *PDR5* gene was then investigated by quantitative realtime-polymerase chain reaction (qRT-PCR) analysis. The results indicated that Rds2 is not responsible

for *PDR5* expression upon *Xylaria* sp. extract treatment although the previous assays showed increased sensitivity of the Δ*rds2* strain to *Xylaria* sp. extract. Thus, the overall results suggested that there are additional drug resistance genes whose expression is controlled by Rds2 and that *PDR5* expression may be regulated by other unknown transcription factors. Nevertheless, the important results of this study showed that *Xylaria* extracts possess promising antifungal activity against *S. cerevisiae*.

Keywords: Antifungal activity / Drug resistance / *PDR5* / Rds2 regulator/ *Saccharomyces cerevisiae* / *Xylaria* extract

หัวข้อวิทยานิพนธ์	การศึกษาบทบาทของยีน *RDS2* และ *PDR5* ที่มีต่อการต้านทานสารสกัดจากเชื้อรา *Xylaria* ในยีสต์ *Saccharomyces cerevisiae*
หน่วยกิต	12
ผู้เขียน	นาวสาวพิชญดา สมบูรณ์
อาจารย์ที่ปรึกษา	ดร. นิษก์นิภา สุนทรกุล
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บทคัดย่อ

การแสดงออกของยีนที่เกี่ยวข้องกับการดื้อยาใน *Saccharomyces cerevisiae* จะถูกควบคุมโดยโปรตีนในกลุ่ม zinc cluster หรือ zinc binuclear cluster เช่น โปรตีน Pdr1 และ Pdr3 ซึ่งเป็นตัวควบคุมพันธุกรรมที่สำคัญของยีนที่ทำหน้าที่ขนส่งยาหลายชนิด เช่น *PDR5, SNQ2* และ *YOR1* จากการศึกษาลักษณะทางฟีโนไทป์ก่อนหน้านี้รายงานว่า zinc cluster Rds2 มีความเกี่ยวข้องกับการไวต่อยาต้านเชื้อรา ketoconazole นอกจากนี้ในสภาวะที่เซลล์ใช้กลีเซอรอลเป็นแหล่งคาร์บอนนั้น Rds2 จับกับโปรโมเตอร์ของยีน *PDR5* ด้วย ดังนั้นในการทำวิทยานิพนธ์นี้จึงมีวัตถุประสงค์เพื่อศึกษาบทบาทของ Rds2 ในการดื้อยา และค้นหาสารจากธรรมชาติที่ออกฤทธิ์ยับยั้งการเจริญของเชื้อยีสต์ โดยใช้สารสกัดจากเชื้อราในสกุล *Xylaria* จำนวน 4 สายพันธุ์ ประกอบด้วย *Xylaria cubensis, Xylaria globosa, Xylaria obovata* และ *Xylaria* sp. มาทดสอบความสามารถในการยับยั้งการเจริญของเชื้อยีสต์ *S. cerevisiae* ด้วยการวัดขนาดของบริเวณใส (clear zone of inhibition) โดยทดสอบกับยีสต์สายพันธุ์ปกติ (wild-type) และสายพันธุ์ที่ลบยีน *RDS2* (Δ*rds2*) และ/หรือ *PDR5* (Δ*pdr5*) ผลการทดลองแสดงให้เห็นว่า สารสกัดจาก *Xylaria* ทั้ง 4 สายพันธุ์สามารถยับยั้งการเจริญของยีสต์ได้ นอกจากนี้การใช้สารสกัดจากเชื้อรา *Xylaria* ร่วมกับยา ketoconazole ส่งผลให้ขนาดเส้นผ่านศูนย์กลางของบริเวณใสเพิ่มขึ้น ผลการทดลองนี้ยังแสดงให้เห็นว่า สารสกัดจาก *Xylaria* sp. มีความสามารถในการยับยั้งการเจริญของยีสต์สายพันธุ์ปกติ, Δ*rds2* และ Δ*pdr5* ดีที่สุดเมื่อเปรียบเทียบกับสารสกัดอื่นๆ โดยให้ค่า MIC_{50} เท่ากับ 0.10, 0.03 และ 0.02 มิลลิกรัมต่อมิลลิลิตร ตามลำดับ และพบว่ายีสต์สายพันธุ์ Δ*pdr5* มีความไวต่อสารสกัดจาก *Xylaria* สูงกว่ายีสต์สายพันธุ์ปกติ และสายพันธุ์ Δ*rds2* การศึกษาบทบาทของ Rds2 ต่อการแสดงออกของยีน *PDR5* ด้วยเทคนิค quantitative realtime-polymerase chain reaction (qRT-PCR) ชี้ให้เห็นว่า Rds2 ไม่ได้ทำหน้าที่ควบคุมการแสดงออกของยีน *PDR5*

ในสภาวะที่มีสารสกัด *Xylaria* sp. อย่างไรก็ตามผลการศึกษาก่อนหน้าได้แสดงให้เห็นว่ายีสต์สายพันธุ์ Δ*rds2* มีความไวต่อสารสกัด *Xylaria* sp. สูงขึ้นเมื่อเทียบกับยีสต์สายพันธุ์ปกติ ดังนั้น Rds2 จึงมีบทบาทในการตอบสนองต่อสารสกัดจาก *Xylaria* sp. โดยอาจทำหน้าที่ควบคุมการแสดงออกของยีนที่เกี่ยวข้องกับการดื้อยายีนอื่นนอกเหนือจาก *PDR5* ขณะที่การแสดงออกของยีน *PDR5* ก็อาจถูกควบคุมด้วยตัวควบคุมพันธุกรรมตัวอื่นเช่นกัน อย่างไรก็ตามข้อสรุปที่สำคัญคือสารสกัดจากเชื้อรา *Xylaria* มีความสามารถในการยับยั้งการเจริญของยีสต์ *S. cerevisiae*

คำสำคัญ: การดื้อยา / ความสามารถในการยับยั้งเชื้อรา / ตัวควบคุมพันธุกรรม Rds2 / สารสกัดจาก *Xylaria* / *PDR5* / *Saccharomyces cerevisiae*

ACKNOWLEDGEMENTS

It would not be possible for this thesis to be accomplished without the assistance of these people. First, I am grateful to my supervisor, Dr. Nitnipa Soontorngun for giving me the opportunity to do my Masters in her laboratory as well as for her advice and assistance. I would also like to thank Dr. Songsak Wattanachaisaereekul for his advice on technical matters for *Xylaria* extraction. Also, I thank the committees for taking the time to make recommendations to complete this study. I am grateful to Piyasuda Thepnok and Pitchya Tangsombatvitich, who spent the time to teach me to understand qRT-PCR analysis and for answering any questions I had. I am also thankful to Sirilak Baramee for giving me guidance with the MIC assays. I am indebted to my friends and others for their help throughout my study. In addition, I am also indebted to my family for their steadfast confidence and words of encouragement throughout the study. This study was financially supported by the National Research Council of Thailand (NRCT).

ABBREVIATIONS

°C	=	degrees Celsius
µg/ml	=	micrograms per milliliter
µl	=	microliter
mg/ml	=	milligrams per milliliter
mg	=	milligram
ml	=	milliliter
ABC	=	ATP-binding cassette
cDNA	=	complementary deoxyribonucleic acid
DBD	=	DNA-binding domain
DNA	=	deoxyribonucleic acid
g	=	gram
hr	=	hour
L	=	liter
PDR	=	pleiotropic drug resistance
PDRE	=	pleiotropic drug resistance element
MIC	=	lowest concentration of extract that inhibited observable cells growth
MIC_{50}	=	lowest concentration of extract that inhibited 50% cells growth compared with untreated cells
MFC	=	lowest concentration of extract that completely killed cells
mRNA	=	messenger ribonucleic acid

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CHAPTER 1 INTRODUCTION

1.1 Background

The wide spectrum of organisms that are resistant to antifungal agents has significantly increased in the past decade. Resistance to antifungal agents has essential effects related to disease, mortality, and healthcare worldwide. Earlier studies revealed some genes that are regulated in response to the presence of drugs. Thus, antifungal drug resistance mechanisms can be divided into two main groups. The first group includes mechanisms used to bypass the effects of the antifungal agents on the cell. These mechanisms cause alterations in the drug targets that block drug binding or increase production of multidrug transporters that directly efflux drugs out of the cell. Another group includes mechanisms that allow cells to cope with drug-induced stress, such as alterations in the target enzyme that minimize the toxicity of the drug [1].

The budding yeast *Saccharomyces cerevisiae* is a simple eukaryote and effective model organism to study the biochemistry and cell molecular biology. There are several reasons why this yeast is used as a model organism including its size, generation time, accessibility, ease of genetic manipulation, conservation of mechanism, and importantly its genome has been completely sequenced. It is commonly used in studies on the mechanisms of resistance to drug and other toxic compounds. It also has a genetic proximity to the critical pathogenic yeast *Candida* species. Hence, *S. cerevisiae* has become a very important model in the research of drug resistance mechanism in fungal pathogens for humans, animals, and plants.

Multi-drug resistance or pleiotropic drug resistance (PDR) refers to a cell's ability to acquire resistance to a diverse range of structurally and functionally unrelated cytotoxic compounds [2]. Proteins implicated in PDR can be separated into three groups, consisting of the ATP-binding cassette (ABC) of the transporter family, major facilitator superfamily (MFS) transporters and transcriptional factors. ABC proteins can transport various compounds, such as ions, heavy metals, anticancer drugs, steroids, mycotoxins, antibiotics, and whole proteins out of the cell while the MF transporters have narrower range of substrates [3]. Transcriptional factors of PDR in *S. cerevisiae* include the bZip protein family (Yap family) and the zinc cluster protein family, also known as binuclear cluster proteins. The latter is a member of class III of the zinc finger protein. They are transcriptional regulators unique to fungi and characterized by the

presence of a zinc cluster motif in the DNA-binding domain with consensus sequence $CysX_2CysX_6$-$CysX_{5-12}CysX_2CysX_{6-8}Cys$ [3, 4].

Diseases caused by fungi are extremely damaging to living organisms. Multidrug resistance or pleiotropic drug resistance mechanism is a mechanism generally found in various organisms, like humans, plants, animals, bacteria, and fungi [5, 6]. Most PDR are involved in the overexpression of drug efflux pump genes. Drug transporters are classified into two groups based on the different energy processes including the ATP-binding cassette (ABC) family or the major facilitator superfamily (MFS) [7, 8]. In *S. cerevisiae*, *PDR5* is a well-known and important transmembrane associated ABC pump that effluxes in various target substrates such as sex hormones, drugs, toxic compound, ions, and other xenobiotics[9].

As stated above, PDR frequently results from the overexpression of cell surface efflux pumps, which expel the drugs, thereby minimizing drug toxicity. In budding yeast, examples of PDR transporters associated with antifungal drug resistance are Pdr5, Pdr10, Pdr15, Pdr11, Pdr12, Aus1, Snq2, and Yor1 [3, 10], which are each located in the plasma membrane. Recently, novel genes are found and named RDS due to their drug sensitivity phenotype. For example, *RDS2* mediates ketoconazole sensitivity [3]. The Rds2 regulator is revealed to bind at *PDR5* promoter of drug transporter gene during the glycerol utilization; however, little is known regarding the role of Rds2 in PDR [4].

At present, the repetitive use of antifungal drugs has also caused an increased level of resistance to these drugs. This has created a necessity to find new antifungal agents and causes numerous interests in bioactive compounds. Forests are the main sources of bioactive compounds. The wood-decay fungal species in the *Xylaria* genus are found to have biological activity in terms of antimycobacterial, antimalarial, and antifungal properties [11]. Therefore, these fungal species are a suitable source to find a new antifungal compound.

The *Xylaria* genus is known to be a decomposer in tropical forests and produces a myriad of secondary metabolites with biological activities [12]. They have been studied extensively in the production of secondary metabolites and bioactive compounds. Some of them are shown to be a valuable biological activities such as antimicrobial,

antifungal, antimalarial, cytotoxicity, anticancer, and inhibitor of HIV-1 protease. For instance, secondary metabolite 7-amino-4-methylcoumarin, Xylarianic acids A and B, Multiplolides A and B, Xylaropyrone, Xylactam, Sordarin, Xylopimarane, Cytochalasins they shows interesting activities [11, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22]. However, the study of fungi in xylariaceous founds in Thailand are limited. Therefore, the aim of this study is to uncover new *Xylaria* species that produce extracts with antifungal activity against *S. cerevisiae.*

In this study, we were interested to investigate the antifungal activity of four *Xylaria* extracts, including *X. cubensis, X. globosa, X. obovata,* and *Xylaria* sp. against *S. cerevisiae.* In addition, it is hypothesized that the Rds2 regulator may be involved in mediating *PDR5* expression. Initially, the antifungal activities of these extracts were carried out by clear zone of inhibition assay followed by MIC and MFC assays. To gain better understanding on the roles of Rds2 regulator on *PDR5* expression, qRT-PCR was performed in the presence of *Xylaria* extracts.

1.2 Objectives

1.2.1 To identify antifungal activity of *Xylaria* extracts

1.2.2 To test for the sensitivity of yeast zinc cluster deletion Δ*rds2* and Δ*pdr5* strains to *Xylaria* extracts

1.2.3 To investigate the involvement of Rds2 on the regulation of *PDR5* gene in mediating resistance to *Xylaria* sp. extract via qRT-PCR

1.3 Scope of work

1.3.1 To test for the sensitivity of yeast zinc cluster deletion Δ*rds2* strain via clear zone of inhibition assay as well as MIC and MFC assays in the presence of four *Xylaria* extracts including *X. cubensis, X. globosa, X. obovata,* and *Xylaria* sp. extracts.

1.3.2 To examine the role of Rds2 in mediating *PDR5* expression in the presence of *Xylaria* sp. extract via quantitative real-time polymerase chain reaction (qRT-PCR) analysis

1.4 Research methodology

1.4.1 Isolation of *Xylaria* extract: *Xylaria* species were cultured on malt extract broth for extraction with EtOAc (ethyl acetate). The EtOAc extracts were evaporated to concentrate the extract.

1.4.2 Strains of *S. cerevisiae* wild-type and deletion strains including Δ*rds2* and Δ*pdr5* were grown overnight in YPD medium (1% yeast extract, 2% peptone, and 2% glucose) and re-cultured to the mid-log phase (an optical density of 0.6–0.8 at OD_{600}). Cells cultures were used to test antifungal activity of four *Xylaria* extracts comprise of *X. cubensis, X. glubosa, X. obovata,* and *Xylaria* sp. via clear zone of inhibition assay. Cells cultures were used to test for antifungal activity of *Xylaria* extracts via MIC assay. Cells that showed invisible growth in 96-well plate were taken to do MFC assay.

1.4.3 The expression of *PDR5* gene target of Rds2 was examined via qRT-PCR in the presence and absence of *Xylaria* sp. extract. *S. cerevisiae* BY4741, Δ*rds2,* and Δ*pdr5* strains were exposed for 2 hrs in the presence of *Xylaria* extract. RNA isolation and purification were done according to the method of hot-phenol expression. cDNA was synthesized from RNA template. Examination of the *PDR5* expression level was investigated via qRT-PCR.

1.5 Benefits

1.5.1 The study of *Xylaria* species led to new knowledge on biological activity of the *Xylariaceae* fungus in Thailand.

1.5.2 Characterization of drug-related regulatory gene *RDS2* and drug transporter *PDR5* provide new knowledge concerning the mechanism of drug resistance. In addition, the knowledge gained from the study with *S. cerevisiae* that is eukaryotic model organism may be useful for the development of new antifungal agents or target in human or plant pathogenic fungi.

1.5.3 Discovery of alternative sources of natural products isolated from *Xylaria* species may provide another option for management of fungal pathogen which may help to reduce the chemically derived-antifungal agents and more environmental friendly.

CHAPTER 2 LITERATURE REVIEW

2.1 Zinc cluster proteins

In eukaryotes, the zinc-containing proteins are members of the largest families of transcriptional regulators, which manifest various functions and structures [23, 24]. They allow for more efficient transcription since zinc atoms can improve the stability of the structure of proteins [2, 25]. These proteins are grouped together because of the represented zinc-finger-like structure [26, 27]. Most zinc finger proteins play significant roles in the transcription and translational processes [2, 26]. Moreover, it also relevant to other functions, including protein-protein interactions, chromatin remodeling, protein chaperoning, lipid binding, and zinc sensing [2]. The name "zinc finger" was first recognized 20 years ago with the TFIIIA transcription factor of *Xenopus* because its three-dimensional look resembles a finger [2, 26, 27, 28].

Zinc finger proteins can be divided into three classes depending on their zinc-binding motif, specifically, the type and order of their zinc-coordinating residues (e.g., Cys_2His_2, Cys_4, and Cys_6 category) [2, 26]. Class I encompasses the Cys_2His_2 (C_2H_2) proteins, also known as the classical zinc finger. The classical zinc finger is the most common transcription factor in eukaryotes. Specially, they only bind to nucleic acids as a monomer (Fig. 2.1A). Secondarily, Cys_4 (C_4) zinc fingers are the group comprised of GATA, LIM, and nuclear receptor proteins. An appropriate example of this class is the mammalian glucocorticoid receptor. Moreover, they play a role in nitrogen metabolism in *Saccharomyces cerevisiae* [26]. Class II proteins bind to DNA as monomers or heterodimers [2]. Class III is made up of zinc clusters, zinc binuclear clusters, or $Zn(II)_2Cys_6(Zn_2C_6)$ proteins; these proteins contain two zinc atoms that specially coordinate cysteine binding (Fig. 2.1B). They hold 55 members; the best example of this class is Gal4. The zinc cluster protein, Gal4, is a transcription factor of *S. cerevisiae* and the best study model [24]. The C_6 proteins can bind DNA as monomers, homodimers, or heterodimers [2]. The consensus amino acid sequences of proteins in these classes are displayed in Table 2.1 [2, 29]. Zinc cluster proteins are involved in many processes, such as the regulation of genes that confer to carbon and nitrogen utilization, secondary metabolite production, or pleiotropic drug resistance mechanism and stress response [2, 24].

2.1.1 Structural and functional domain

The structure of zinc cluster proteins contains three functional domains, including an N-terminal DNA binding domain (DBD), a regulatory domain, and a C-terminal activation domain (Fig. 2.2) [2]. The DBD can be divided into three parts, comprised of zinc finger, linker, and dimerization regions. In most cases, the DBDs are positioned at the N-terminus, except Ume6 of *S. cerevisiae* and Czf1 of *Candida albicans.* The metal-binding motif of DBD plays an important role in the DNA binding and protein function. The metals within this motif are important in stabilizing protein folding and functions. These metals may be zinc or other metals that can function in the same manner. Generally, amino acids within the metal-binding motif are six cysteine residues, but are sometimes repeated by proline, resulting in higher flexibility, while lysine residue maintains a stable structure. Other amino acids that can work include arginine, histidine, and glutamine. A sequence of amino acids surrounding it and the loop create two short alpha helices. The linker region is located C-terminally in the zinc cluster motif and various forms in zinc cluster proteins. This region delivers an inflexible scaffold and enables it to bind to a specific site on DNA with great specificity. The last element in DBD is the dimerization region, which locates the C-terminal after the linker. It is a highly conserved coiled-coiled structure since it consists of heptad repeats, like leucine zippers that are responsible for dimerization and protein-protein interaction.

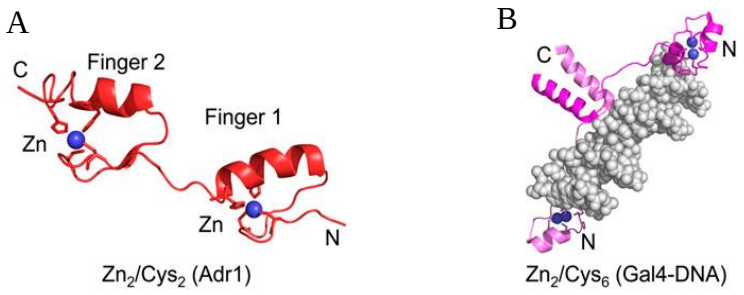

Figure 2.1 Crystal structures of DNA-binding domains of yeast transcription factors. The zinc atom is showed in blue. (A) C_2H_2 zinc fingers of Adr1 bind as a monomer and (B) C_6 zinc finger of Gal4 binds as a homodimer [26]

Table 2.1 Three major classes of eukaryotic zinc fingers [2].

Zinc finger class	Subclass(es)	Consensus amino acid sequence	Example
I(C_2H_2)	FOG(C_2HC)	Cys-X_{2-4}-Cys-X_{12}-His-X_{3-5}-His	*Xenopus* TFIIIA
II(C_4)	GATA, nuclear receptor, LIM (C_3H)	Cys-X_2-Cys-X_n-Cys-X_2-Cys-X_n-Cys-X_2-Cys-X_n-Cys-X_2- Cys	Glucocorticoid receptor
III(C_6)		Cys-X_2-Cys-X_6-Cys-X_{5-12}-Cys-X_2-Cys-X_{6-8}-Cys	*S. cerevisiae* Gal4

The regulatory domain is also called the middle homology region. This region separates the DBD from the C-terminal acidic region. Although it does have an important role in the regulation of transcription, it is not always found in all zinc cluster proteins. Moreover, the regulatory role for this region has been suggested by several deletion studies. The C-terminal acidic domain acts as an activation domain. It is not a conserved domain and the function or structure of this region is not well defined [2, 29].

Figure 2.2 Functional domains of zinc cluster proteins. Zinc cluster can be divided into three domains: the DBD, the regulatory domain, and the acidic region. Moreover, the DBD contains three regions: the zinc finger, the linker, and the dimerization region [2].

2.1.2 DNA binding specificity

As stated above, zinc cluster proteins play a role as transcriptional regulators [30]. The binding site and DNA targeting of zinc cluster proteins are under the influence of several factors. However, zinc cluster proteins often recognize trinucleotide sequences or the CGG triplets, in single or repeated forms, symmetrically and asymmetrically, in major grooves of DNA [2, 24, 26]. The CGG triplets exist in various elements. Even though some targets have very similar sequences, they each have specific regulatory tasks, and, hence, need to contain a vast repertoire of binding sites to enhance binding specificity. The most important factors for the DNA-binding specificity of zinc cluster proteins are direction of CGG triplets and the distance between them, which make the binding more specific [2]. Zinc cluster proteins can bind as monomers, homodimers,

and heterodimers to CGG triplets, which can be oriented in everted, inverted, or direct repeats (Fig. 2.3).

Furthermore, spacing between CGG triplets is crucial for specificity, and for correct binding to trinucleotide sequences on target genes of zinc cluster proteins [29]. For instance, Gal4 binds to CGG triplets with inverted direction and that separated by 11 bp (CGG-N_{11}-CCG) in contrast to Put3, which binds to CGGs spaced by 10 bp (CGG-N_{10}-CCG) [2]. Both zinc cluster proteins show a different number of spacers that enhance binding specificity. Some zinc cluster proteins can form heterodimers between members within this family for increasing potential binding sites, which come from special and different combinatorial elements [2, 29].

2.1.3 Mechanism of action

The transcription activity of zinc cluster proteins can be controlled with various strategies, including nuclear cytoplasmic shuffling, DNA binding, phosphorylation, unmarking of the activation domain, and by external stress [29]. As mentioned previously, most of zinc cluster proteins are transcription regulators. Formation of monomers, heterodimers, and homodimers are found. For example, zinc cluster proteins involved in pleiotropic drug resistance (PDR) networks are able to heterodimerize with different specificity for DNA elements. Moreover, zinc cluster proteins generate heterodimers with transcription factor of other families. For example, certain zinc cluster proteins, like Arg81 (ArgRII), regulate genes implicated in arginine metabolism by dimerizing with ArgRI and Mcm1, members of the MADS box family. Interestingly, zinc cluster proteins can regulate the transcription of target genes by their own or in correlated networks with other regulator in the same class by working through DNA recognition sites, which can be one or more [2].

In order to function as transcriptional regulators, the expression of several zinc cluster proteins is also regulated by other zinc cluster proteins in the same class. Some zinc cluster regulators can also be self-regulated via formation of a positive feedback loop [2]. Importantly, the role of zinc cluster proteins is also dependent on its location. Locations are classified into two groups. The first group is made up of zinc cluster proteins that constantly appear in the nucleus; the second group is composed of zinc cluster proteins in the cytoplasm.

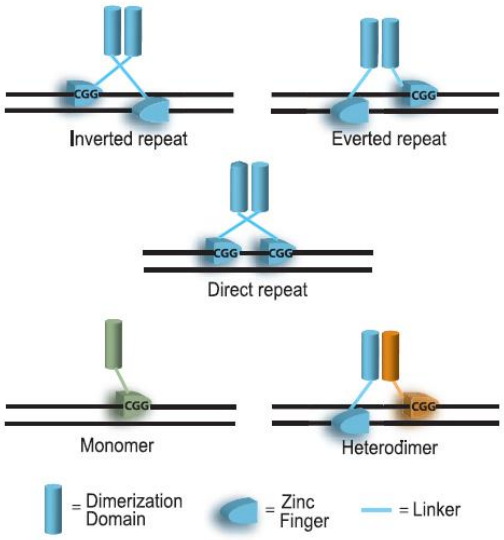

Figure 2.3 A model for zinc cluster protein DNA recognition. The alignment CGG triplets cause the three different types of zinc finger, including inverted, everted, and direct repeats. Zinc cluster proteins can works alone as monomer on DNA target (in green) or act together as homodimers (two molecules in blue), and/or heterodimers (one molecule in blue and one in orange) [2].

Zinc cluster proteins are transcription factor that belong to the first group that are constitutively localized within the nucleus. They are very important for binding to target promoters, but need metabolic intermediates or effector molecules in activation. In contrast to the second group, proteins are initially localized in the cytoplasm, and are transported into the nucleus by the α/β importin heterodimer through nuclear pores. However, there are many transport mechanisms required to carry out this work [2, 29]. Numerous zinc cluster proteins are stimulated using phosphorylation or dephosphorylation processes. Promoter occupancy of these transcriptional regulators is also controlled by outer factors that lead to disparity in binding of target sites [29]. However, sometimes they need a helper for the working in some processes such as chromatin-remodeling complex, the histone-modifying enzyme, and/or transcriptional co-factors since eukaryotic DNA is usually tightly packed into chromatin, which limits DNA accessibility to transcription factors.

As mentioned before, zinc cluster proteins are involved in many cellular processes, such as sugar metabolism, gluconeogenesis and respiration, amino acid metabolism and vitamin synthesis, mitosis, meiosis, chromatin remodeling, nitrogen utilization, and

peroxisome proliferation—as well as the stress response and PDR (see above). However, this study is interested primarily in the role of zinc cluster proteins on PDR.

2.2 Pleiotropic drug resistance (PDR)

Prokaryotes and eukaryotes are resistant to toxic compounds such as drugs. This phenomenon is part of the ability of cells to resist compounds that are toxic to cells, which can vary in both structure and function [2, 3, 10]. Cells that have obtained PDR have accordingly shown higher levels of expression of drug efflux pumps or protein transporters to expel drugs or toxic compounds out of cells. PDR creates complex problems for the treatment of many diseases via hampering of the capability of either the body's own malignant cells or of alien pathogenic organisms to develop PDR and, by this way, the cells become tolerant to drugs [3]. Additionally, PDR is a phenomenon that is similar to the multidrug resistance (MDR) phenomena that occurs in other organisms, parasites, fungal pathogens, bacteria, even tumor cells[7]. Hence, *S. cerevisiae* has become a good model organism to better understand the molecular mechanism of PDR. The efficiency of PDR is dependent on three types of proteins, including 1) ATP-binding cassette (ABC) proteins, 2) major facilitator superfamily (MFS) proteins, and 3) transcription factors. ABC and MFS transporter proteins increase exportation of drugs from cells under the control of mutated transcription factors [3, 10, 31].

2.2.1 ATP-binding cassette (ABC) proteins

ABC proteins are found in various organisms, from bacteria to humans, and are involved in many main processes of cells [3]. Their functions include peptide pheromone secretion, regulation of mitochondrial functions, vacuolar detoxification, stress adaptation, and, interestingly, pleiotropic drug resistance [7]. ABC transporters work by hydrolysis of ATP to translocate compounds—such as ions, sugars, amino acids, steroids, phospholipids, bile acids, peptides, drugs, and whole proteins of cells—across cell membranes [29]. Moreover, ABC transporters can transport dual exogenous xenobiotics and endogenous toxic metabolites.

The structure of the ABC transporter domain consists of a nucleotide-binding domain (NBD) at less one and many transmembrane-spanning segments (TMS). Normally, TMSs and NBDs are arranged in a repeated forward $(TMS_6\text{-}NBD)_2$ or reverse (NBD-

$TMS_6)_2$ configuration, depending on the type of ABC transporter. NBDs contain a highly conserved ATP-binding motif (for nucleotide binding domain) (Fig. 2.6). They are necessary for fueling ATP hydrolysis to function such as membrane transport or other [29], whereas TMSs identify detached substrate specificities of personal ABC proteins and main roles in the architecture of substrate translocation pores. For instance, Pdr5 is an ABC transporter with reversed topology (NBD-$TMS_6)_2$ [32].

The most well-known ABC transporters are Pdr5 and Snq2. An overexpression of Pdr5 and Snq2 lead to PDR because they export xenobiotics that are different structurally or functionally across the plasma membrane [7]. Pdr5 encodes for the multidrug transporters of cycloheximide resistance [3], whereas Snq2 has been shown to be an ABC transporter responsible for resistance to the mutagen 4-nitroquinoline oxide [33]. Other examples of plasma membrane ABC transporter of *S. cerevisiae* include Pdr10, Pdr15, Pdr11, Pdr12, Aus1, Snq2, and Yor1 (Fig. 2.4) [7, 29]. Therefore, their overexpression may indirectly confer antifungal resistance through functions of ABC transporters.

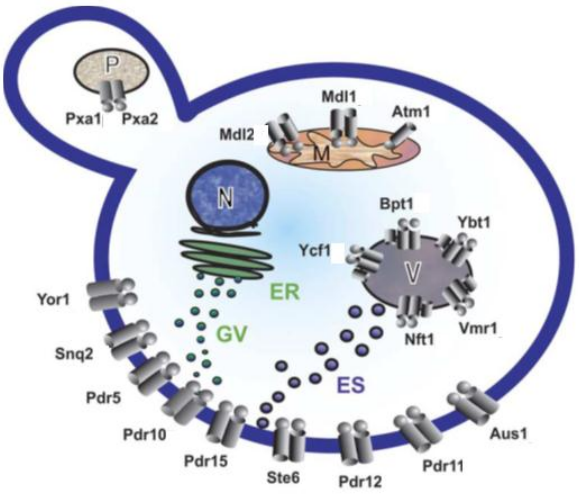

Figure 2.4 The yeast ABC membrane transporter proteins. The model represents the subcellular localization of major ABC transporters at the cell membrane, vacuole, mitochondria and peroxisomes. N, nucleus; V, vacuole; ER, endoplasmic reticulum, GV, Golgi vesicles; ES, endosomes. M, mitochondrion, P, peroxisome [7].

2.2.1.1 Pleiotropic drug resistance 5 (Pdr5)

In the presence of toxins, the cells can survive by developing mechanisms to reduce or eliminate those toxins out of the cell. The model yeast, *S. cerevisiae*, shows the pleiotropic drug resistance (PDR) and uses this process to protect itself from various xenobiotics. The PDR is a highly conserved phenomenon that can be found in both the prokaryote and eukaryote [2]. It is certain that the PDR causes difficulties in the treatment of infection diseases, particularly fungal infection. There are many mechanisms of PDR. For example, PDR occurs by the overexpression of membrane transporter genes to pump out cytotoxic compounds from the cell. The well-known PDR gene is *PDR5* that encodes for the ATP-binding cassette transporter protein, Pdr5 [9]. Pdr5 is very important in the elimination of toxins by using energy from the breakdown of ATPs [32, 34]. It is a single polypeptide chain with 1511 amino acids and 170 kDas [34]. The structure includes at least six highly hydrophobic transmembrane domains (TMDs) and two nucleotide binding domains (NBDs) or $(TMS_6 – NBD)_2$ [32, 35]. It not only expels azole and its derivatives but also extrudes a variety of structurally unrelated compounds [35]. Previous studies have found that there are many Pdr5 substrates, such as fluconazole, tritylimidazole, clotrimazole, cycloheximide, rhodamine 6-G and tetrapropyltin. [35, 36, 37]. The mutation or loss of function of *PDR5* causes the cell to become more sensitive to drugs because it is unable to efflux drugs. On the other hand, overexpression of *PDR5* causes the cell to become more resistant to these drugs [9]. Furthermore, Mamnun, Y. M. *et al.* [9] showed that *PDR5* expression depends on the growth phase, and is higher in the exponential phase but rapidly reduces when cells are out of the exponential phase. Therefore, this is support for Pdr5 function in cellular detoxification during cell growth.

PDR gene expression is tightly controlled by regulatory zinc cluster proteins [2]. The Zn_2Cys_6-containing transcriptional regulatory protein is one member of three zinc finger proteins. This protein is found in *S. cerevisiae*, fungi or amoebas [36]. Zinc cluster proteins that regulate the overexpression of *PDR5* are Pdr1 and Pdr3, while Rdr1 functions as a *PDR5* repressor [2]. Pdr1 and Pdr3 (pleiotropic drug resistance 1,3) act as activators of *PDR5* (Fig. 2.5) [38, 39]. They are the master regulators conferred to the PDR network that can form heterodimers and homodimers [2, 7, 32]. Importantly, they regulate the *PDR5* transcription by binding the same site on the PDREs (pleiotropic drug resistance elements) [2, 7, 37]. However, Pdr1 is the key regulator of Pdr5 which

operates the resistance to numerous xenobiotics [32]. Moreover, Rdr1 is a *PDR5* transcription regulatory protein that acts as a repressor [36, 40]. The deletion mutant of *RDR1* affects the resistance to cycloheximide [40].

Additionally, Pdr5 is also involved in tolerance to ketoconazole [41]. Ketoconazole is an inside group of antifungal drug azoles, which acts by inhibiting the ergosterol biosynthesis through cytochrome P450 inhibiting. This protein refers to *ERG11* [42]. Interestingly, the ketoconazole sensitivity test revealed the new zinc cluster regulator, Rds2 (Regulator of Drug Resistance), which is involved in the sensitivity to ketoconazole of *S. cerevisiae* [3]. Importantly, ChIP-chip analysis under ethanol used as an alternative carbon source revealed that Rds2 is bound at the promoter of *PDR5* as well [43]. This data indicates that the expression of *PDR5* may be controlled by Rds2.

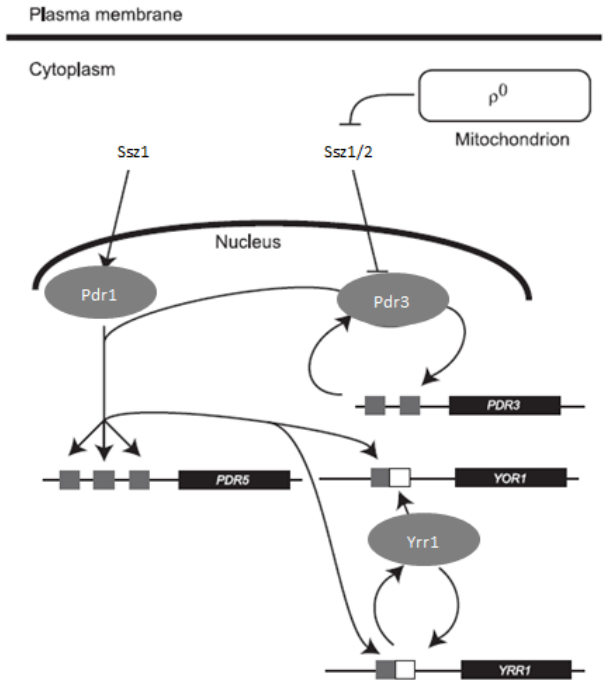

Figure 2.5 Regulation of *PDR5* expression by Pdr1 and Pdr3 regulators. Pdr1 and Pdr3 can form hetero- or homodimer to bind on PDREs to control the expression of *PDR5*. The solid black arrows show positive control and the blunt arrows indicate negative control. The grey squares show the Pdr1/Pdr3 response element (PDREs) [5].

2.2.2 Major facilitator superfamily (MFS) proteins

In addition to ATP power proteins or ABC transporters, cells enable the expulsion of toxic compounds, such as drugs, across plasma membranes through the proton motive force (Fig. 2.6) [44]. The major facilitator superfamily proteins, or MFSs, are protein transporters found in various organisms, ranging from bacteria to higher eukaryotes [45, 46]. Interestingly, thus far, MFS transporters have more than 300 private proteins [8]. The superfamily of MF protein has various members that can be classified into families, including sugar, amino acid, multidrug, allantoate, uracil/uridine/allantoin, monocarboxilated, sulfate, ammonia, phosphate, purine/cytosine, and calcium permease homologues [45, 46].

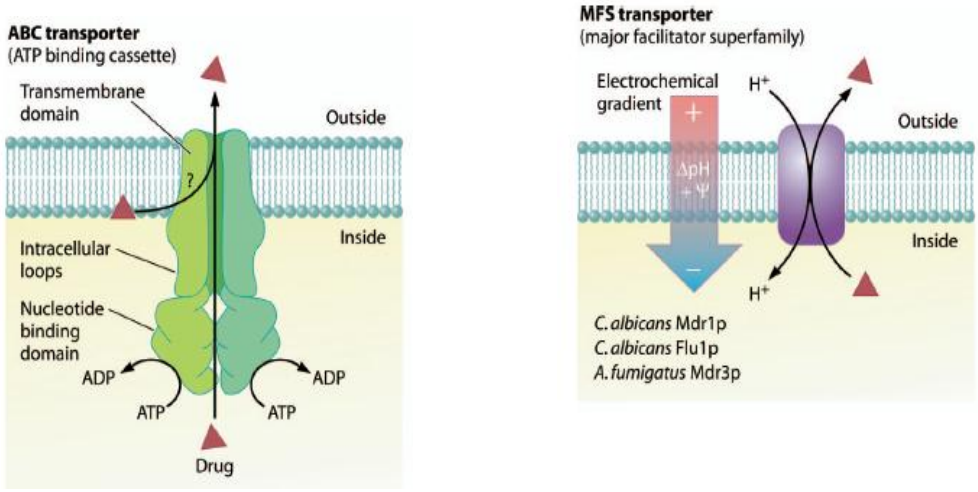

Figure 2.6 The domain arrangements of two main classes of drug transporters are ABC and MFS transporters. The ABC transporters pump out xenobiotics using the hydrolysis of ATP, while the MFS proteins use the proton-motive force to efflux compounds out of the cell [44].

However, two subfamilies of MFS proteins that are tolerant to drugs can be identified using the transmembrane spans (TMS) number in the TMD. One of these subfamilies is DHA1, which refers to drug:H+ antiporter 1 with 12-TMS and DHA2, which is composed of 14-TMS [29, 46]. Within the 14-TMS and 12-TMS family, several fungal proteins are involved in multidrug resistance. For instance, the *Candida albicans CaMDR1* gene, which belongs to *DHA1*, was originally identified as conferring

resistance to benomyl and methotrexate. Accordingly, overexpression and gene knockout studies have indicated that *CaMDR1* is also involved in endurance to cycloheximide, benztriazoles, 4-nitroquinolone-*N*-oxide, and sulfometuron methyl. *C. albicans* without the *CaMDR1* gene have a smaller the risk [8, 29]. *S. cerevisiae* possesses both DHA1 and DHA2 families, such as *AQR1*, which confer the excretion of excess amino acid. DHA2 members are *AZR1* and *SGE1*, which are involved in diverse processes. *AZR1* plays in weak organic acids, ketoconazole, and crystal violet resistance. Likely, *SGE1* is answerable for unbridled crystal violet, ethidium bromide, 10-*N*-nonyl acridine orange, malachite green, and methyl methanesulphonate [6]. Nevertheless, ABC transporters have greater specificity for substrates than MFS transporters. Hence, the association between azole resistances is of great clinical significance.

2.2.3 Transcription regulators of PDR

To maintain the stability of the cell, the proper expression of transporters including ABC transporters and MFS transporters is required. These transporters are expressed under the control of transcription regulators in a PDR network (Fig. 2.7). There are many transcription factors shown to regulate the expression of genes encoding for ABC or MFS transporters. The transcription factors can be divided into two major families: zinc cluster proteins and the bZip protein family (Yap family).

Zinc cluster proteins or binuclear $Zn(II)_2Cys_6$ zinc finger proteins are a subclass of zinc finger proteins [2, 10]. Zinc cluster proteins involve themselves in PDR networks by regulating drug transporter genes, thereby conferring drug resistance (Fig. 2.7). These higher levels of expression are often due to hyperactive or gain-of-function mutation in the transcription factors. The zinc cluster proteins Pdr1 and Pdr3 are among the master regulators connected to drug resistance in budding yeast. Pdr1 was first categorized by an amount of dominant multidrug-resistant alleles that were mapped to its gene's location, while Pdr3 was first recognized as a gene that conferred conflict to the mitochondrial inhibitor mucidin [2]. Both proteins belong to the large Gal4 family of fungal transcription regulators, which share 36% of amino acid identity on structural motifs from all sequences [32, 39].

Zinc cluster proteins can form homo- and heterodimers by connecting the dimerization domain of both proteins together where closely located to the DNA binding site. Prd1/Pdr3 are good examples for explaining the control of gene expression. They regulate transcription PDR target genes by binding *cis*-acting elements on promoters, namely PDREs (pleiotropic drug resistance elements) [7]. Most members of Gal4 family recognize PDREs consensus element sequence 5'-TCC/aC/tGG/cA/g-3'enclosing the everted repeat of GGC [32, 39]. The quantity and localization of PDREs are related to the diversity of start codon on promoters. Moreover, the homo- or heterodimers combination affinities between PDRE of Pdr1/Pdr3 can be adjusted. This event can be used to describe a variety of target genes for Pdr1 and Pdr3 (Table 2.2) [32]. Importantly, there are two PDREs in *PDR3* promoter, so Pdr3 can autoregulate via PDREs in their own promoters and can respond to mitochondrial signals. In general, transcription of *PDR5, SNQ2, PDR10, PDR15,* and *YOR1* are controlled by Pdr1/Pdr3 and Yrr1. When *PDR5, SNQ2,* and *YOR1* are deleted, there are other drug efflux pumps with regulated expression level by Pdr1 [29]. However, several hyperactive Pdr3 mutants indicated expression of *PDR5* and *SNQ2*, as well as *PDR3* is increased [2]. The promoters of *PDR5, SNQ2,* and *YOR1* genes contain one or several PDREs.

Yrr1, another zinc cluster protein, with identified mutations leading to increased cell cycle inhibitor reveromycin A, 4-nitroquinoline-1-oxide, sulfometuron methyl and oligomycin resistance, is identified through genetic screens. The overlapping target of Yrr1 with Pdr1/Pdr3 purveys some significant evidence of cross talk between regulators of PDR in *S.cerevisiae* because their PDRE sequences show similar regulation to Yrr1's YRRE sequence [2]. Yrr1 plays a role in drug resistance via positive overexpression on *YOR1* and *SNQ2* promoters (Fig. 2.8) [39, 47]. Importantly, Yrr1 has a putative Yrr1 response element (YRRE), which contains the consensus sequence T/ACCGC/TG/TG/TA/TA/T and PDRE in its promoter [29]. Hence, Yrr1 can resist reveromycin A, 4-nitroquinoline-1-oxide, sulfometuron methyl, and oligomycin via regulation of these elements.

Moreover, Stb5 and Rdr1 are other zinc cluster proteins related to the regulation of *PDR5* and/or *SNQ2* [10]. Stb5 can control the expression of drug efflux pump genes such as *PDR12* and *ATR1* in the presence of diamide because it is a direct positive

regulator of *SNQ2*. Hence, it acts as both an activator and repressor by formulating with other zinc cluster regulators for homodimers or heterodimers (Fig. 2.8).

Pdr5 is another plasma membrane transporter of PDR. Overexpression of Pdr5 leads to exportation of wide spread toxic compounds that are different in structure and function across the plasma membrane. Besides, Pdr5 plays an important role during cell growth because Pdr5 can extrude accumulated intracellular cytotoxic metabolites, which is confirmed by the disruption of *PDR5* and *SNQ2* [33]. Hence, Pdr5 levels are increase in an exponential growth phase whereas they quickly decrease when cells enter stationary growth phase [7]. Interestingly, several hyperactive Pdr3 mutants also induce increased expression of *PDR5* and *SNQ2* and indicate that Pdr1 and Pdr3 are activators of *PDR5*. In contrast, Rdr1 is the repressor of *PDR5*, the same as Stb5 (Fig. 2.8) [2, 7].

Notably, among the zinc cluster regulators, Pdr12 confers resistance to weak organic acid, not transported hydrophobic drugs [7]. The expression of *PDR12* is controlled by War1, a main modulator of weak acid stress via induction of transcription of *PDR12*. Moreover, Stb5 can regulate the *PDR12* expression in the presence of diamide [2]. Pdr15 is the closest homologue of the master described [48]. Specially, Pdr15 contains two elements that respond to the environment, including pleiotropic drug resistance elements and stress response elements (STREs). These elements are controlled by Pdr1/Pdr3 and Msn2, respectively. Thereby, the subsequent transcriptional induction of *PDR15* demonstrates a cross-talk between PDR and general stress response—e.g., heat shock, high osmolarity and weak acid stress. The Cys_2His_2 zinc finger protein Msn2 is a master regulator of common stress response pathways, and so is the activator of Pdr15 via bypass of the HOG pathway that transduces extracellular stress signals. After, the Msn2 and Hog1 are phosphorylated as well as activated and undergo rapid nuclear translocation effects to export a stress response [7, 48]. Hence, Pdr15 levels are also powerfully elevated in cells suffering diauxic shift.

Other zinc cluster proteins that regulate the drug resistance of plasma membranes include Pdr8, Rds1, Rds2, and Rds3. However, functions of Pdr8 remain unclear. At the present, Pdr8 regulates some genes involved in PDR, for example *YOR1*, *PDR15*, and *AZR1* [2]. In the path of Rds1 to Rds3, regulators of drug sensitivity are recently found. Rds1 and Rds3 are identified to be involved in resistance to cycloheximide,

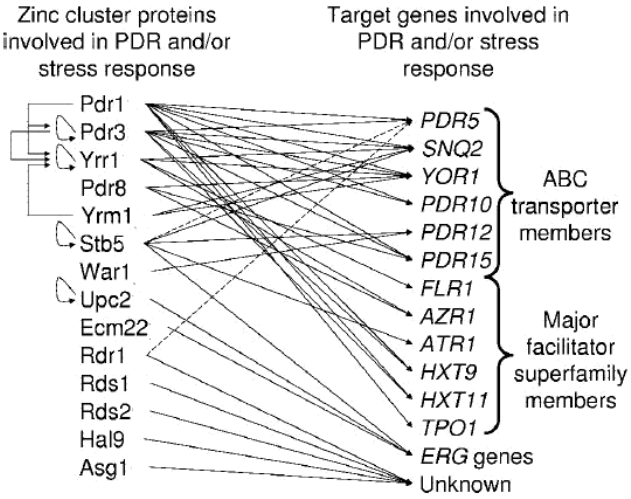

Figure 2.7 Zinc cluster proteins associated with PDR network in *S. cerevisiae*. PDR genes are showed as the only major transporter in groups of ABC and MF transporter. ERG genes are also considered to be additional zinc cluster targets. The map shows direct targets of zinc cluster proteins (solid line) and direct or indirect targets are shown in dashed lines [2].

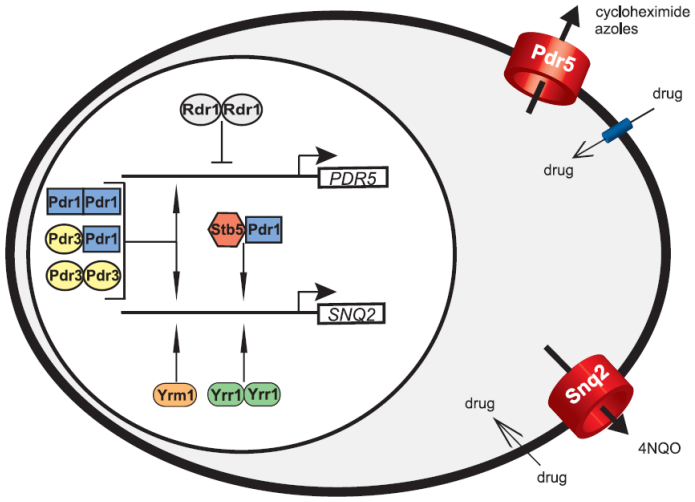

Figure 2.8 The cooperatively coordinate function of zinc cluster proteins involved in the PDR network of *S. cerevisiae*. Zinc cluster proteins can form homodimers to regulate the expression of target genes like Pdr1 (in blue), Pdr3 (in yellow) and Stb5 (in red). Some of these can also form heterodimers with differ regulators, for instance Pdr3, Rdr1 (in gray), and Yrr1 (in green). Of course, the difference in the dimer will make a difference in the expression of genes that are regulated [2].

Table 2.2 Regulators of ABC transporters or ABC genes in the yeast *S. cerevisiae* [2, 7].

Regulator	Cellular function	ABC target genes
$Zn(II)_2Cys_6$ sub family		
Pdr1	Regulation of PDR	*PDR5, PDR10, PDR11, PDR15, SNQ2, YOR1*
Pdr3	Regulation of PDR	*PDR5, PDR10, PDR15, SNQ2, YOR1*
Yrr1	Regulation of PDR	*YOR1,SNQ2*
Rdr1	Negative regulation of *PDR5*	*PDR5*
Stb5	Regulation of PDR, Pdr1-heterodimer	*PDR5, SNQ2*
Pdr8	Regulation of PDR	*PDR15, YOR1*
Yrm1	Specific inhibitor of Yrr1	*YOR1, SNQ2*
War1	Regulation of weak acid stress response	*PDR12*
Ecm22 and Upa2	Regulation of sterol biosynthesis	*PDR11, AUS1*
Rds1	Repressor of PDR genes	unknown
Rds2	Regulator of drug sensitivity	unknown
Cys_2His_2 sub family		
Msn2	Regulation of general stress response	*PDR15*
b-ZIP		
Yap1	Regulation of oxidative stress response	*YCF1, SNQ2, PDR5*
Yap8	Modulation of arsenite resistance	*YCF1*
General TF in other sub family		
Ngg1	Inhibitation of Pdr1 activity	

PDR, pleiotropic drug resistance; TF, transcription factor.

while Rds2 show strong resistance to antifungal azole ketoconazole, the inhibitor of the *ERG11* gene product involved in ergosterol synthesis [3]. The expression of *ERG* genes important to the ergosterol biosynthetic pathway are regulated by zinc cluster proteins, for example Upc2 and Ecm22 [7]. The *ERG* genes contain the sterol response element in their promoters in the location for positive regulation of Upc2/Ecm22. Interestingly, Upc2 also plays an important role in anaerobic exogenous sterol accumulation, and obtains cooperation from *AUS1* and *PDR11* for the uptake of free sterols under decreased ergosterol biosynthesis environments [2, 7].

2.2.3.1 Rds2 transcription factor

The yeast *S. cerevisiae* has transcription regulators that contain two zinc atoms [2]. The zinc binuclear cluster or zinc cluster proteins that are involved in multidrug or pleiotropic drug resistance (PDR) have more than 50 members [49]. These proteins play an important role as transcription regulators of the expression of drug efflux pump genes, resulting in the extrusion of drugs from cells. The new zinc cluster regulator, Rds2 (for regulator of drug sensitivity), is one zinc cluster regulator with identified sensitivity to the antifungal agent ketoconazole that disrupts ergosterol biosynthesis [3, 50]. Ergosterol has the same function with regard to cholesterol and is a main component of yeast and fungi membranes. Ketoconazole interferes with ergosterol synthesis by acting as an inhibitor of the *ERG11* gene product that converts lanosterol into ergosterol [1, 42, 51, 52]. Moreover, the transcription factor Rds2 also increases cell susceptibility to calcofluor white, a fluorescent stain that binds to chitin in the cell wall of fungi. However, tests of antibiotic sensitivity of *S. cerevisiae* lacking *RDS2* reveal that it is sensitive to bleomycin, hyromycin B, and amphotericin B, while having resistance to quinidine. Interestingly, Rds2 confers resistance to osmotic stress, NaCl and LiCl [50]. So, it is a possibility that Rds2 controls the expression of the plasma transmembrane protein that confers the detoxification system, either directly or indirectly. One example of this includes pleiotropic drug resistance. Soontorngun and coworkers (2008) have reported that the binding site of Rds2 at the promoter of pleiotropic drug resistance genes under glycerol conditions include *PDR5* and *PDR16* (belong to the ABC transporter) and *QDR3, TPO1,* and *AQR1* (members of the MFS transporter) [50]. These genes work to efflux various xenobiotic compounds such as cycloheximid, fluconazole, ketoconazole, amphotericin B, polyamine, quinidine, or short-chain monocarboxylic acids [34, 53, 54, 55, 56, 57, 58, 59].

Recently, Rds2 has been identified as a zinc cluster regulator that is involved in regulating genes in many cellular processes such as gluconeogenic genes[4, 49, 50], glyoxylate cycle genes, respiration genes, TCA cycle genes and transcription factors [51, 60]. It can act as either an activator or inhibitor of genes depending on the needs of the cells [4, 43, 60]. Rds2 binds to promoters of *PCK1*, *FBP1*, and *LSC2*, and acts as a positive regulator of these gluconeogenic genes [60, 61]. The respiration gene *HAP4* is another crucial gene that positively regulates gene expression by Rds2 [3, 4, 62]. Hap4 works as an activator subunit of the Hap2/3/4/5 complex in controlling the expression of respiratory genes [62]. For negative regulation, Rds2 functions by binding on the promoter of *OPI1*, a gene in the phospholipid biosynthetic pathway [60]. If the cell lacks Opi1, then derepression of *GUT1* occurs as well as gene conference to glycerol utilization [63]. In addition, it is possible that Rds2 regulates *SIP4* with Cat8. Rds2, Sip4, and Cat8 are all controlled by Snf1 kinase [4, 43, 60]. These relationships are summarized in Figure 2.9. However, target genes and strict roles of the Rds2 regulator implicated in PDR networks remain unclear (Fig. 2.7).

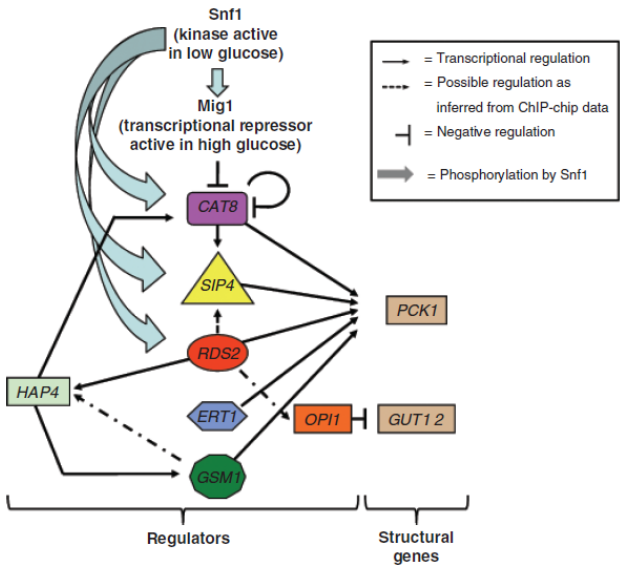

Figure 2.9 A model for the regulatory network of the Rds2 zinc cluster regulator under nonfermentable carbons [60].

2.3 Antifungal agents

2.3.1 Antimicrobial agents affecting fungal sterols

Drug resistance in fungi is of important concern. It can cause tremendous damage to plants, animals, and humans. It is especially difficult to treat patients who come in contact with both immunocompromised and immunocompetent individuals. In addition, the similarity of the targets of the actions of drugs between humans and fungi make the performance of available clinical drugs limited. Antifungal drugs mainly play the inhibitory role via the ergosterol biosynthesis, the (1,3)-β-D-glucan biosynthesis, or other key components of the fungal cell wall [1]. However, due to these reasons, antifungal drugs also have toxicity to human cells, because of the similarity between the two sterols on cell membranes: ergosterol in fungi and cholesterol in human cell membranes. The three major groups of antifungal agents in clinical use, namely azoles, allylamine, and thiocarbamates, are prized for their antifungal activities with regard to inhibition of synthesis of, or direct interaction with, ergosterol [64].

2.3.1.1 Azole-based antimycotic agents

Azole drugs have been found to have effective antifungal properties of N-substituted imidazoles in the late 1960s. The first compounds include miconazole and econazole, which are followed by ketoconazole, fluconazole, and itraconazole which are updated to combat human fungal infections [64]. These are called triazoles; they are heterocyclic synthetic compounds that exert potential in ergosterol biosynthesis by the inhibition of enzyme cytochrome $P450_{14DM}$ (also known as lanosterol 14α-demethylase), which is encoded by *ERG11* and catalyses at a late step (Fig. 2.11). Inhibition of 14α-demethylase leads to the depletion of egosterol and sterol precursors accumulated instead. These sterols are toxic compounds, including 14α-methylated sterols (lanosterol, 4,14-dimethylzymosterol, and 24-methylenedihidro-lanosterol), and lead to the formulation of a plasma membrane with an altered structure and function (Fig. 2.10A) [5, 64, 65, 66]. Other compounds that target enzymes in the ergosterol biosynthesis pathway include allylamines, thiocarbamates, and morpholines. These compounds target the enzyme in the first step squalene epoxidase, that is encoded by *ERG1* to convert squalene to ergosterol [29].

The mechanisms of fungal cell resistance to azoles include alteration of the target enzyme, upregulation of multidrug transporters, and cellular stress responses. Alteration of the target enzyme is an alteration of the target enzyme, Erg11 (Fig. 2.12A). Erg11 has important functions in the synthesis of ergosterol, which is a major sterol in the cell membrane of *S. cerevisiae* (and other yeasts). The alteration in the *ERG* pathway leads to disruption of the cell membrane. Resistance may occur which can be caused by mitotic recombination, gene conversion, and the formation of an isochromosome, which confers resistance by over expression of the *ERG11* gene and the expression of the multidrug transporters gene, regulated by the Tac1 transcription factor [1].

On the other hand, PDR occurs from the overexpression of either of the two families of multidrug transporters that pump drugs out of the cell: ABC transporters and MFS transporters (Fig. 2.12B). In *Candida* spp. the fluconazole resistant cells are caused from upregulation of Mdr1, a member of the MFS transporter family. Recently, identification was completed of the constitutive *MDR1* overexpression caused from Mrr1, a key transcription factor regulating *MDR1*, and hyperactivating mutations. Nevertheless, the most-frequent azoles resistant to fluconazole and other azoles of cells involved in Cdr1 and Cdr2 belong to the ABC transporter family. The expression of these two ABC transporters regulated the key transcription factor that revealed Tac1. Tac1 is a common regulator of *CDR* to respond to drug exposure such as azole [1]. Interestingly, the resistance to ketoconazole and itraconazole , members of azole antifungal drugs by overexpression of *ERG16* and *CDR1* is correlated with increased opportunity of cells to pass cross-resistance to other azoles [29].

Other mechanisms protect cells from antifungal drug potential are connected to preventing the toxic sterol intermediate accumulation by losing the Erg3 function in the ergosterol biosynthesis after Erg11 is inhibited by azole (Fig. 2.12C). However, some mechanisms may incorporate resistance to the drugs resulting in increased efficiency in coping with stress. For example, regulation targets, in addition to *MDR1* of the transcription factor Mrr1, result in induction of oxidoreductase. As a result, maybe it helps that cells can withstand toxic molecules generated from the presence of drugs. A part from *CDR1* and *CDR2*, the transcription factor Tac1 serves the regulation of some genes that encode for enzymes in oxidative stress response and lipid metabolism such as glutathione peroxidase, sphingosine kinase, and phospholipid flippase [1].

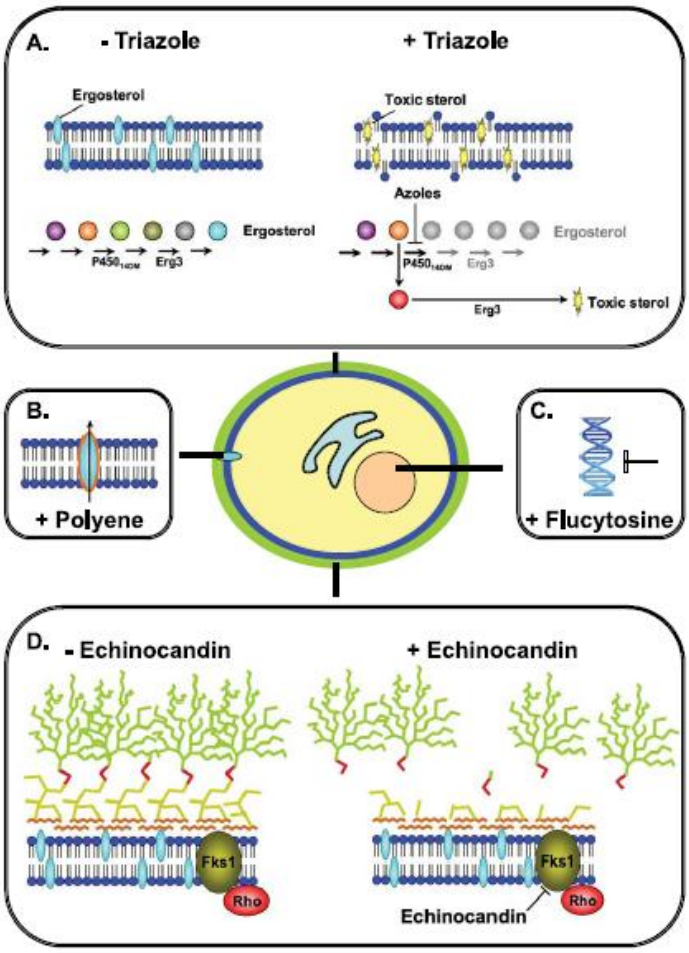

Figure 2.10 Antifungal drugs and their targets. (A) Triazoles exert its antifungal activity through the inhibition of ergosterol biosynthesis via blocking the cytochrome $P450_{14DM}$ activity. This causes the accumulation of a toxic sterol intermediate in the fungal cell membrane and leads to cell membrane stress. (B) Ergosterol can bind with polyenes resulting in formation of membrane-spanning channels. Hence, fungal cells leak cellular components and osmotic cellular. (C) Flucytosine is an antifungal drug that targets DNA and RNA synthesis by inhibition of thymidilate synthase. (D) Echinocandins inhibit (1,3)-$\boldsymbol{\beta}$-D-glucan synthase and disrupts the cell wall integrity resulting in cell wall stress (+, present; −, absent) [1].

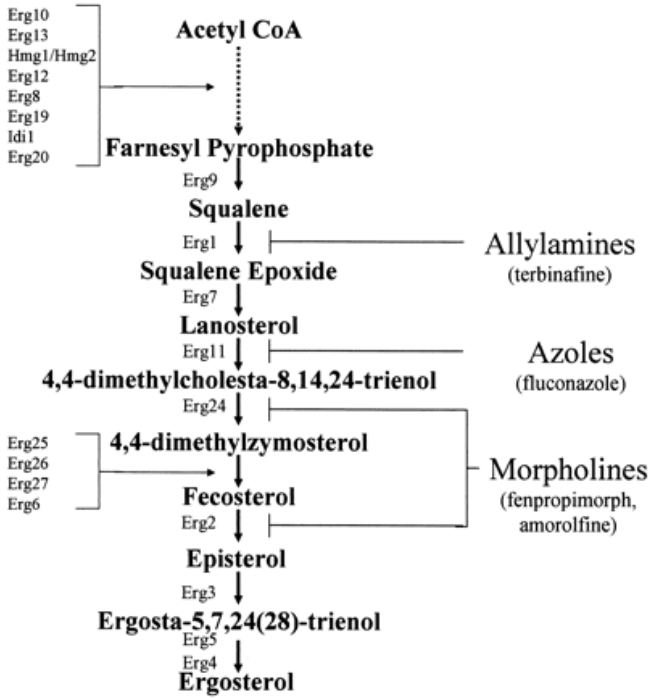

Figure 2.11 Ergosterol biosynthesis and proteins that are involved in the process. Ergosterol is known to be the major component in the fungal cell membrane, and many antifungal drugs designed to target ergosterol biosynthetic pathways in different enzymes. All carbon atoms in ergosterol come from acetate, which is converted to acetyl CoA [29].

In all eukaryotes, the Ca^{2+}-calmodulin-activated protein phosphatase calcineurin is a key regulator of cell signaling that mediates critical responses to antifungal drugs (Fig. 2.12D). Calcineurin dephospholylated transcription factor Crz1 is connected to azole resistance of *C. albicans*. In contrast to *S. cerevisiae*, downstream effectors of calcineurin are Hph1 and Hph2 of the endoplasmic reticulum that mediate the response to triazole [1]. Importantly, the function of calcineurin is dependent on the molecular chaperone Hsp90. The Hsp90 interacts with the catalytic subunit of calcineurin and is a key regulator of cellular signaling in eukaryote cells.

S. cerevisiae is accepted to be genetically close to *Candida* spp., which has resistance to the azole drugs with PDR (pleiotropic drug resistance) [5]. PDR is a phenomenon caused by the work of mechanisms within the cell so that the cell can tolerate the toxicity of the drugs [1, 5, 6, 67, 68]. The PDR protein, Pdr5 has a prominent role in the resistance to azole [3, 34, 36, 39, 54, 68]. The Pdr5 protein acts to transport drugs out of the cell to reduce the toxicity of the drug inside the cells, which makes the cells resistant to the drug. However, *PDR5* gene expression is controlled by proteins in a group of zinc cluster proteins like Pdr1 and Pdr3, for example [9, 38, 39].

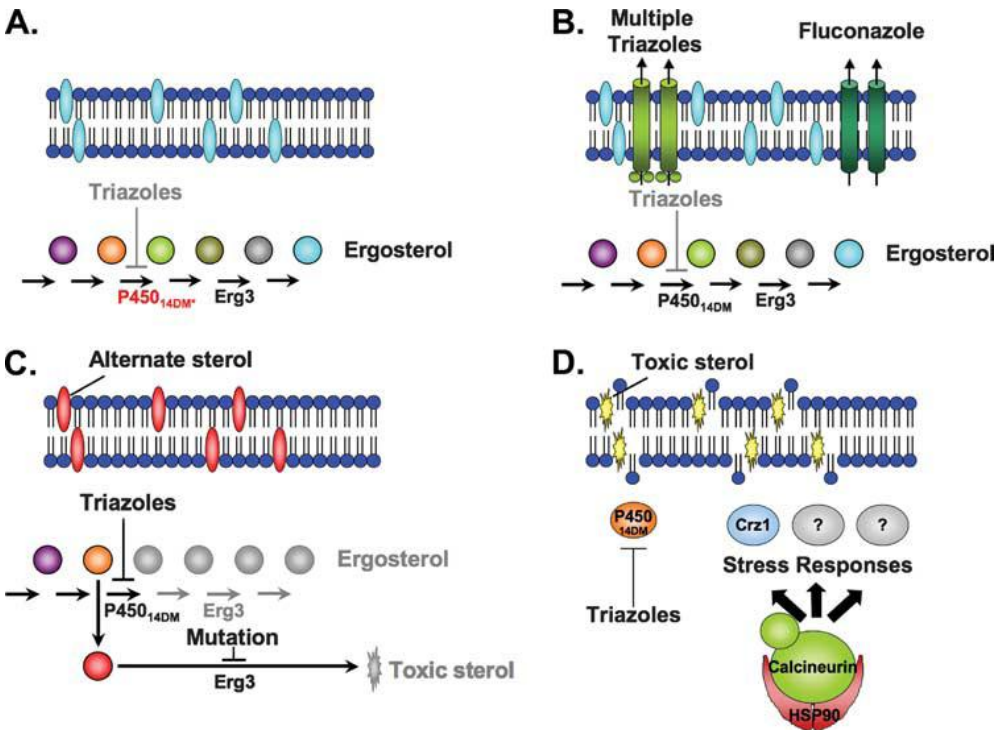

Figure 2.12 Triazole resistance in *C. albicans*. (A) Decreasing of effects from triazole cause the increasing of its target, cytochrome $P450_{14DM}$ by mutation or overexpression of *ERG11*. (B) Reducing intracellular drug concentration by overexpression of PDR genes, including the ABC transporter family and the major facilitator transporter that transports drugs out of the cell. (C) Avoiding toxic sterol accumulation in cell membrane by mutation of *ERG3*. The accumulation of toxic sterol in cell membrane is a common pathway to functions of the triazole drug. (D) Resistance to triazole, whether in wild-type or mutant strains were connected to Hsp90, a calcineurin stabilizer. This is an important protein because its downstream effectors are necessary to cell stress response [1].

Additionally, *S. cerevisiae* can modify ergosterol synthesis as well as *Candida* spp. As mentioned above, changes in ergosterol synthesis can cause resistance to azole drugs. *S. cerevisiae* has two members of the zinc cluster transcription factor, Upc2 and Ecm22, which act as activators of *ERG11*. Both transcription factors cause upregulation of *ERG11* that result in azole drug resistance [1, 32, 37, 68, 69, 70, 71].

2.3.1.2 Polyenes

All organisms contain sterols in their outer membrane that are sensitive to polyene, such as yeast, algae, and protozoa; but, non-sterols in the outer membrane make the organisms resistant. The importance of sterols is showed in an earlier study, which show that the presence of sterol in growth culture cause poorer action of polyenes [64]. The

polyenes cannot dissolve in aqueous solution, so most of the polyenes, such as amphotericin B, interact with the fungal membrane. Amphotericin B is inserted into the lipid bilayer of the fungal membrane containing ergosterol, which results in hydrophobic linking of sterol and eight amphotericin B molecules and the formation of aqueous pores (Fig. 2.13) [1, 29, 64]. This alteration of the membrane leads to leakage of intracellular biomolecules and ions, resulting in cell death due to an increase of pores in the cell membrane. Polyenes are also believed to cause oxidative damage. Although amphotericin B is the most effective antifungal drug available, it is toxic to human or host cells because of the similarity between cholesterol and ergosterol.

The polyene resistance mechanism is hypothesized to be caused by changes, either quantitative or qualitative, of sterol content of the cells. The probability of this hypothesis is gathered 3 ways: (1) adjustment in composition of total cell sterol by decreasing ergosterol, the sterol target of amphotericin B; (2) modifying the polyene-binding sterols by replacing ergosterol with other sterols, for example substitution of ergsterol, cholesterol, or stigmasterol by a 3-hydroxy or 3-oxosterol; or (3) alteration of identity and location of existing ergosterol [64]—each result in reduction of the binding of polyene. Nonetheless, sometimes Erg3 is attributed to the reduction of ergosterol because it has been reported that *ERG3* affects hypersensitive amphotericin B in the deletion of mutants [1].

2.3.1.3 Allylamines

Allylamines are antifungal agents that are distinct from other ergosterol-inhibiting antifungal agents, both functionally and chemically. They are comprised of two antifungal agents, terbinafine and naftifine. Inhibition of allylamines is in squalene epoxidation of the ergosterol synthesis pathway (Fig. 2.14) [64]. The result of inhibition of squalene epoxidation is the increasing of squalene number to more than ergosterol products, and the death of the cell in the end. This is because cell membrane permeability is disrupted by the loss of cellular organization. Some studies have reported that terbinafine can be a substrate of *CDR1*, resulting in improved resistance to allylamines [64]. In *S. cerevisiae, ERG1* encodes for squalene epoxidase, which is the early step of lanosterol 14∞-demethylase. Importantly, the *ERG1* product, squalene epoxidase, is the target for terbinafine [72, 73]. The presence of allylamines and terbinafine in *S. cerevisiae* cells leads to changing ergosterol concentration in the cell

membrane, which inhibits growth [73]. Presently, resistance to terbinafine is rare. However, resistance can be achieved when using this drug for a long time. *S. cerevisiae* is a eukaryote model organism, so it is especially suited to study the mechanisms of resistance that may occur. Leber, *et al*. [73] explained that the resistance to terbinafine confers to a single mutation in the *ERG1* gene, since squalene epoxidase properties are changed.

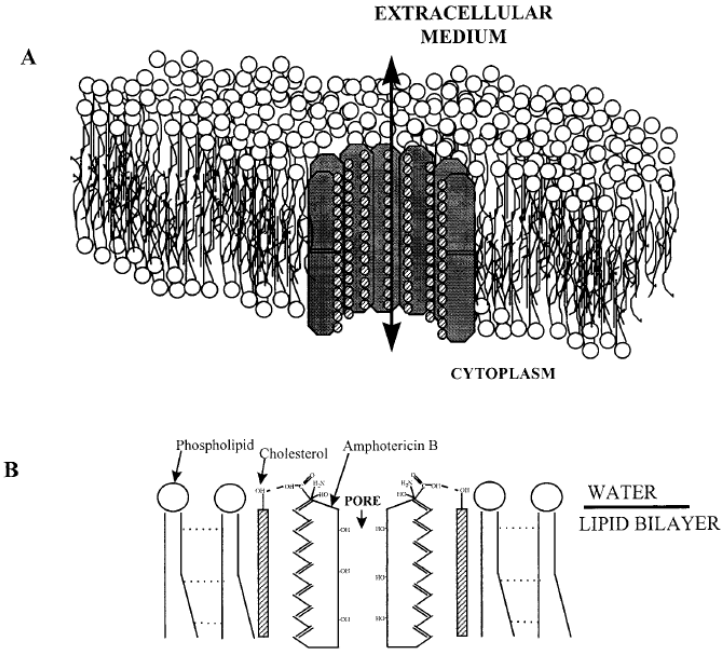

Figure 2.13 Mechanism of action of amphotericin B on a phospholipid bilayer in the cell membrane. (A) Amphotericin B molecules formed channels by binding sterols and leaking cell membrane. (B) Molecular orientation in an amphotericin B-cholesterol pore. The dotted lines between the hydrocarbon chains of phospholipids represent short-range London-Van der Waals forces. The dashed lines represent hydrogen bonds formed between amphotericin B and cholesterol molecules [64].

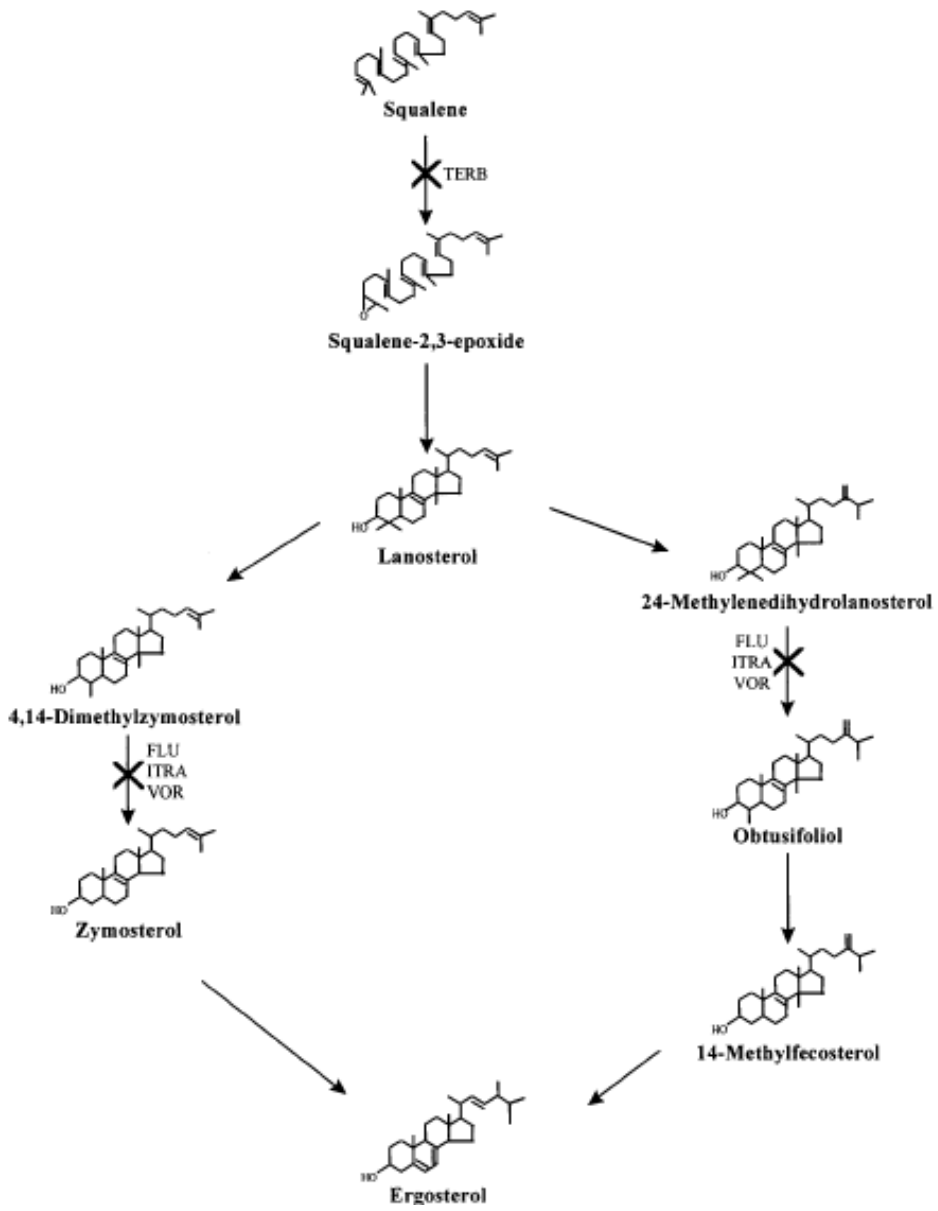

Figure 2.14 The terbinafine target works on an ergosterol synthesis pathway. The first step is a target of terbinafine and is an easy target for other ergosterol inhibition agents. TERB, terbinafine; FLU, fluconazole; ITRA, itraconazole; VOR, voriconazole [64].

2.3.2 Compounds targeting fungal cell walls

2.3.2.1 Inhibitors of glucan synthesis

The fungal cell wall contains compounds, including mannan, chitin, and α- and β-glucans, that are exclusive to the fungal kingdom, and so provide selective toxicity advantages. The fungal (1,3)-β-glucan synthases are specific inhibitors of echinocandins, one third of the inhibitors of glucan synthesis that includes aculeacins and papulacandins [74]. Echinocandins are lipopeptides that have fungicidal activity against *Candida* and *Aspergilus* species, both *in vitro* and *in vivo* [64, 75]. Echinocandins are massive lipopeptide molecules that are comprised of three derivatives, caspofungin, micafungin, and anidalafungin [74, 75]. The (1,3)-β-glucan synthesis studied in *S. cerevisiae* revealed that *FKS1* and *FKS2* genes have an important role in (1,3)-β-glucan synthesis. The mutation of the *FKS1* gene can be resistant to caspofungin. However, studies in yeast have also revealed that they play as a multicomplex enzyme subunit [74]. The multi-subunit enzyme complex contains at least two subunits, which are Fks1 and Rho1 (Fig. 2.15), encoded by *FKS1* and

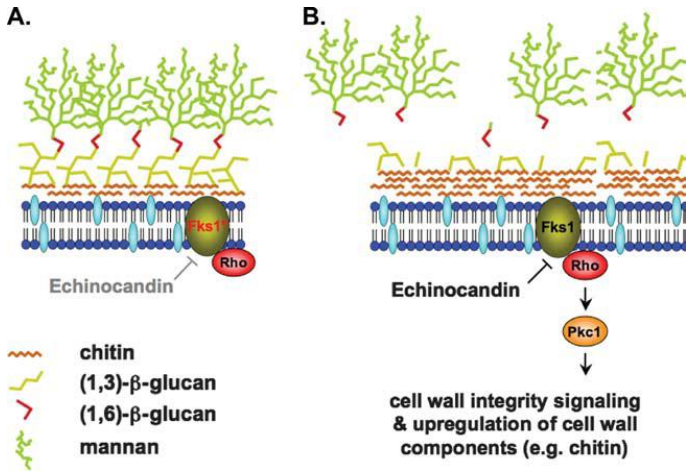

Figure 2.15 Echinocandin resistance mechanisms. (A) Decreasing of the side effects of echinocandins by mutation of (1, 3)-$\boldsymbol{\beta}$-D-glucan synthase that is the target of echinocandins encoded by *FKS1*. (B) Adjustment in cell wall composition by conferring with Rho1, a positive regulator of glucan synthase. It can mediate stress responses, including activation of the PKC cell wall integrity pathway and upregulation of cell wall components synthesis, such as chitin [1].

RHO1, respectively. Rho1 seems to act as a key product of the synthesis of (1,3)-β-glucan. Thus, inhibition of the (1,3)-β-glucan synthesis of this compound can cause cell death [5]. Nevertheless, the first resistance to echinocandin was reported in *S. cerevisiae*. Although the drugs are really good in killing *Canidida* spp., it was found that resistance occurred after many years of use. Resistance to the echinocandins were expected to be caused by mutations in the *FSK1* at either the HS1or HS2 hot spot regions [66, 67].

2.3.3 Compounds inhibiting nucleic acids

2.3.3.1 5-Fluorocytosine

5FC is a fluorinated pyrimidine with inhibitory activity against many types of yeast that act by interfering with pyrimidine metabolism and RNA, DNA, and protein synthesis (Fig. 2.10c). 5FC is converted to 5-fluorouracil (5FU) by cytosine deaminase and then converted to 5-fluorouridylic acid (FUMP) by UMP pyrophosphorylase for incorporating into RNA. Furthermore, 5FU can also inhibit thymidylate synthase as it is converted to 5-fluoro-deoxyuridine monophosphate. However, 5-Fuorocytosine can gain resistance from cells very quickly [75]. Resistance to 5FC may result from decreased uptake or loss of enzymatic activity responsible for conversion to FUMP [64]. For *S. cerevisiae* and *C. glabrata,* resistance may be caused by a defect in the function of the cytosine permease [69]. In other *Candida* spp., resistance may occur from mistakes in 5-FUTP and 5-dUMP conversion. On the other hand, resistance to 5-Fluorocytosine comes from a mutation in the *FUR1* gene that encodes uracil phosphoribosyltransferase, which leads to decreased conversion of 5-FC to 5-FU [66, 67, 76, 77].

2.4 Natural products

After the discovery of antifungal agents such as amphotericin B, azole, etc., the treatment of fungal infections seems to be effective [64]; However, some decades ago, it has become an increasingly important issue due to the emergence of drug resistance in patients [78]. Drug resistance problems are increasing worldwide. Apart from the limitations of drug targets that like humans, fungi have the ability to develop resistance mechanisms. Some examples include alterations in drug imports, intracellular drug processing, target enzymes, or alterations in efflux pumps [69]. Hence, it has become a

serious problem because of the difficulty in developing a new drug for replacement. There is a need to find novel sources of antifungal drugs. Today, natural products are still the most important reserve supply for finding new drugs.

Plants are a relatively cheap source of biological material available for selecting molecules with desired biological activity and have been used in traditional oriental therapies for a long time. Plants have an almost limitless ability to synthesize aromatic substances of different functional groups, mostly phenols or their oxygen-substituted derivatives. Plants are a rich source of a wide variety of bioactive secondary metabolites used for treating various diseases. Some plants are used for their odors (terpenoids), pigments (quinines and tannins), and flavors (terpenoid capsaicin from chili pepper)—those found to be endowed with medicinal properties [79].

The limitations of the amount of antifungal drugs and the widespread systemic fungal infections have made it necessary to investigate better antifungal drugs. As alternative of plants, microorganisms are capable of producing secondary metabolites that are considered bioactive compounds. Studies have explained the capability of secondary metabolites as antimicrobials, antivirals, and antitumor drugs, as well as agricultural and pharmacological agents [78]. Interestingly, some secondary metabolites were developed to be used as medicines, such as strobilurin, benzylpenicilin, etc. [78].

2.4.1 Antifungal agents derived from *Xylaria* species

Xylaria is a genus of fungi (Xylariaceae, Xylariaes, Sordariomycetes, Ascomycota) that has famous members of mycobiota in tropical forests [12]. Species in the genus *Xylaria* are saprobic or weakly parasitic in woody plants. However, some *Xylaria* species can be found in sawdust, leaf mold, dung, or in soil. Most species in this genus form upright, clavate or strap-like dark stomata surrounded by perithecia containing ascospores [80]. They can play diverse functions in terrestrial ecosystems, including decomposers, plant pathogens, endophytes, and symbionts with termites [12, 81, 82]. They also possess a wide range of bioactive compounds as well. Chinese traditional medicine uses the *Xylaria nigripes*, also known as *Wu Ling Shen*, to improve immunity and hematopoiesis [83, 84].

As stated above, *Xylaria* as decomposers have to produce extracellular enzymes to decompose plant structures [12, 79]. There is much evidence for *Xylaria* fungi showing

the ability to produce cellulotytic and lignolytic enzymes, which play roles in hydrolysis, solubilisation, and/or minoralization of plant litters [12, 82]. Importantly and interestingly, *Xylaria* species function as endophytes that live in host life cycles without causing any disease symptoms in the host [81, 82]. Fungal endophytes are a potential source to produce a wide range of biologically active metabolites. A secondary metabolite is defined as a chemical compound that is produced by a partial species in a genus, an order, or even phylum, and exhibits a difference in potential [85]. Secondary metabolites are important compounds as they can be antimicrobial, antibacterial, antifungal, antioxidant, or anticancer agents, for example. *Xylaria* has definitely been agreed upon to be an important secondary metabolite source [82, 85].

Fungi of the genus *Xylaria* are known to be a full source of secondary metabolites. Many of the secondary metabolites possess biological activity, and remarkable antifungal activities have been reported for xylarin and xylaramide [86, 87]. Ramesh *et al*. [88] have studied the *Xylaria* sp. strain R005 to investigate the antibacterial activity against the multidrug-resistant *Staphylococus aureus* and *Pseudomonas aeruginosa*. The fruit body of *Xylaria* sp. was used to determine the susceptibility of *S. aureus* and *P. aeruginosa* that are in the broad spectrum of Gram-positive and Gram-negative bacterial infections, respectively. *Xylaria* sp. R005 has shown antibacterial activity against *S. aureus* and *P. aeruginosa* with MIC values of 225 µg/ml to 625 µg/ml and 120 µg/ml to 625 µg/ml, respectively [88]. *Xylaria* sp. also possesses benzoquinone metabolites, 2-chloro-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione and xylariaquinine, which exhibits antimalarial activity against the *Plasmodium falciparum* K1 strain with MIC_{50} values of 1.84 and 6.68 µM, respectively [18].

Natural compounds, sordarin and sodaricin, are inhibitors of fungal protein synthesis [78, 89, 90]. Sordarin has a famous antifungal agent target protein synthesis at elongation factor-2 or EF2, which is extracted from *Sordarin araneosa* [78]. The other compound, sodaricin, is extracted from *Podospora pleiospora* [17, 89]. Importantly, *Xylaria* sp. PSU-D14 is found to produce sordaricin derivertive, sordaricin, and xylarin [17]. The antifungal compound xylarin is reported to have the capability to inhibit yeast growth [78]. Also, xylarin is found with *X. longipes* and identified as an inhibitor of protein synthesis (Fig. 2.16c) [78, 87, 91]. Sometimes, the antifungal activity of *Xylaria* extracts has better activity when combined with antifungal agents. Wu *et al*. [92]

reported synergistic antifungal activity against *Candida albicans* SC5314 with 0.004 µg/ml ketoconazole, but there is no activity when used alone (Fig. 2.16d). On the other hand, 7-amino-4-methylcoumarin extracted from *Xylaris* sp. YX-28 is tested for antibacterial and antifungal activity against microorganisms involved in food-borne diseases. This compound represented strong activity against many microorganisms, e.g., *Staphylococcus aureus, Escherichia coli, Salmonella typhimurium* and *Candida albicans* with MIC values of 16 µg/ml, 10 µg/ml, 20 µg/ml, 15 µg/ml, and 15 µg/ml, respectively [13].

Moreover, *Xylaria* sp. BCC 1067 is also a potential producer of polyketides and other bioactive compounds. The extract of this fungus contains two different polyketides: depudecin and 19,20-epoxycytochalasin Q (PK-nonribosomal peptide (NRP) hybrid; anticancer and anti-malaria in) [80]. Synthesis bioreductive esters of *Xylaira* sp. show that some compounds, 7 and 8, are strongly cytotoxic to NCI-H187 [93]. This efficiency can be further developed as anticancer drugs. The ability to inhibit cancer cells is reported in *Xylaria* sp. strain F1642 and other strains as well [94, 95]. The antioxidant activity is also interesting for *Xylaria* sp. [96, 97].

Xylaria nigripes or *Wu Ling Shen* is an important fungus for use in traditional Chinese medicine. It has the ability to improve immunity and hematopoiesis [83, 84]. *X. nigripes* has been reported to possess potential antioxidant, antiradical, and hepatoprotective activity [83, 84]. Recently, *X. nigripes* was studied and defined as an inhibitor of chronic inflammation and infection processes (NF-kB inhibitor) [84, 98].

Thailand is placed in a tropical zone with a diverse ecology system and bioresources. However, antimicrobial activity from endophytic fungi isolated from mangroves in Thailand has rarely been reported. Recently, the endophytic fungus *Xylaria cubensis* was reported to inhibit the growth of test microorganisms with low values of MIC when compared with the antibiotic drugs, vancomycin and gentamicin [99]. Sutjaritvorakul *et al*. [100] investigated the antimicrobial activity of endophytic fungi from Nan Province, Thailand. They found that *Xylaria* sp.1 can inhibit the growth of all tested microorganisms including *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aerogenosa, Escherichia coli,* and *Candida albicans*. Part of the *Xylaria* sp. strain BCC 4297 exhibited cytotoxicity activity against cancer cell lines with xylopimarane [101]. However, xylopimarane was investigated in human cancer cell lines KB such as oral

carcinoma, breast cancer and small-cell lung cancer [20]. *X. hypoxylon* has also been researched for its cytotoxicity, in which it is revealed that xylarone and 8, 9-dehydroxylarone are compounds that present this activity (Fig. 2.16e, f, respectively) [102]. *X. cubensis* and *X. obovaa* revealed that cytochalasin is an extract that shows cytotoxic activity [22]. Moreover, *X. cubensis* also possesses antifungal activity against *C. gloeosporioides* TC01 [103].

Xylaropyrone, a new compound from *Xylaria feejeensis* MU18, shows modulate *S. cerevisiae* growth inhibition with the MIC value of 32 μg/ml [15]. The well-known *Xylaria* fungi, *Xylaria polymorpha*, was reported to hold many bioactive compounds such as antifungal agents [104] (xylarinic acid a and b) (Fig. 2.16g, h, respectively) [14] and antioxidant agents (xylarinols a and b) [19].

Moreover, multiplolides A and B compounds from the *Xylaria multiplex* can also oppose fungi at 7 and 2 mg/ml [105]. For all the reasons above, the crude extracts of 10 *Xylaria* strains are tested for antifungal activity. These strains include *Xylaria* sp., *Xylaria obovata, Xylaria globosa, Xylaria arbuscula, Xylaria apiculata, Xylaria cubensis, Xylaria hypoerythra, Xylaria grammica, Xylaria multiplex,* and *Xylaria nigripes* taken from the Pilot Plant Development and Training Institute, King Mongkut's University of Technology Thonburi, Bangkuntien Campus. These are listed in Table 3.1 and Figure 3.1.

Natural products still remain to be potential sources for finding new and suitable drug molecules. The genus *Xylaria* has been known to be a great asset of novel bioactive metabolites with high activity against pathogenic microorganisms. Therefore, it could be evaluated as supplement food or drugs for future health care improvement.

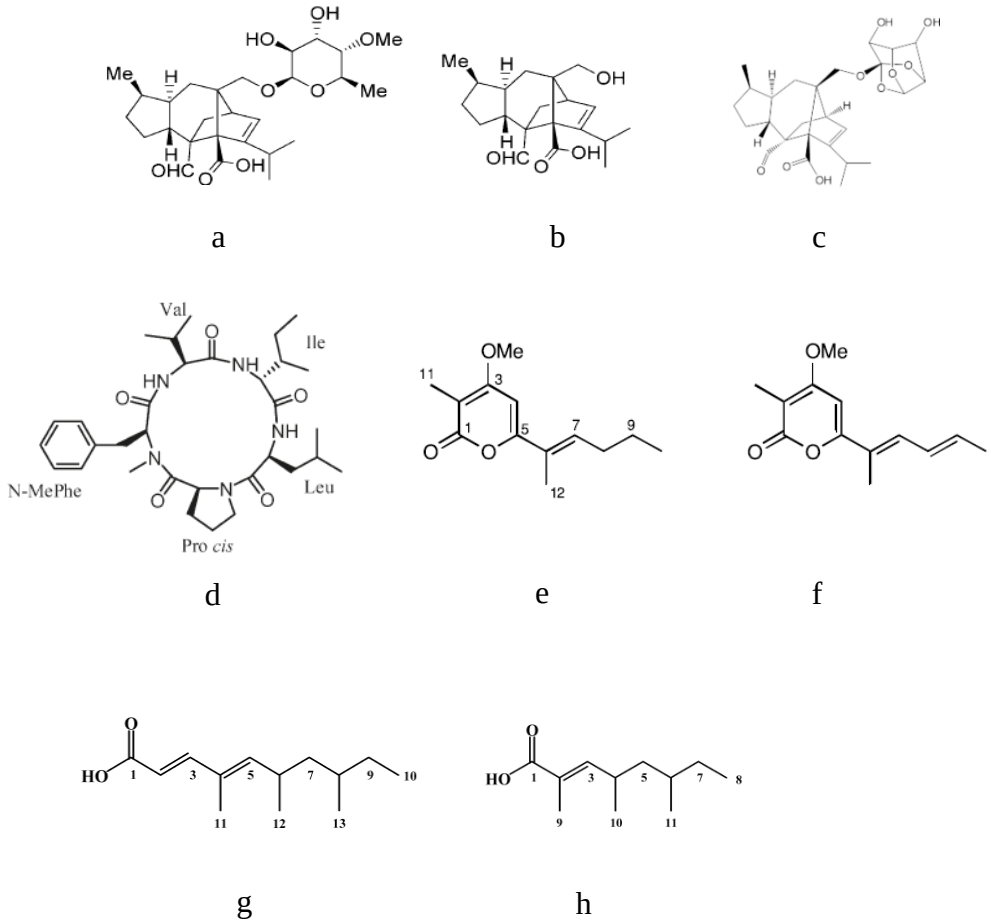

Figure 2.16 *Xylaria* compounds, the protein synthesis inhibitors sordarin (a), sordaricin (b), and xylarin (c) from *X. longipes.* Like sordarin, sordaricin and xylarin exhibit high anti-fungal activities towards yeasts. Compound d, proline-containing cyclopentapeptide, from *Xylaria* sp. It has no antifungal activity against *Candida albicans* SC5314 when used alone. The combination of the *Xylaria* compound with 0.004 µg/ml ketoconazole show markedly synergistic antifungal activity. [78, 89, 91]. Compounds xylarone (e) and 8,9-dyhydroxyl arone (f) are found in *X. hypoxylon.* And the compounds from *X. polymorpha,* xylarinic acid A (g) and B (h) [14]**.**

CHAPTER 3 MATERIALS AND METHODS

3.1 Yeast strains

Two groups of yeast comprised of the model yeast eukaryotic organism, *S. cerevisiae* and the pathogenic yeast *C. albicans* were studied. *S. cerevisiae* strains were BY4741 (*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and mutant strain with deletion in *YPL133c* (*rds2Δ::kanMX4*) and in *PDR5* (*pdr5Δ::kanMX4*). The ORF of *YPL133c*, encoding for zinc cluster protein Rds2, was deleted in BY4741 reference strain [106]. Disruption of ORF *YPL133c* was performed by replacing with *kanMX4* marker that confers resistance to geneticin. The *C. albicans* SGY243 (*ade2/*ade2Δura3*::ADE2/ura3::ADE2*) and its deletion strain Δ*cwt1* (SGY243 *cwt1Δ::kanMX4*) was also included in this study [107]. *CWT1* is homologue gene with zinc binuclear cluster protein *RDS2* in *S. cerevisiae.*

3.2 Media preparation and yeast cell growth

The culture media for yeast cell growth (YPD media) is comprised of 1% yeast extract (Himedia Laboratories, India), 2% bacto peptone (Himedia Laboratories, India), 2% glucose (Himedia Laboratories, India), and 2% of agar (Algas marinas S.A. Alagmar, Chile) was added for making solid YPD media. Five mililiters of the medium were added into a culture test tube and then the yeasts were inoculated and incubated at 30°C and 100 rpm overnight. Ketoconazole was obtained from Sigma and the stock solution was prepared in 70% ethanol at a concentration of 5 mg/ml and stored at -20°C. The *Xylaria* extract was stored by dissolving in 100 µl of methanol. The *Xylaria* were grown in malt extract broth media (CM0057, Oxoid ltd., England) and the solid malt extract media was performed by adding 1.5% of agar c (Bio Basic Canada Inc., Canada).

3.3 *Xylaria* culture and extraction

The *Xylaria* species used in this study were listed in Table 3.1. They were obtained from *Xylaria* collection (the Pilot Plant Development and Training Institute at King Mongkut's University of Technology Thonburi, Bangkuntien Campus). A small piece of *Xylaria* was transferred onto plates of malt extract agar (MEA) and cultured for one week (Fig. 3.1). These plates were incubated at 25°C, except for *Xylaria* sp. which was incubated at 30°C. After that, *Xylaria* was subcultured to 50 ml of malt extract broth (MEB) that was contained in a 250 ml flask for 25 days at 25°C (Fig. 3.2A). After the

incubation period, the *Xylaria* culture was filtered to separate the mycelia and filtrate. The filtrates were taken for extraction with two folds of EtOAc (ethyl acetate). The EtOAc layer was collected from the separation funnel, anhydrous magnesium sulfate ($MgSO_4$) was added and removed by filtration with Whatman No.1 filter paper (Fig. 3.2B). The filtrates were then evaporated at a pressure of 110 mbar and 44°C to remove the solvent. After one night, these crude extracts were then evaporated again with 5 ml of methanol same. If the solvent could not be completely removed, crude extracts were taken for freeze-dry. Finally, dried crude extracts were kept at 4°C and dissolved with methanol for future uses (Fig. 3.2C).

Table 3.1 List of *Xylaria* species used in this study, source, and corresponding bioactive compounds produced.

No.	Organism	Source	Metabolites	References
1	*Xylaria* sp. BCC 1067	Petiole (Nenga pumila), Northern Thailand	Depudecin	[108]
			Preussomerin G	[108]
			Preussomerin I	[108]
			Phaseoilnone	[108]
			Phomenone	
			19,20-epoxycytochalasin Q	[108]
			(E)-methyl 3-(4-methoxyphenoxy) propeonate	
			Xyrrolin	[109]
2	*Xylaria apiculata* BCC 1136	Twig, Central Thailand	n/a	
3	*Xylaria arbuscula* BCC 1156	Twig, Central Thailand	n/a	
4	*Xylaria cubensis* BCC 1027	Wood, Central Thailand	n/a	
5	*Xylaria globosa* BCC 31425	Wood, Northern Eastern Thailand	n/a	
6	*Xylaria grammica* BCC 22680	Wood, Northern Thailand	n/a	
7	*Xylaria hypoerythra* BCC 2646	Wood, Northern Eastern Thailand	n/a	

Table 3.1 List of *Xylaria* species used in this study, source, and corresponding bioactive compounds produced (continue).

No.	Organism	Source	Metabolites	References
8	*Xylaria multiplex* BCC 1177	Wood, Northern Thailand	n/a	
9	*Xylaria nigripes* BCC 26565	Soil, Central Thailand	n/a	
10	*Xylaria obovata* BCC 28742	Wood, Northern Thailand	n/a	

n/a, not available

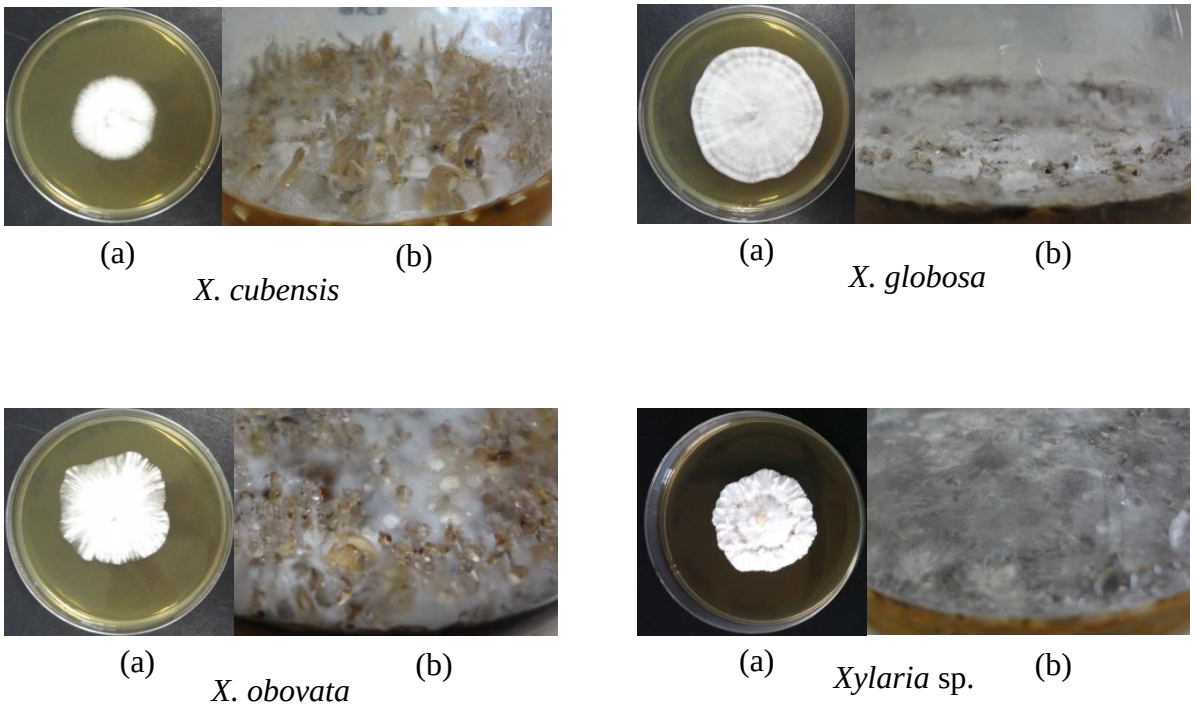

Figure 3.1 Cultivation of *Xylaria* spp. on MEB agar (a) and as fruiting bodies on malt extract broth media (b).

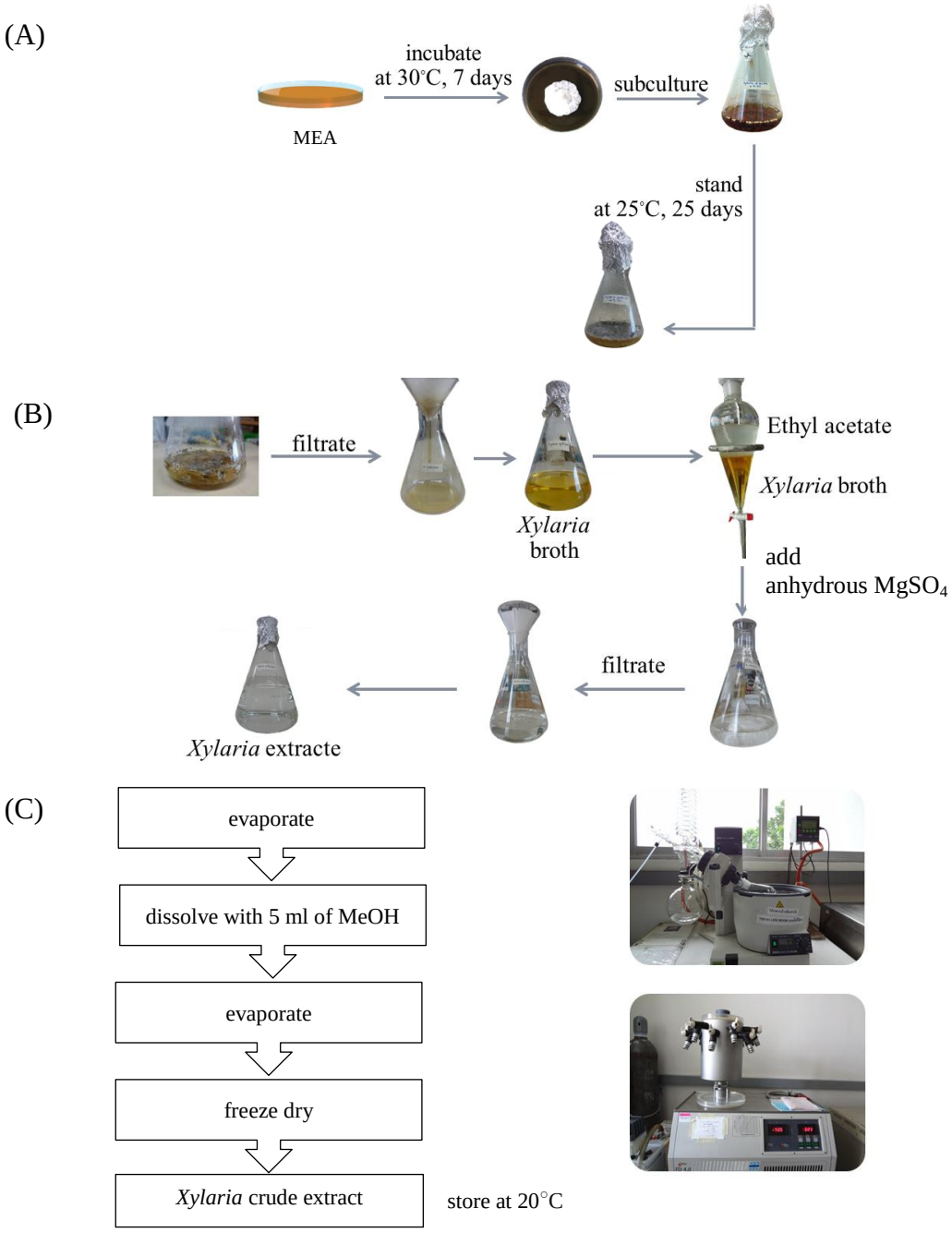

Figure 3.2 Cultivation, extraction, and evaporation for Preparation of *Xylaria* extracts: *Xylaria* were cultivated and maintained on malt extract agar (1.5% of malt extract and 2% of agar) at 30°C. The *Xylaria* contained in agars were cut into small pieces and transferred to 50 ml of malt extract broth media and allowed to grow for 25 days at 25°C (A) The culture was filtered to separate mycelia and broth (filtrate). Filtrates were then extracted with two folds of ethyl acetate (EtOAc). The EtOAc layer was recovered, anhydrous magnesium sulfate ($MgSo_4$) was added and the filtrate was filtered with paper filter (Whatman No.1) (B) The EtOAc solvent was evaporated to dryness and left for a night in darkness. 5 µl of methanol was added and evaporated again to provide crude extract. The freeze drying was used to completely dry crude extracts (C) *Xylaria* crude extract stored at -20°C.

3.4 Biological assay

3.4.1 Clear zone of inhibition

Growth of yeasts *S. cerevisiae* and *C. albicans* in the presence of the antifungal drug ketoconazole and/or *Xylaria* extracts were investigated. Yeast cells were first grown in YPD media to an optical density OD_{600} of 0.6-0.8 and then diluted to an optical density OD_{600} of 0.1. Then, 100 microliters of cell dilutions were spread onto YPD plates. Afterwards, the 5 µl *Xylaria* extracts that dissolved in the methanol were spotted onto a paper disk at the center of the plates. The zones of inhibition were measured after 3 days of incubation at 30°C under dark conditions. The azole antifungal drug, ketoconazole, was served as a positive control. The combined activity between ketoconazole and *Xylaria* extracts were also studied (see Appendix).

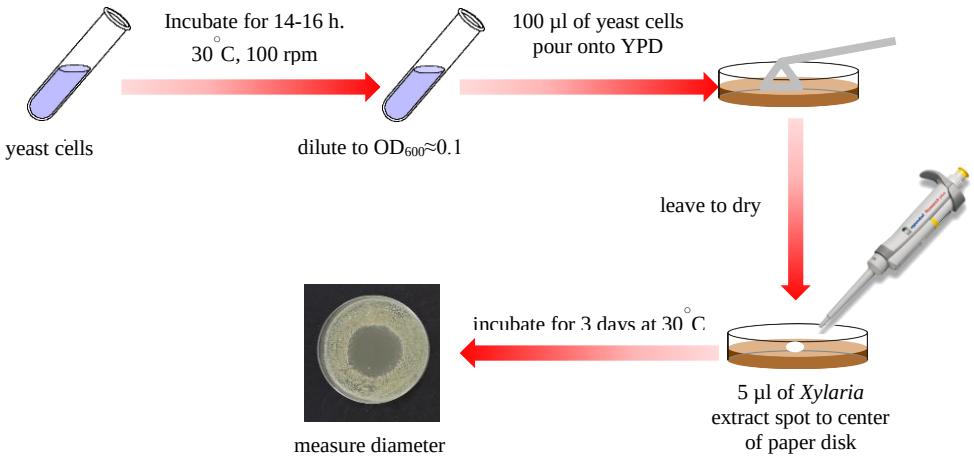

Figure 3.3 Determine the antifungal activity of compounds against *S. cerevisiae* and *C. albicans* strains using a clear zone of inhibition assay.

3.4.2 MIC assay/antifungal test

The sensitivity of yeast *S. cerevisiae* to *Xylaria* extracts were performed using minimal inhibition concentration (MIC) assay. The lowest concentration of extract that impaired growth of yeast *S. cerevisiae* to 50 percents compared with untreated cells was indicated to be a MIC_{50} value. Briefly, yeast cells were inoculated overnight and diluted to OD_{600} of 0.1, re-inoculated and grown to OD_{600} of 0.6-0.8. Yeast cells cultures were then diluted by 1000 folds in fresh YPD media. Fifty microliters of final cell suspension were applied to each well of a sterile 96-well plastic flat-bottom plate (costar® 3599, USA) and extract solutions were then added to each well at a series of *Xylaria* extracts

concentrations. The final volume was adjusted to 100 microliters with sterile water. The plates were incubated for 24 h at 30°C and the OD_{600} was measured by using an automated microplate reader at 600 nm (M965+Microplate Reader, Metertech Product, Taiwan). The antifungal drug ketoconazole was also included in this assay as a positive control (Fig. 3.4).

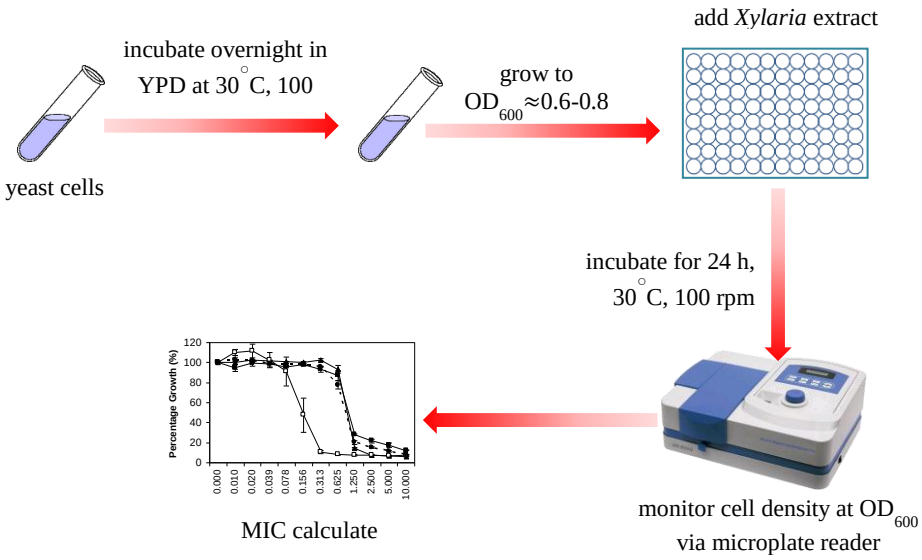

Figure 3.4 Determination of the antifungal activity of compounds via minimal inhibition concentration (MIC) assay. The MIC assays were performed in YPD media containing *Xylaria* extracts at various concentrations using a microtiter plate.

3.4.3 MFC assay/ antifungal test

The minimal fungicidal concentration (MFC) was evaluated to investigate the concentration of *Xylaria* extracts required to kill the cells [110]. After determination of MIC values, the well that showed no visible growth was diluted to 1000X of starting OD_{600} of the culture. Then, 30 µl of dilution was spread on YPD plate. These plates were then incubated under 30°C until growth was observed in the control plates, containing no *Xylaria* extract [111]. The concentrations of *Xylaria* sp. extract were used as 0.03, 0.06, 0.1, 0.5, 1.0, and 5.0 mg/ml to determine the MFC values (Fig. 3.5).

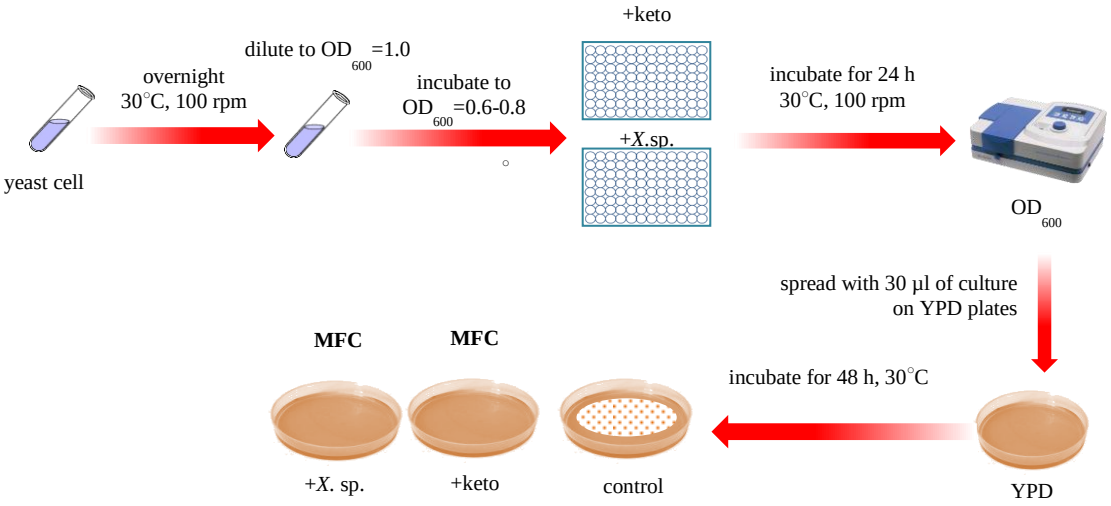

Figure 3.5 Diagram shows steps performed in the minimal fungicidal concentration (MFC) assay used to investigate the concentration of *Xylaria* extracts that kill the cells.

3.5 RNA isolation and purification

3.5.1 Induction of *PDR5* expression

The level of *PDR5* or *YOR153w* expression was observed in the absence or presence of *Xylaria* sp. extract and ketoconazole. Yeast cells were grown overnight at 30°C, 100 rpm and re-inoculated in 40 ml of YPD media to OD_{600} of 0.1. When cells reached the OD_{600}=0.7, they were diluted and transferred to a 10 ml tube that contained 4 µg/ml of ketoconazole, or 0.5 mg/ml of *Xylaria* sp. extract, and YPD alone. These cultures were incubated for 2 h. After that, cells were spun for 5 min at 2600 rpm and washed with DEPC water twice. Remaining pellets were transferred to a new Eppendorf tube and spun for 1 min at 1400 rpm, 4°C to remove the remaining liquid. Cell pellets were stored at -80°C for RNA extraction.

3.5.2 RNA extraction

The yeast cells were taken out of a -80°C freezer and thawed on ice. Then 150 µl of Acid Phenol-Chloroform was added (ratio 5:1) (pre-warmed 10 min at 65°C) and 150 µl of TES were added. Then, the mixture of cells was disrupted with glass beads by vortexing the tube at the highest angle setting (≈ 20° angle). After, they were incubated at 65°C (a water bath) for 1 h, and subjected to a vortex for 20 sec (10 sec upright, 10 sec at angle) every 10 min. They were spun for 20 min at 14,000 rpm. Then, the RNA was extracted with 750 µl of phenol-Chloroform (5:1) and 750 µl of chloroform-

isoamyl. Supernatant was transferred to new tubes with 50 µl of 3 M Sodium Acetate, pH 5.2 and 800 µl of 70% Ethanol (pre-cooled to 20°C, filled to top of tube) and stored at -20°C for longer than 1/2 h to precipitated RNAs, spun down for 20 min at 14000 rpm, 4°C. A small amount of aspirate was left, and RNAs were subsequently washed with 500 µl of 70% ethanol, spun down, aspirated to remove the last bit and RNAs left to air dry for 30 min, and dissolved in DEPC treated water and kept at -20°C before uses.

3.5.3 RNA purification

Briefly, RNAs were purified (Qaigen kit). The RNA samples were adjusted to a volume of 100 µl with RNase-free water. 350 µl Buffer RLT and 250 µl of ethanol were added and mixed well, transferred to a RNeasy Mini spin column, then centrifuged for 15 sec at 10000 rpm. 350 µl of Buffer RW1 was added to RNeasy spin column and centrifuged for 15 sec at 10000 rpm. A mixed solution of DNase 1 and Buffer RDD was applied to the column and centrifuged briefly twice. Then, columns were placed on the benchtop for 15 min, 350 µl of Buffer RW1 was added to the RNeasy spin column, and centrifuged for 15 sec at 10000 rpm. After that, 500 µl of Buffer RPE was added and centrifuge for 15 sec at 10000 rpm twice. RNeasy spin columns were removed to new collection tube, 30 µl of RNase-free water was added to the column membrane and was left standing for 10 min, and for 15 sec at 10000 rpm. Lastly, 30 µl of RNase-free water was added to each collection tubes.

3.5.4 RNA gel and electrophoresis

Gel preparation:

This method was made for a Large-size gel (20-40 samples), with a final volume of 300 ml and a weight of 3 g of agarose. 30 ml of 10X MOPS was added and completed with 207.5 ml of DEPC water. Then, it was microwaved, cooled down and followed by an addition of 62.5 ml of formaldehyde and 5 µl of 10 mg/ml ethidium bromide. The gel was then poured into the gel box and left to be solidified at room temperature.

RNA sample gel preparation:

The RNA sample for gel loading contained 5.5 µl RNA, 1.0 µl 10X MOPS, 3.5 µl of formaldehyde, 10 µl formamide, and 2 µl 5X loading dye. The final volume was 22 µl. The sample was heated at 65°C for 15 min. Then, a quick spin was done and the sample was kept on ice until loading. The gel migrated within the chemical hood. It was washed overnight with distilled water and a picture of the gel was taken.

3.6 qRT-PCR

3.6.1 First-Strand cDNA Synthesis

The cDNA synthesis was started by combining 5 µg of total RNA, Primer 1 µl (50 µM of oligo$(dT)_{20}$, or 2 M gene-specific primer (GSP), or 50 ng/µl random hexamers), 10 mM dNTP mix, and up to 10 µl DEPC-treated water. All tubes were incubated at 65°C for 5 min and then placed on ice for at least 1 min. Preparation of the following cDNA Synthesis Mix was done by adding each component in the indicated order (for 1 reaction): 10X RT buffer 2 µl, 25 mM $MgCl_2$ 4 µl, 0.1 M DTT 2 µl, RNaseOUT™ (40 U/µl) 1 µl, and Superscript™III RT (200 U/µl) 1 µl (for control reaction, add DEPC-treated water instead Superscript™III RT (200 U/µl)). 10 µl of cDNA Synthesis Mix was added to each RNA/primer mixture, mixed gently, and collected by brief centrifugation. All solutions were incubated as follows: 50 min at 50°C for oligo$(dT)_{20}$, GSP primed, and for Random hexamer primed, incubated for 10 min at 25°C, followed by 50 min at 50°C. After incubation, the reactions were terminated at 85°C for 5 min and chilled on ice. The reactions were collected by brief centrifugation and followed by the addition of 1 µl of RNase H to each tube and incubated for 20 min at 37°C. cDNA synthesis reaction can be stored at -20°C or used for PCR immediately.

3.6.2 PCR

The cDNAs were used as a substrate for quantitative real time-PCR (qRT-PCR). The reaction mixtures contained 4 µl of the appropriate dilution of cDNA, 6 µl of 2X Brilliant SYBR green master mix (Kapabiosystem), and 10 µl of primer master mix (PMM). Amplification was performed by using MxPro QPCR machine and MxPro QPCR software for analysis. Then, the reaction mixtures were placed in a preheated (94°C) real-time thermal cycler. The initial denaturation step was performed (94°C for 2 min.). This was followed by a performance of 40 cycles PCR: this entailed denaturing

at 94°C for 15 sec; annealing at 55°C for 30 sec; and extending at 68–72°C for 1 min. Upon completion, reactions were maintained at 4°C. An internal control, the *S. cerevisiae* actin gene *ACT1* was amplified. All reactions were performed in triplicated. For analysis, 10 µl of each sample was used for agarose gel electrophoresis and ethidium bromide staining in 1XTAE. Primer sequences used in qRT-PCR are noticed in Table 3.2. The relative mRNA level of target gene was calculated using $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (C_{t,PDR5}-C_{t,ACT1})_{\Delta} - (C_{t,PDR5}-C_{t,ACT1})_{WT}$ [112].

Table 3.2 Sequences of *PDR5* primer used in qRT-PCR**.**

Primer name	**Primer sequence (5'→3')**
PDR5	GTGCTCATAAATGCCCTGCT and TGAACGGCCCTGTACTCTTC
ACT1	ATTATATGTTTAGAGGTTGCTGCTTTGG and CAATTCGTTGTAGAAGGTATGATGCC

CHAPTER 4 RESULTS AND DISCUSSION

4.1 Function of Rds2 regulator in PDR

Pleiotropic drug resistance protein Pdr5 is an important plasma membrane associated pump that belongs to a group of ATP-binding cassette (ABC) transporters [2]. ABC transporters can efflux a variety of unrelated compounds from intracellular such as ions, sex hormone, wastes, and toxin [32]. Here, we were interested in the identification of *Xylaria* extracts with promising antifungal activity. In addition, the ability of Pdr5 transporter to efflux a variety of substrates makes it an appealing candidate resistance to *Xylaria* extracts. Previous work also showed that Rds2 is a PDR regulator and is bound to *PDR5* promoter during the glycerol shift [3, 4]. Thus, we began by testing for sensitivity of yeast deletion strains Δ*rds2* and Δ*pdr5* strains to crude extracts isolated from the *Xylaria* species via different antifungal assays.

4.1.1 Clear zone of inhibition

Pleiotropic drug resistance or PDR is the ability of cells to tolerate the drugs or substances that are toxic to the cells [32]. The PDR occurs by the over expression of PDR genes related to pump drugs out of cells, such *as PDR5, PDR12, PDR15, SNQ2, YOR1, FLR1,* and *AZR1* of *S. cerevisiae* [2]. In this experiment, we selected *PDR5* to test for *Xylaria* extract resistance because overexpression of *PDR5* gene allows cells to withstand a wide variety of toxic compounds and drugs [113]. As previously reported, *S. cerevisiae* strain lacking *PDR5* showed impaired growth when grown in the presence of ketoconazole [41]. This is good evidence, indicating the pertaining roles of *PDR5* during growth under toxic environment. The results of drug susceptibility tests with an antifungal drug ketoconazole in the yeast *S. cerevisiae* BY4741 background and the deletion strain Δ*pdr5* was verified again. We confirmed that Δ*pdr5* strain exhibited increased sensitivity to ketoconazole (Fig. 4.1).

We hypothesized that *PDR5* may confer resistance to *Xylaria* extracts and function to efflux these toxic extracts out of cells, and its expression may be controlled by the transcription factor Rds2. To examine that the antifungal activity of *Xylaria* extracts against *S. cerevisiae* BY4741 and deletion Δ*rds2* and Δ*pdr5* strains, a clear zone of inhibition assay was carried out. Four *Xylaria* extracts including, *Xylaria* sp. BCC1067, *X. cubensis* BCC 1027, *X. globosa* BCC 31425, and *X. obovata* BCC 28742 were tested.

Briefly, the yeast cells were grown overnight on YPD media and diluted to OD_{600} of 0.1 and spread onto YPD plates, containing sterile paper disks that were soaked with 5 µl *Xylaria* extract at the center of the plate. The zones of inhibition were measured after 3 days of incubation at 30°C under dark conditions. The azole antifungal drug, ketoconazole, was served as a positive control and the combined activity between ketoconazole and *Xylaria* extracts were also studied.

The inhibition zones formed by *Xylaria* crude extracts and ketoconazole were showed in Table 4.1. The results showed that all four extracts inhibit the growth of three yeast strains at different degree (Table 4.1). As showed in the Table 4.1, all *Xylaria* spp. presented similar antifungal activity against *S. cerevisiae* BY4741 with an inhibition zone, ranging from 6.0 mm to 8.8 mm. Because these fungi are in the same genus, the majority components of the secondary metabolites produced are likely to be of similar types. Moreover, the same concentrations were used to test for antifungal activity against *S. cerevisiae.* In addition, these *Xylaria* were cultured under the same conditions, so the materials they produced are likely to be the same.

Among all extracts, *Xylaria* sp. crude extract was the best growth inhibitor with a clear zone of inhibition of 8.8 mm (Table 4.1). We also performed this experiment with *S. cerevisiae* deletion Δ*rds2* and Δ*pdr5* strains because it is hypothesized that the *PDR5* gene may be involved in tolerance to antifungal activity of *Xylaria* crude extracts via the control of Rds2. The results of Δ*rds2* strain showed that it is most sensitive to *X. cubensis* crude extract with a clear zone of inhibition of 10.5 mm (Table 4.1). All extracts have strong antifungal activity against Δ*pdr5* strain, except for *X. globosa* crude extract (Table 4.1). These clear zone diameters range from 9.5 mm to 20.5 mm (Table 4.1). It is clearly seen that Pdr5 is a major protein, responsible for removal of these extracts. Not surprisingly, since previous studies have demonstrated that Pdr5 transports numerous compounds out of the cell, including toxic compounds [54].

Ketoconazole drug is widely used in clinical settings; however, high rate of resistance of fungal pathogen is found. One of the ketoconazole resistance mechanisms is the overexpression of the drug efflux pumps or PDR transporter genes. So, we were interested to see the effect of the combination of ketoconazole and *Xylaria* crude extracts against yeast growth. Clearly, the combination of *Xylaria* crude extracts and

ketoconazole enhanced the antifungal activity against all tested strains when compared with *Xylaria* or ketoconazole alone (Table 4.1).

In *S. cerevisiae*, *X. cubensis* crude extract showed the best antifungal activity with a diameter of clear zone of inhibition of 23.0 mm (Table 4.1). Extracts of *Xylaria* sp., *X. obovata,* and *X. globosa* showed different clear zone values of 19.0 mm, 17.0 mm, and 9.5 mm, respectively (Table 4.1). The results showed little difference of inhibited growth of deletion Δ*rds2* strain when compared with BY4741 wild-type strain. However, the most potent antifungal activity continued to be of *X. cubensis* extract with 23.5 mm of clear zone of inhibition (Table 4.1). Interestingly, deletion Δ*pdr5* strain was very sensitive to all *Xylaria* extracts and *Xylaria* sp. extract is the most potent antifungal activity with a diameter of clear zone of inhibition of 34.0 mm (Table 4.1). Other extracts showed similar diameters of clear zone with 30.5 mm, 30.0 mm, and 30.0 mm for *X. cubensis*, *X. globosa,* and *X. obovata,* respectively (Table 4.1).

The activity found in this study may be resulted from the presence of toxic components of *Xylaria* extracts which remains to be identified. Ketoconazole inhibited ergosterol biosynthesis, which is a major sterol in fungal cell membranes and an accumulation of alternative toxic sterols led to disrupted cell membrane integrity [114]. One possibility for enhanced antifungal activity in the combinatorial test is that altered plasma membrane components caused by ketoconazole administration administration might facilitate the entry of *Xylaria* extracts into the cell. Some of *Xylaria* components, for example sordarin, act as a protein synthesis inhibitor target elongation factor 2 in fungal cells resulted in stopping cellular processes [78]. However, each *Xylaria* extract might contain different bioactive compounds so the potential growth inhibitory growth effect may be resulted from different mechanism.

In fact, the study of antimicrobial activity of *Xylaria* spp. has been investigated. Recently, Ramech *et al*. [88] reported that the extracts of *Xylaria* sp. strain R005 exhibited the antibacterial activity against both gram-positive and gram-negative bacteria such as *Staphylococcus aureus* ans *Pseudomonas aeuginosa*, with zones of inhibition values of 22.5 mm and 24.3 mm, respectively.

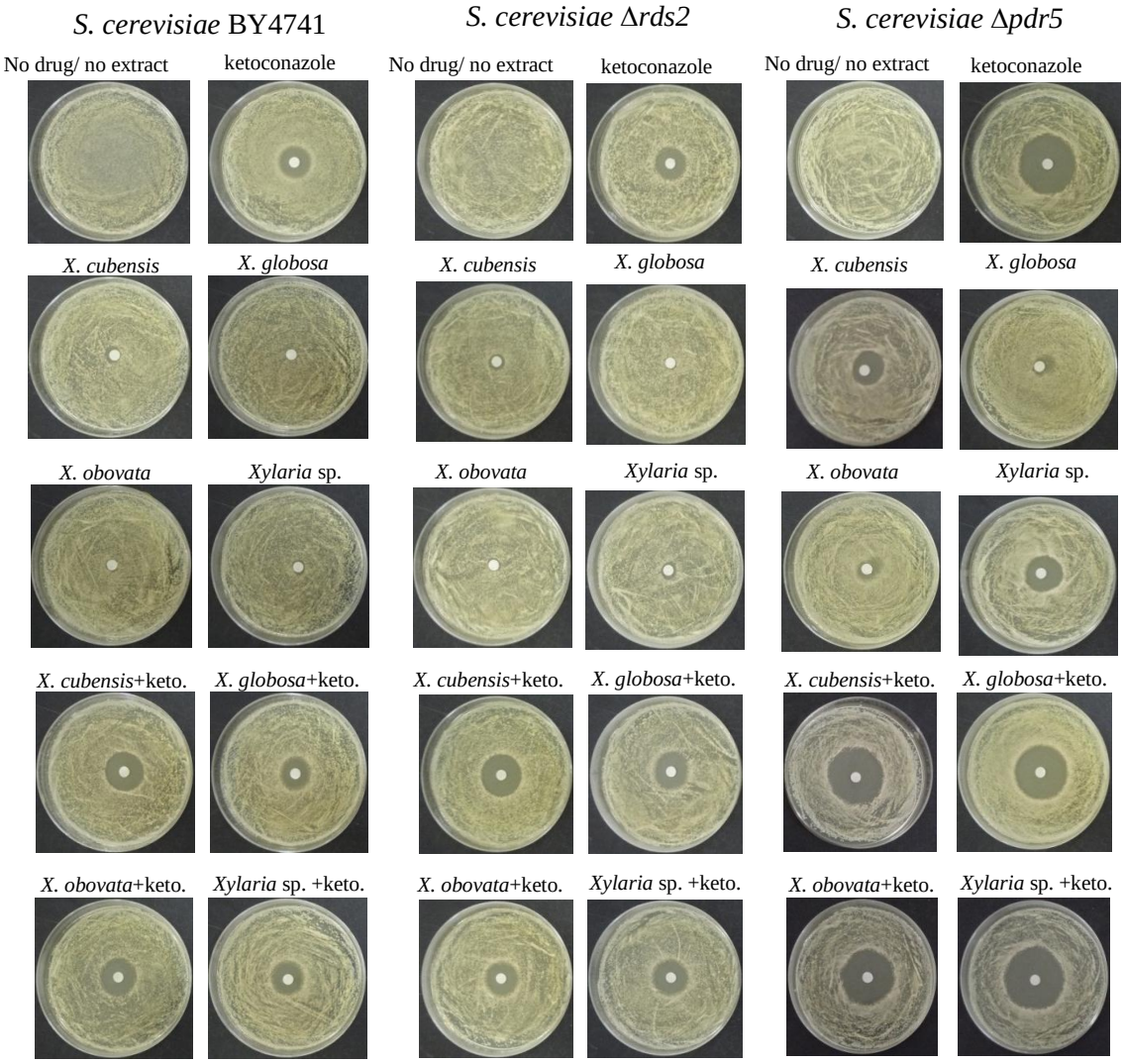

Figure 4.1 The antifungal activity of *Xylaria* extracts against *S. cerevisiae* BY4741, Δ*rds2*, and Δ*pdr5* strains were examined with a clear zone of inhibition assay. The antifungal activity from the combination of *Xylaria* extracts and ketoconazole was also investigated. *Xylaria* extracts were dissolved in methanol. 5 µl of 40 g/l crude extract was used to test and combine with 5 µl of 1,000 µg/ml ketoconazole for combination.

Although little antifungal activity tested with phenotypic (see Appendix A) was found when testing with the extract alone, there was a stronger antifungal activity against the *S. cerevisiae,* when combined with 4 µg/ml of ketoconazole (Fig. 4.1 and Table 4.1). We suggested that these *X. cubensis, X. globosa, X. obovata,* and *X.* sp. crude extracts may have synergistic antifungal activity with ketoconazole against *S. cerevisiae.* Similarly, as reported by other research group, the *Xylaria* sp. strain R005 exhibited strong synergistic antibacterial activity against *Pseudomonas aeruginosa* (strain 3) when used with the antibiotic ciprofloxacin [88]. Further experiments such as

crosscheck analysis can be carried out to investigate the synergistic effect between *Xylaria* extract and ketoconazole

4.1.2 MIC assay/antifungal test

In the past, we have conducted clear zone of inhibition experiment to identify *Xylaria* extracts that possess antifungal activity against *S. cerevisiae* BY4741 and their deletion strains, Δ*rds2* and Δ*pdr5*. Four *Xylaria* extract comprised of *Xylaria* sp. BCC1067, *X. cubensis* BCC 1027, *X. globosa* BCC 31425, and *X. obovata* BCC 28742 were found to present good antifungal activity (Fig 4.2 and Table. 4.2). However, the clear zone of inhibition assay was only a preliminary step for testing their actual antifungal activity and it is essential to have other evidences to support. In this case the minimum inhibitory concentration (MIC) assay is considered a better option to test for the antifungal activity as this method is more sensitive than the methods above.

Next, the MIC assay is used for testing the effectiveness of *Xylaria* extracts against yeast strains. Dilution of overnight yeast cell culture was re-inoculated at 30°C with a starting OD_{600} of 0.1 and the cells were grown until OD_{600} reached 0.6-0.8. Then, cells were again diluted to OD_{600} of 1000 folds of the initial OD_{600} of 0.1. After that, dilution of cells was added into a 96-well plastic flat-bottom plate that contained *Xylaria* extracts at various concentrations. The final volume was adjusted to 100 microliters with sterile distilled water. The plates of cells were incubated for 24 h at 30°C to monitor the growth inhibition by using an automated microplate reader at OD of 600 nm. The antifungal drug, ketoconazole, was included in this assay as a positive control.

With our results, we defined MIC_{50} the lowest concentration of *Xylaria* extract to reduce 50% growth of *S. cerevisiae* that was observed on the optical density check at 600 nanometers after incubation at 30 $^{\circ}$C for 24. After the end of 24 hours, the graph showed that all *Xylaria* extracts including *X.cubensis* BCC 1027, *X. globosa* BCC 31425, *X. obovata* BCC 28742, and *Xylaria* sp. BCC 1067 were effective in inhibiting the growth of *S. cerevisiae* BY4741 and deletion Δ*rds2* and Δ*pdr5* strains with increasing extract concentrations (Fig. 4.2). Results showed inhibited growth of *S. cerevisiae* BY4741, Δ*rds2* and Δ*pdr5* strains under the presence of *Xylaria* extracts. Also, we further tested and determined the minimal inhibition concentrations to validate the antifungal activity against *S. cerevisiae* of these *Xylaria* extracts.

Table 4.1 Antifungal activities of *Xylaria* extract against *S. cerevisiae* BY4741and its deletion strains Δ*rds2* and Δ*pdr5* via clear zone of inhibition assay.

Compounds tested	Clear zone of inhibition (mm.)[a]								
	BY4741			**Δ*rds2***			**Δ*pdr5***		
A. *Xylaria* extracts									
Xylaria cubensis BCC 1027	8.0	±	0.0	10.5	±	0.7	20.0	±	0.0
Xylaria globosa BCC 31425	6.0	±	0.0	7.0	±	0.0	9.5	±	0.7
Xylaria obovata BCC 28742	8.5	±	2.1	8.0	±	1.4	20.0	±	1.4
Xylaria sp. BCC 1067	8.8	±	0.4	8.5	±	0.7	20.5	±	0.7
B. Combination of *Xylaria* extractswith ketoconazole									
Xylaria cubensis BCC 1027	23.0	±	1.4	17.0	±	2.1	30.5	±	2.1
Xylaria globosa BCC 31425	9.5	±	3.5	15.5	±	2.1	30.0	±	0.0
Xylaria obovata BCC 28742	17.0	±	0.0	20.0	±	0.0	30.0	±	0.0
Xylaria sp. BCC 1067	19.0	±	1.4	10.5	±	0.7	34.0	±	1.4
C. Ketoconazole									
H_2O	15.5	±	0.7	8.5	±	0.7	30.5	±	2.1

[a]diameter of disk (6 mm.)
[b]used 5 µl of 40 mg/ml *Xylaria* extracts and then combined with 5 µl of 1 mg/ml ketoconazole
[c]used 5 µl of 1 mg/ml ketoconazole
Xylaria extracts were dissolved in methanol before use.

Table 4.2 The haft minimal inhibition concentration MIC_{50} of *Xylaria* extracts against *S. cerevisiae* BY4741, Δ*rds2* and Δ*pdr5* strains.

Compounds	MIC_{50} (mg/ml)		
	BY4741	**Δ*rds2***	**Δ*pdr5***
Xylaria cubensis BCC 1027	0.10	0.08	0.09
Xylaria globosa BCC 31425	0.09	0.06	0.20
Xylaria obovata BCC 28742	0.08	0.06	0.08
Xylaria sp. BCC 1067	0.10	0.03	0.02

MIC_{50} is minimal concentration where 50% yeast cells were inhibited.
The experiments showed antifungal activity of *Xylaria* extracts against *S. cerevisiae* deletion *RDS2* and *PDR5* in term of MIC_{50} from two susceptibility independent experiments.

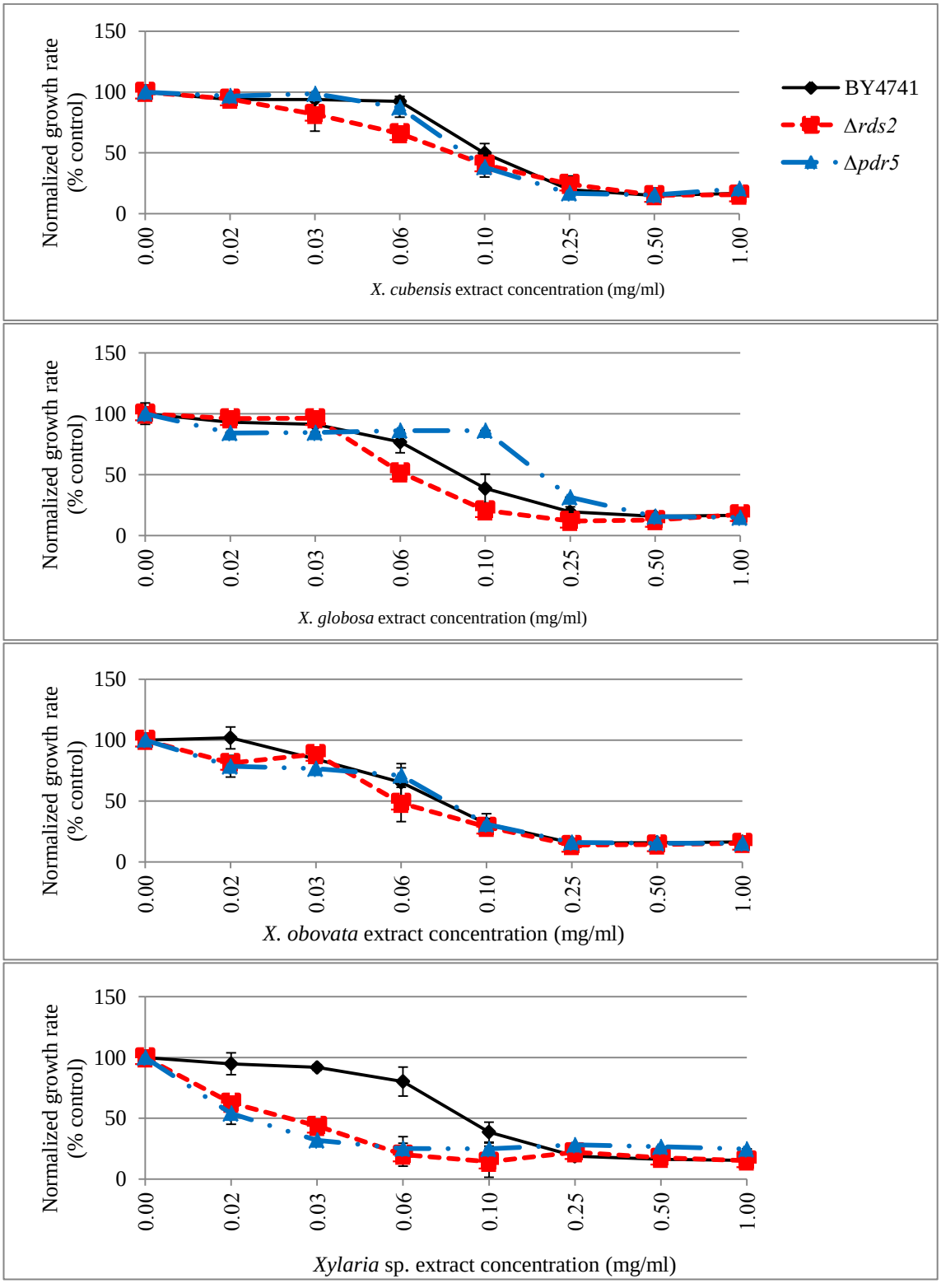

Figure 4.2 The antifungal activity of *Xylaria* extracts against *S. cerevisiae* BY4741 (—◆—) and deletion *RDS2* (- -■- -) and *PDR5* (—▲·) strains. The strains were grown in YPD overnight at 30°C and re-inoculated to OD_{600}=0.6-0.8. Cells were diluted to OD_{600}=0.001 before being treated with extracts. Cells were used for 50 µl of final volume in 96-well plate. OD_{600} values were detected at 24 h of incubation at 30°C and 100 rpm. The solid black line is BY4741 as well as red and blue dash lines are Δ*rds2* and Δ*pdr5* strains, respectively.

The minimal inhibitory concentration (MIC_{50}) was compared in *S. cerevisiae* cells that were grown in the absence of extract or antifungal drug in the media. The crude extract of *Xylaria* sp. exhibited best antifungal activity against the model yeast *S. cerevisiae* BY4741, Δ*rds2*, and Δ*pdr5* strains at the MIC_{50} value of 0.10, 0.03, and 0.02 mg/ml, respectively (Table 4.2). Thus, the lack of *RDS2* or *PDR5* genes resulted in increased sensitivity to *Xylaria* sp. extract. Finally, we showed that the extract of these *Xylaria* including *X. cubensis*, *X. globosa*, *X. obovata*, and *Xylaria* sp. have the potential ability to inhibit the growth of the model yeast, *S. cerevisiae*. We also recommend to further test for the antimicrobial activity against other microorganisms as these extracts may have the ability to resist the growth of microorganisms other than *S. cerevisiae*. Recently, Pongcharoen *et al*. [17] investigated antifungal activity of MIC value of 32 µg/ml of *Xylaria* sp. PSU-D14 against *C. albicans* ATCC90028 (CA28). Moreover, before that, Phongpichit *et al.* [115] also reported antifungal activity against pathogenic yeast *C. albicans* (CA28) with a MIC value of 0.128 mg/ml. In 2008, a study presented the bioactive compound 7-amino-4-methylcoumarin that was extracted from *Xylaria* sp. YX-28 and found in *Ginkgo biloba* L. (Ginkgoaceae). This extract possesses broad-spectrum antimicrobial activity against numerous food-borne and food spoilage microorganisms [13]. In summary, a search for natural compounds with bioactivity is useful, urgent and important for antifungal drug development.

4.1.3 MFC assay/antifungal test

The results from MIC assay were used to examine the minimal fungicidal concentration of *Xylaria* sp. extract. The experiment was performed with *Xylaria* sp. extract because this extract shows the best antifungal activity against *S. cerevisiae* BY4741, Δ*rds2*, and Δ*pdr5* strains. The minimal fungicidal concentration (MFC) value was defined as the lowest concentration of *Xylaria* sp. extract that represented no visible turbidity in 96-well plate and without colony development on YPD plate [111]. Shortly, the culture yeast cells from MIC assay grown in the presence of *Xylaria* sp. extract were taken to measure the colony number, including MFC value. 30 µl of cells culture in each well were spread onto the top of the YPD agar plate. Plates were incubated at 30°C till the growth of untreated plates could be observed. Yeast colonies were then counted and calculated for cell survival.

Results indicated that killing activity of *Xylaria* sp. extract was more effective at killing the deletion strains than the parental strain. The strongest activity was observed for *S. cerevisiae* deletion Δ*pdr5* strain with MFC values at 50%, 20% and 0% viability was found to be 0.12, 0.39, and 0.50 mg/ml of *Xylaria* sp. extract, respectively (Table. 4.3). The Δ*rds2* strain showed close sensitivity to *Xylaria* sp. extract when compared with the Δ*pdr5* strain at viability percentages of 50 and 20 with MFC values of 0.13 and 0.48 mg/ml, respectively (Table. 4.3). In contrast, the MFC value of *Xylaria* sp. extract to completely kill *S. cerevisiae* wild-type strain was double that of Δ*pdr5* strain. The results of MFC values of *Xylaria* sp. extract against *S. cerevisiae* strains were in the range of 0.55-1.00 mg/ml (Table. 4.3).

Interestingly, the deletion strains were more sensitive to *Xylaria* sp. extract than the wild-type strain. It is known that Pdr5 is a membrane associated protein and a first line to protect cells from toxic environments. The expression of *PDR5* may affect the intracellular concentration of *Xylaria* sp. extracts through exportation of these toxic extracts. The transcription factor Rds2 also conferred resistance to this extract. A previous study has reported Rds2, zinc cluster regulator, as a transcription factor that confers drug sensitivity [3]. Results showed that deletion of *RDS2* increases sensitivity to *Xylaria* sp. extract (Table. 4.3). It can be explained that Rds2 may regulate the expression of some PDR genes, involved in exportation of extracts. In support, it is found that the Pdr*5* and Rds2 are involved in the cell's ability to tolerate to stress of *Xylaria* sp. extract (Table. 4.3).

Table 4.3 Minimal fungicidal concentration (MFC) values of *Xylaria* sp. extract against *S. cerevisiae* wild-type (WT) and deletion Δ*rds2* and Δ*pdr5* strain.

%Viability	***Xylaria* sp. extract MFC (mg/ml)**		
	WT	**Δ*rds2***	**Δ*pdr5***
50	0.55	0.13	0.12
20	0.87	0.48	0.39
0	1.00	1.00	0.50

4.1.4 Gene expression analysis

When the cells encounter a toxic environment, the gene expression is rapidly altered to protect the cells and ensure survival. We postulated that *Xylaria* sp. extract possibly triggers the *PDR* network in *S. cerevisiae* by acting on Rds2 in order to induce the expression of *PDR5*. Therefore, the quantitative real time polymerase chain reaction (qRT-PCR) was performed to examine the expression level of *PDR5* in the presence of *Xylaria* sp. extract. The expression level was compared between *S. cerevisiae* deletion Δ*rds2* strain and BY4741 parental strain.

To test the hypothesis of Rds2 regulation on *PDR5* in response to stress produced by *Xylaria* sp. extract, we studied the gene expression profile after 2 hr of *Xylaria* sp. extract exposure. qRT-PCR analysis was monitored using SYBR Green I and performed as previously described (in materials and methods). Expression levels of the gene were normalized using a housekeeping gene, *ACT1*. The mRNA fold enrichments of the experiment were calculated using the comparative ΔΔCt method [112].

The results obtained from the *PDR5* expression study can be divided into two major parts. First, the expression of the *PDR5* gene in *Xylaria* sp. extract treatment was induced minimaly by ∼2-folds. In another part, under normal condition (untreated), expression of *PDR5* was not changed upon deletion of *RDS2* gene (-1.3-fold). There was also no change of *PDR5* expression (1.2-fold) in strain lacking *RDS2* under treatment with *Xylaria* sp. extract. It can be concluded that *PDR5* expression is induced in response to *Xylaria* sp. extract stress but the control of its expression is governed by other transcription factors not by Rds2.

In view of this, Rds2 have no relevance in the regulation of *PDR5* expression. Therefore, it probably is that *PDR5* expression is regulated by other transcription factors. This suggestion is supported by a study that showed the roles of Pdr1 and Pdr3 which are known to be master regulators of *PDR5* gene in other stress response [36]. Thus, further investigation on their roles in conferring *Xylaria* resistance is warrant.

CHAPTER 5 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The emergence of drug resistance in fungal pathogens makes it necessary and urgent to find new drugs for replacement. Bioactive compounds have been of much interest due to the rich and powerful growth inhibition of pathogenic fungi. Wood-decay fungi *Xylaria* species is one interesting example. In this work, we investigated the antifungal activity of *Xylaria* spp. against *S. cerevisiae* BY4741 and deletion strains Δ*rds2* and Δ*pdr5* which lack PDR regulator and transporter, respectively.

The four *Xylaria* spp. consisting of *Xylaria* sp. BCC 1067, *Xylaria cubensis* BCC 1027, *Xylaria globosa* BCC 31425, and *Xylaria obovata* BCC 28742 were examined for their antifungal activity against *S. cerevisiae* via clear zone of inhibition assay. This assay showed that these *Xylaria* crude extracts can produce clear zones on the *S. cerevisiae* field. For example, *Xylaria* sp. crude extract showed the best antifungal activity against *S. cerevisiae* BY4741 with 8.8 mm and *C. albicans* with 25.5 mm of diameter of clear zone of inhibition. We also investigated the *Xylaria* effect on strains lacking *PDR5*. This gene is a drug resistance gene and is a potential direct target of Rds2. The result of the clear zone of inhibition test showed that Δ*pdr5* is more sensitive than wild type and Δ*rds2* strains with a clear zone of inhibition of 23.5–25.5 mm for *Xylaria* extracts alone and 25.0-28.5 mm for combined *Xylaria* extracts and ketoconazole (Table 4.1 and Fig. B.1).

Next, we used the MIC assay for antifungal activity to obtain the MIC_{50} values of each *Xylaria* extract. At the present, the *Xylaria* extract by itself showed some antifungal activity against *S. cerevisiae*. For the MIC assay we also tested the deletion Δ*rds2 and* Δ*pdr5* strains. The results were similar to the clear zone of inhibition test that Δ*pdr5* strain is more sensitive to *Xylaria* extract than the wild-type and Δ*rds2* strain. Especially, we found that *Xylaria* sp. extract best inhibit growth of Δ*rds2* and Δ*pdr5* strains (Table 4.2 and Fig. 4.1). The increased sensitivity of *S. cerevisiae* strain lacking the *PDR5* gene indicated that Pdr5 is involved in the *Xylaria* extract effect. Therefore, Pdr5 is associated with the transport of *Xylaria* extracts out of the cell. However, cells lacking Rds2 can grow better than the Δ*pdr5* strain. For this fact, we suggested that other zinc cluster regulators could compensate for its loss.

To prove this hypothesis, we performed experiments, qRT-PCR, to observe the expression level of the *PDR5* gene in the Δ*rds2* strain in the presence of *Xylaria* sp. An increase in the expression of *PDR5* gene confirmed the connection of the *PDR5* in the resistance to *Xylaria* sp. extract. However, the lack of *RDS2* had no significant effect on *PDR5* expression level in presence of *Xylaria* sp. extract. In contrast, the results from earlier experiments showed that Rds2 has a role in the sensitivity to *Xylaria* sp. extract (Table 4.1, 4.2, and 4.3).

Overall results indicate the involvement of the *PDR5* gene in the tolerance to the toxic stress by *Xylaria* sp., which is showed by remarkable sensitivity in clear zone of inhibition, MIC, and MFC assays (Table 4.1, 4.2, and 4.3, respectively). These results support the suggestion that, upon *Xylaria* sp. treatment, the expression of *PDR5* gene is regulated by other transcription factors, for instance Pdr1, Pdr3, or Stb5. Rds2 also acts as transcription regulator of other PDR transporters.

5.2 Recommendations

Increases in the resistance to antifungal drugs of pathogenic fungi and a discovery of new drugs or other compounds for effective replacement have been of high importance. Natural sources of bioactive compounds receive a special interest because they are a rich and powerful source of antifungal agents. *Xylaria* is a fungus that is found in many tropical forests of Thailand. It plays diverse functions in the forest and also produces secondary metabolites with many biological activities. Recently, *Xylaria* has become an interesting source to study PDR and antifungal activity. Nevertheless, there is little information about *Xylaria* spp. in Thailand. In the present study, we found that some *Xylaria* species, including *Xylaria* extracts *Xylaria* sp. BCC1067, *X. cubensis* BCC 1027, *X. globosa* BCC 31425, and *X. obovata* BCC 28742 possess antifungal activity against the tested *S. cerevisiae* and pathogenic yeast *C. albicans.* This is good evidence to support the importance of this fungus in drug development.

Here, we also found that the expression of *PDR5* during the presence of *Xylaria* sp. extract was not dependent on the Rds2 regulator. We have noted that *PDR5* expression would likely to be controlled by other transcription factors in response of *Xylaria* sp. extract. At the same there may be other target genes that control functions by Rds2 in the presence of *Xylaria* sp. extract. Therefore, we proposed that these cases should be

studied in the next experiments. For identification of Rds2 target gene, ChIP-chip assay could be carried out. The gene expression study was also interesting to identify the transcription regulator of *PDR5*, using other yeast deletion strains. Finally, the information obtained in these studies will increase understanding of the drug resistance mechanism in *S. cerevisiae.*

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APPENDIX

APPENDIX A
PHENOTYPIC ANALYSIS

Introduction

The antifungal drug ketoconazole is a common azole drug that used in clinical treatment. Because azole drugs are extensively and repetitively used, the resistance rate is very high [1]. Akache, *et al*. [3] found that the ketoconazole resistance of the *S. cerevisiae* is associated with the new zinc cluster protein, Rds2 (Regulator of Drug Resistance). Thus, in this study we are interested to characterize role of *S. cerevisiae* Rds2 on drug resistance in presence of *Xylaria* extracts.

The *Xylaria* extracts of 10 species, including *Xylaria apiculata* BCC 1136, *Xylaria arbuscula* BCC 1156, *Xylaria cubensis* BCC 1027, *Xylaria globosa* BCC 31425, *Xylaria grammica* BCC 22680, *Xylaria hypoerythra* BCC 26466, *Xylaria multiplex* BCC 1177, *Xylaria nigripes* BCC 26565, *Xylaria obovata* BCC 28742, and *Xylaria* sp. BCC 1067 were screened for antifungal activity against *S. cerevisiae* (BY4741 background). The experiment was performed using the wild-type and the Δ*rds2* cells. The azole antifungal drug, ketoconazole, was included as a positive control. The susceptibility of *S. cerevisiae* BY4741 and Δ*rds2* strains to these *Xylaria* extracts were initially carried out by spot test on plates containing either *Xylaria* extract alone or *Xylaria* extract combined with ketoconazole.

Material and Method

The sensitivity of *S. cerevisiae* to *Xylaria* extracts was studied by using spot assay. First, cells were grown in YPD media overnight and four folds serially dilutions of cell cultures were done at starting OD_{600} of 0.1. 10 µl of each dilution was then spotted on different YPD plates: containing no ketoconazole nor *Xylaria* extract, containing ketoconazole, containing *Xylaria* extract, or containing a mixture of both ketoconazole and *Xylaria* extract. Cells were incubated for 3 days at 30°C to monitor the growth (Fig. A.1).

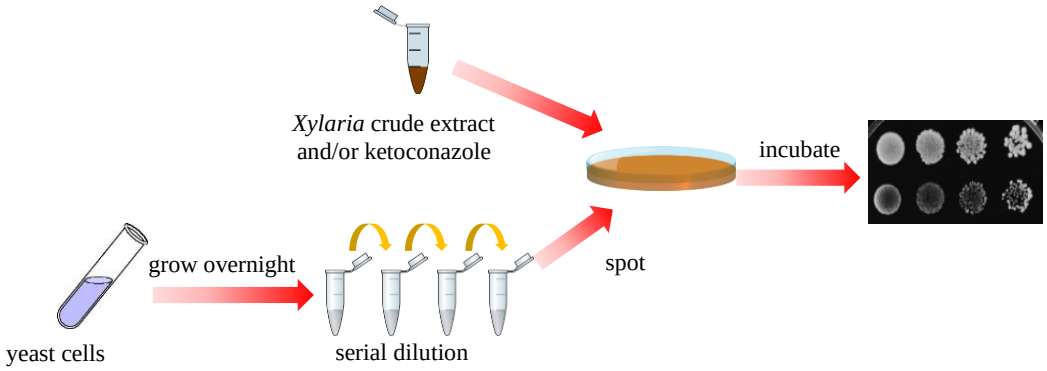

Figure A.1 Schematic for the phenotypic analysis via spot assay to test for sensitivity of *S. cerevisiae* strains to *Xylaria* extracts.

Results and Discussion

To investigate the antifungal activity of *Xylaria* extracts, we set up a *S. cerevisiae* model system consist of wild-type BY4741 as well as deletion Δ*rds2* and Δ*pdr5* strains to definedthe sensitivity to ketoconazole as first (Fig. A.2). The results from spot tests showed that extracts obtained from all species of *Xylaria* exhibited antifungal activity when combined with the antifungal drug, ketoconazole, whereas there was no activity for the *Xylaria* extract alone at 0.0175% concentration.

The susceptibility test of combined ketoconazole drug allows us to divide *Xylaria* extracts into three groups according to their antifungal activity against *S. cerevisiae* (Fig. A.3). First group is comprised of four *Xylaria* species, *Xylaria cubensis* BCC 1027, *Xylaria globosa* BCC 31425, *Xylaria obovata* BCC 28742, and *Xylaria* sp. BCC 1067, that shown to inhibit growth of *S. cerevisiae* (group A). In the second group, extracts exhibited no change in the ability to prevent the growth of the *S. cerevisiae*. This group contained *Xylaria apiculata* BCC 1136, *Xylaria arbuscula* BCC 1156, and *Xylaria multiplex* BCC 1177 (group B). The last one was *Xylaria grammica* BCC 22680, *Xylaria hypoerythra* BCC 26466, *Xylaria nigripes* BCC 26565 which showed enhanced growth of *S. cerevisiae* (group C). Abilities to inhibit the growth of *S. cerevisiae* BY4741 and Δ*rds2* strain of all *Xylaria* crude extracts are summarized in Table A.1.

The genus *Xylaria* has been reported as a rich source of secondary metabolites with numerous characteristic properties such as antimicrobial, antioxidant, antifungal,

antiHIV-1, anticancer, anti-protozoan, and antimalarial activity [13, 88, 108, 109]. In this study, the strong antifungal activities against *S. cerevisiae* were observed only when the *Xylaria* extract was mixed with ketoconazole. This may be because the *Xylaria* extract concentrations used in the assay may not be enough to inhibit *S. cerevisiae* growth by itself. Nevertheless, our results showed potential of *Xylaria* sp. BCC1067, *Xylaria cubensis* BCC 1027, *Xylaria globosa* BCC 31425, and *Xylaria obovata* BCC 28742 to enhance the antifungal activity of ketoconazole (Fig. A.3). It is well-known that ketoconazole works by targeting the ergosterol synthesis and causes altered membrane stress conditions, resulting in cell death. The synergistic effect of the azole drug has been reported for the combination of lactoferrin with fluconazole to enhance the antifungal activity of fluconazole against *Candida* spp. [116]. The mechanism of combined effect may be due to the activity of ketoconazole to alter membrane component and increase uptake of *Xylaria* extracts. However, the combination effect of each *Xylaria* extracts with ketoconazole can also cause by different mechanism because of the distinct components found in *Xylaria* extracts. For this fact, further study about the composition of these substances should be done in the future studies.

Conclusion

Among *Xylaria* extracts, the phenotypic assay showed three different phenotypes, sensitivity, non-difference, and resistance. Finally, four *Xylaria* extracts in sensitive phenotype group including *Xylaria* sp. BCC1067, *Xylaria cubensis* BCC 1027, *Xylaria globosa* BCC 31425, and *Xylaria obovata* BCC 28742 showed potential antifungal activity when combined with ketoconazole.

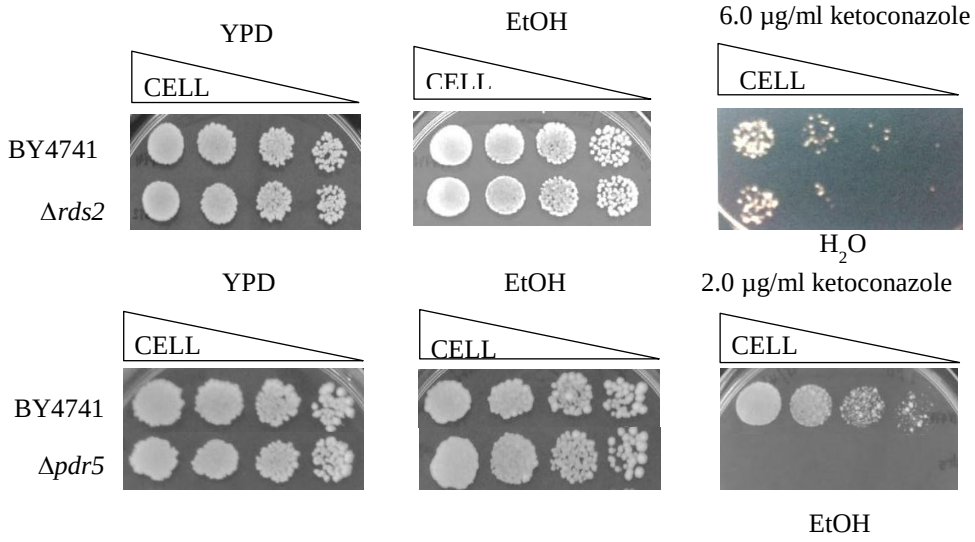

Figure A.2 Spot assay showed sensitivity of *S. cerevisiae* BY4741, Δ*rds2*, and Δ*pdr5* to antifungal drug ketoconazole. Cells culture of OD_{600}=0.6-0.8 were serially diluted 4-folds in water, and spotted onto appropriate YPD agar plates. Data are representative results from two independent experiments, phenotypic and susceptibility assays.

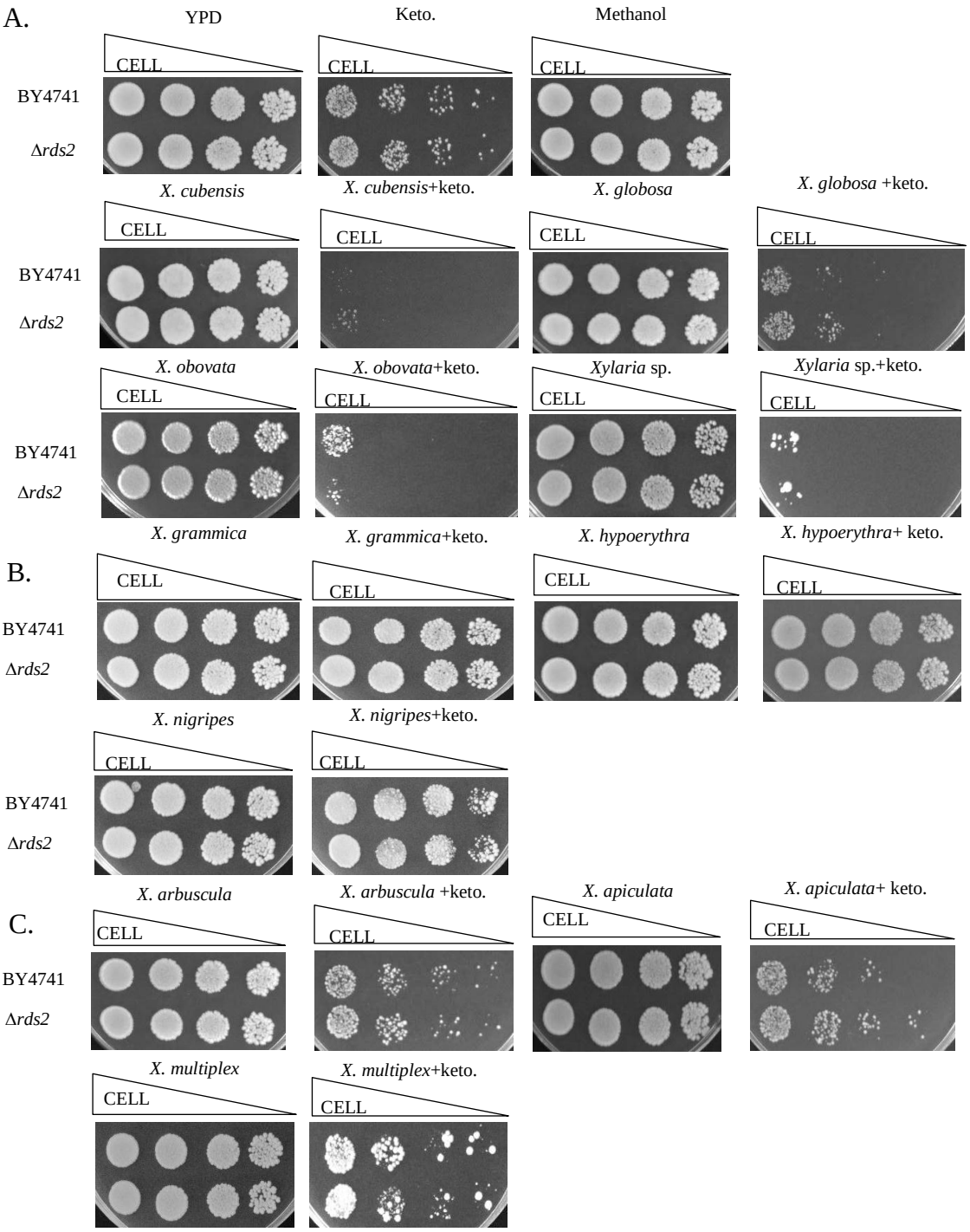

Figure A.3 The effect of *Xylaria* extract alone on growth of *S. cerevisiae* BY4741 and Δ*rds2* strain. *S. cerevisiae* Δ*rds2* (deletion of gene encoding for regulator of drug sensitivity) mutant. Exhibited sensitivity to some *Xylaria* crude extract was shown when combined with ketoconazole. The results were examined by compared growth of the deletion Δ*rds2* strain with wild-type strain. Data are representative results, using 0.0175% *Xylaria* crude extracts and 4 μg/ml of ketoconazole. Cells cultured of OD_{600}=0.6-0.8 were serially diluted 4-fold in water, and were inoculated onto YPD agar plates. A, sensitive; B, resistance; C, no difference

Table A.1 Effects of *Xylaria* extracts alone and in combination against *S. cerevisiae* BY4741 and deletion strain Δ*rds2*.

Compounds tested	***Xylaria* alone**		***Xylaria* + ketoconazole**	
	BY4741	**Δ*rds2***	**BY4741**	**Δ*rds2***
Xylaria extracts				
Xylaria cubensis BCC 1027	xxx	xxx	x	x
Xylaria globosa BCC 31425	xxx	xxx	x	x
Xylaria obovata BCC 28742	xxx	xxx	x	x
Xylaria sp. BCC 1067	xxx	xxx	x	x
Xylaria grammica BCC 22680	xxx	xxx	xxx	xxx
Xylaria hypoerythra BCC 26466	xxx	xxx	xxx	xxx
Xylaria nigripes BCC 26565	xxx	xxx	xxx	xxx
Xylaria arbuscula BCC 1156	xxx	xxx	xx	xx
Xylaria multiplex BCC 1177	xxx	xxx	xx	xx
Xylaria apiculata BCC 1136	xxx	xxx	xx	xx

x mean that the cells were showed sensitivity, xx mean no detected difference in sensitivity, xxx mean that the cells test were showed resistance. Spot tests were carried out by 4 µg/ml of ketoconazole and 0.0175% of *Xylaria* extracts. *Xylaria* extracts were dissolved in methanol before use. The antifungal drug ketoconazole was used as positive control.

APPENDIX B
THE SENSITIVITY OF *CANDIDA ALBICANS* TO *XYLARIA* EXTRACTS

Introduction

In this test, we used the yeast *C. albicans,* which has close genetic relationship to *S. cerevisiae* to test as well. The genus *Candida* is a major fungal cause of bloodstream infections in humans. *Candida albicans* is primary *Candida* species that associated with candidemia and used to study mechanism multidrug resistance [5]. The *CWT1* is a gene, encoding for putative transcriptional factor of *Candida albicans* and is a homologue of the zinc cluster protein Rds2 in *S. cerevisiae* [117, 118]. The lack of Cwt1 in *C. albicans* leads to defects in cell wall architecture and pleiotropic resistance to drugs. PDR is also detectable in *S. cerevisiae* and the pump of PDR are closely related in the two yeast species as well [117].

Materials and Methods

The clear zone of inhibition of the *C. albicans* strains SGY243 wild-type and Δ*cwt1* strains was performed in same protocol with *S. cerevisiae* as descripted in chapter 3. Briefly, *C. albicans* cells were routinely grown on rich media (YPD), containing 1% yeast extract (Himedia Laboratories, India), 2% bacto peptone (Himedia Laboratories, India), 2% glucose (Himedia Laboratories, India) at 30^{o}C. Firstly, yeast cells were grown overnight in liquid YPD at 30 ^{o}C and 100 rpm, re-inoculated and re-grown until the OD_{600} was 0.6-0.8. 100 µl of cultures of cells were spread on YPD plates. A sterile paper disk was placed on top of the plate at center. After that, 5 µl of *Xylaria* crude extract and/or ketoconazole were dropped onto the center of the disk. Plates were incubated for three days. Antifungal activity was assessed by measuring the diameter of the clear zone of inhibition.

Results and Discussion

It is well known that *Candida* spp. is important pathogenic fungi with rapidly enhanced rate of drug resistance strains. In present study, we tested antifungal activity of *Xylaria* crude extracts against *Candida albicans* SGY243 (Fig. B.1). Our results indicated that

these *Xylaria* crude extracts showed higher antifungal activity against *C. albicans* than *S. cerevisiae.* Diameters of clear zones were increased to 25.5 mm for *Xylaria* sp. extract, 25.0 mm for *X. globosa* and *X. obovata,* and 23.5 mm for *X. cubensis* (Table B.1). It is possible that *Xylaria* extracts may simply be better imported into the *C. albicans* than *S. cerevisiae* cells. It was reported that *S.cerevisiae* with *C. albicans* have differences in the composition of cell wall, such as mannan content [119]. Therefore, it is possible that this difference results in cell wall component in both yeast species different sensitivity to the extracts.

The deletion strain Δ*cwt1,* homologue gene with Δ*rds2,* showed resistant to all extracts than SGY243 strain. In this part, crude extract of *Xylaria* sp. is the best antifungal agent to inhibit growth of all strains except for deletion Δ*cwt1* that is the best inhibited by *X. cubensis.* The increasing of antifungal activity of combination of ketoconazole and *Xylaria* extracts were also found for *C. albicans.* The strain showed small variation of sensitivity with the best was *X. cubensis* extract with 35 mm of diameter of clear zone as well as 34.5 mm for *X. globosa* and 28.5 mm for *X. obovata*. Although, the combination whit ketoconazole can improve the antifungal activity but not much for deletion Δ*cwt1* strain. The most observed effect was for *X. obovata* with diameter of clear zone of 20.0 mm. The other extracts showed that diameter of clear zone with 18.5 mm, 15.5 mm, and 10.5 mm for *X. cubensis, X. globosa,* and *Xylaria* sp., respectively. Probably, antifungal activity is affected by cell wall composition the antifungal activity of *Xylaria* extracts. In 2003, study in cell wall composition and architecture of *C. albicans* has reported that Cwt1 has decrease of β-1,6-glucan content in cell wall [50]. In fact, cell wall is the first line and crucial for protecting cells from toxic and environment. Thus, the altering of cell wall components can make lethal effect to the cell. It supports our results that the Δ*cwt1* is more resistant to the *Xylaria* extracts.

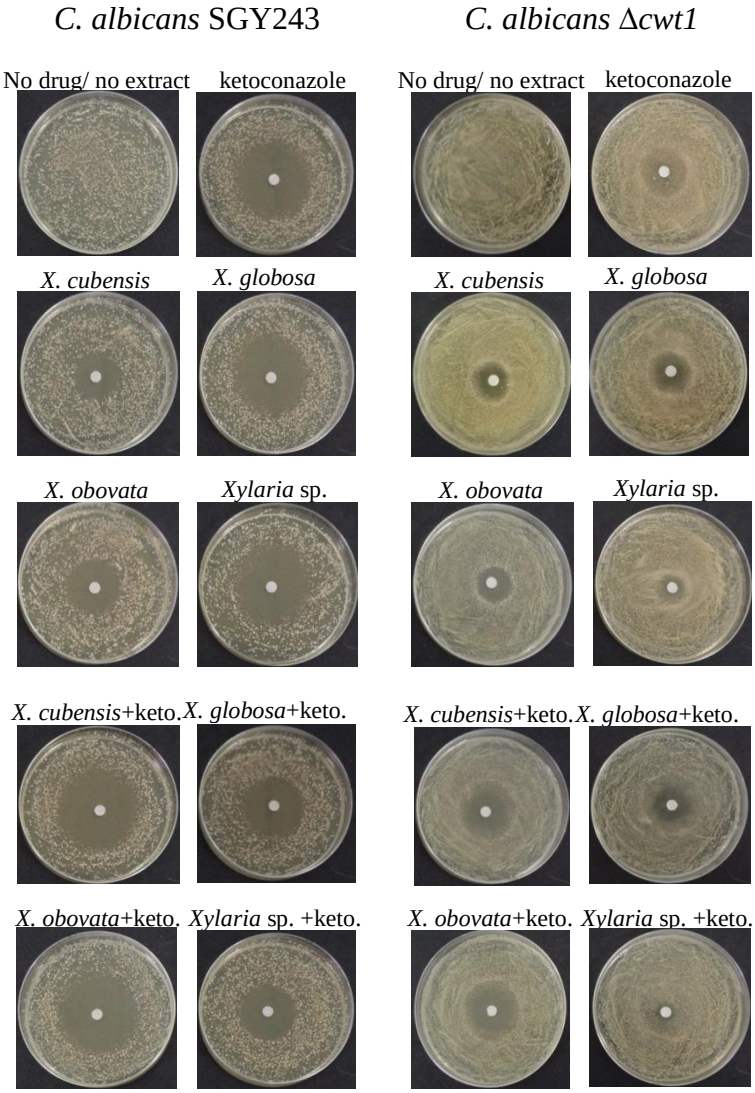

Figure B.1 The antifungal activity of *Xylaria* extracts against *C. albicans* SGY243 and Δ*cwt1* strains were examined with clear zone of inhibition assay. The antifungal activity from combination of *Xylaria* extracts and ketoconazole was also investigated. *Xylaria* extracts were dissolved in methanol. 5 µl of 40 g/l crude extract was used to test and combined with 5 µl of 1,000 µg/ml ketoconazole for combination testing.

Table B.1 Antifungal activities of *Xylaria* extracts against *C. albicans* SGY243 and its deletion strain via clear zone of inhibition assay.

Compounds tested	Clear zone of inhibition (mm.)[a]					
	SGY243			**Δ*cwt1***		
A. *Xylaria* extracts						
Xylaria cubensis BCC 1027	23.5	±	2.1	18.5	±	18.5
Xylaria globosa BCC 31425	25.0	±	0.0	14.5	±	0.7
Xylaria obovata BCC 28742	25.0	±	0.0	16.0	±	5.7
Xylaria sp. BCC 1067	25.5	±	0.7	10.5	±	0.7
B. Combination of *Xylaria* extractswith ketoconazole						
Xylaria cubensis BCC 1027	35.0	±	0.7	17.0	±	2.1
Xylaria globosa BCC 31425	34.5	±	0.7	15.5	±	2.1
Xylaria obovata BCC 28742	34.5	±	2.1	20.0	±	0.0
Xylaria sp. BCC 1067	28.5	±	2.1	10.5	±	0.7
C. Ketoconazole						
H_2O	31.5	±	1.4	8.5	±	0.7

[a]diameter of disk (6 mm.)
[b]used 5 µl of 40 mg/ml *Xylaria* extracts and then combined with 5 µl of 1 mg/ml ketoconazole
[c]used 5 µl of 1 mg/ml ketoconazole
Xylaria extracts were dissolved in methanol before use.

Conclusion

As results, three crude extracts of *Xylaria* including *X. cubensis, X. obovata,* and *Xylaria* sp. had antifungal activity against *C. albicans* strains. Thus, it suggests the potential application of *Xylaria* extracts as natural antifungal agents. Importantly, the bioactive compounds are needed additional studies to explain the mechanism of antifungal activity for this pathogenic yeast.

APPENDIX C

MINIMAL FUNGICIDAL CONCENTRATION

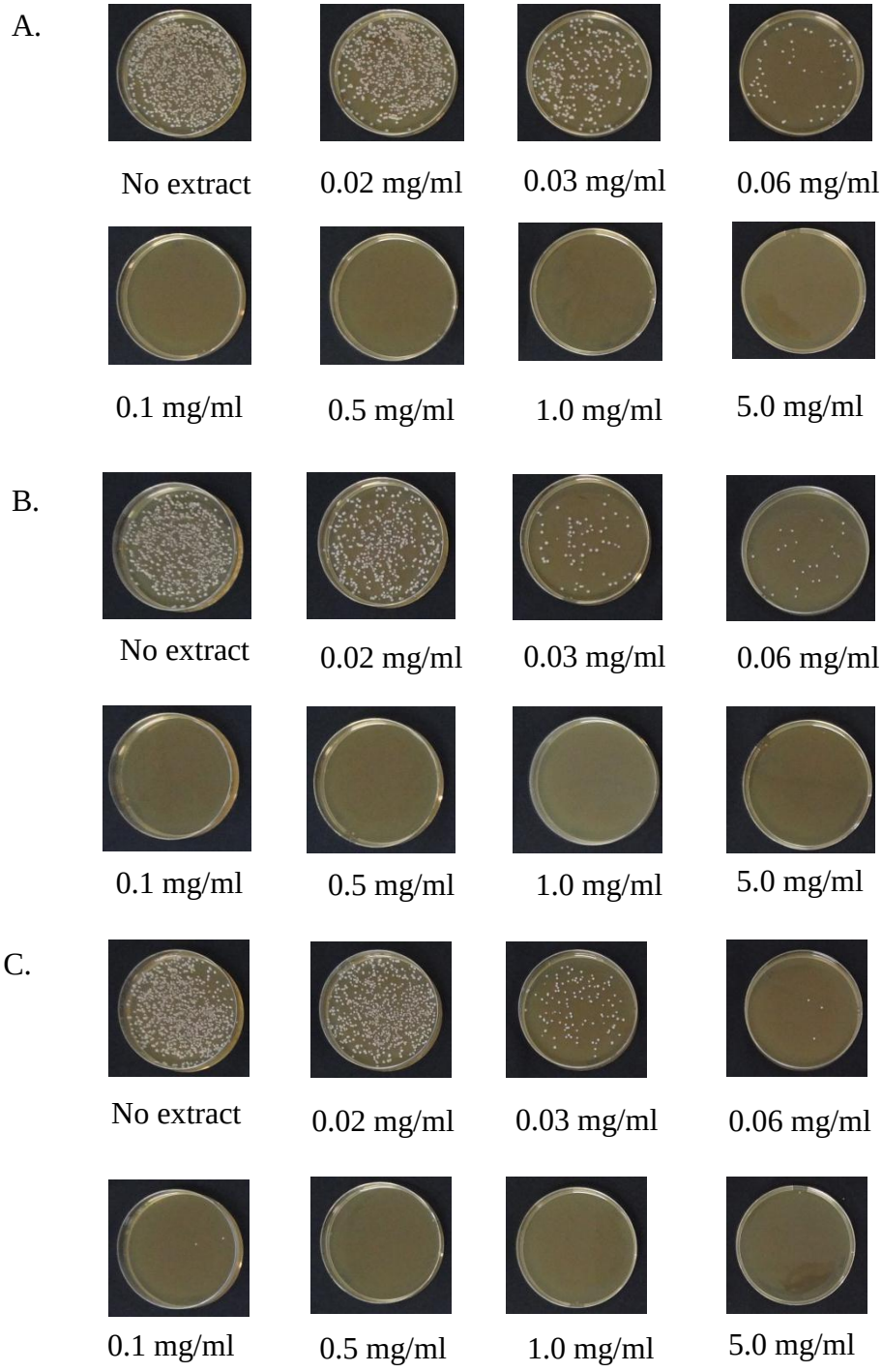

Figure C.1 Cell viability plate assay to determine minimum fungicidal concentration (MFC) of *S. cerevisiae* in presence of *Xylaria* sp. extract in 96-well plate for 24 h. After the MIC investigation the cells in invisible growth wells were diluted to 1000x and spreaded on YPD plates. (A), BY4741 wild-type; (B), deletion Δ*rds2* strain; (C), deletion Δ*pdr5* strain.

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PUBLICATIONS Somboon, P. and Soontrongun, N., 2013, "Identification of wood-decay fungi *Xylaria obovata* extract with antifungal activity", **Burapha University International Conference 2013**, July 4-6, Burapha University, Thailand. (Proceeding)

Soontrongun, N. and Somboon, P., 2013, "Discovery of natural extracts of fungal *Xylaria* species with potent antifungal activities and therapeutic prospects", **The 7th South East Asian Technical University Consortium**

(SEATUC) Symposium, March 4-6, Institut Teknologi Bandung, Indonesia. (Proceeding)

Somboon, P. and Soontrongun, N., 2012, "Investigation of antifungal activity from *Xylaria* spp. extract against *Saccharomyces cerevisiae*", **The Fourth International Conference on Natural Products for Health and Beauty (NATPRO4)**, November 28-30, Chiang Mai, Thailand. (Poster)

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