รายงานวิจัยฉบับสมบรูณ์

โครงการทุนส่งเสริมนักวิจัยรุ่นใหม่

การศึกษาสารธรรมชาติที่ออกฤทธิ์ยับยั้งเชื้อราและกลไกการดื้อยา โดยใช้ระบบยีสต์ deletion

Identification of natural antifungal products and inhibitory mechanisms via yeast deletion system

โดย

ผศ.ดร.นิษก์นิภา สุนทรกุล

หัวหน้าโครงการวิจัยผู้รับทุน หลักสูตรเทคโนโลยีชีวเคมี

คณะทรัพยากรชีวภาพและเทคโนโลยี

มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าธนบุรี

มีนาคม 2560

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สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา และสำนักงานกองทุนสนับสนุนการวิจัย

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บทคัดย่อ

งานวิจัยเกี่ยวข้องกับการตรวจสอบศักยภาพการต้านเชื้อราของสารสกัดจาก *Xylaria* sp. BCC 1067 ต่อยีสต์ *Saccharomyces cerevisiae* ซึ่งใช้เป็นโมเดลในการศึกษาในครั้งนี้ วัสดุและวิธีการ: การพิจารณาค่าความเข้มข้นของสารสกัดในการยับยั้งเชื้อราที่ต่ำสุดต่อค่าความเข้มข้นของสารสกัดในการฆ่าเชื้อราของ *Xylaria* sp. BCC 1067 ปริมาณรีแอคทีฟออกซิเจน (ROS) ภายในเซลล์และเปอร์เซ็นต์ของความอยู่รอดของเซลล์ ในสายพันธ์ยีสต์ที่มีการลบยีนออก ผลลัพธ์: สารสกัดมีฤทธิ์ในการต้านเชื้อราและมีกิจกรรมร่วมกันกับยาต้านเชื้อรา ketoconazole โดยความสามารถในการต้านอนุมูลอิสระขึ้นอยู่กับหน้าที่การทำงานของตัวควบคุมพันธุกรรมที่เรียกว่า Yap1 (Yap1 anti-oxidative stress) และโปรตีนที่เกี่ยวข้องกับการดื้อยาในกลุ่ม Pdr ความสามารถของเซลล์ยีสต์ในการทนต่อสารสกัด มีความสัมพันธ์กับระดับของปริมาณรีแอคทีฟออกซิเจนภายในเซลล์ ความไวต่อยาที่เพิ่มขั้นและการรอดชีวิต ในยีสต์บางสายพันธุ์ที่ꟻกลบยีนที่เกี่ยวข้องออกไป บทสรุป: กระบวนการขับสารต้านเชื้อราจากสารสกัด *Xylaria* sp. BCC 1067 อาศัย Pdr เป็นกลไกหลักในการป้องกันเซลล์ *S. cerevisiae* และแสดงให้เห็นว่า *Xylaria* sp. BCC 1067 เป็นแหล่งต้านเชื้อราใหม่ที่เป็นประโยชน์ น่าสนใจและมีศักยภาพดี สำหรับการใช้ร่วมกับยา azole ในอนาคต

คำสำคัญ: ฤทธิ์ต้านเชื้อรา, *Xylaria* sp. BCC 1067, สภาวะเครียด, *PDR5*, *PDR1*, *YAP1*

Abstract

To investigate antifungal potential of *Xylaria* sp. BCC 1067 extract against the model yeast *Saccharomyces cerevisiae*. **Materials & Methods:** Minimal inhibitory/ fungicidal concentration of *Xylaria* sp. BCC 1067 extract, reactive oxygen species (ROS) levels and cell survival were determined, using a collection of yeast deletion strains. **Results**: The extract showed antifungal effect and synergistic activity with ketoconazole. Yap1 anti-oxidative stress and Pdr multi-drug systems were critical for cell tolerance to antifungal extract as shown by induced intracellular ROS levels, increased sensitivity and rescued growth by antioxidant of deletion strains. **Conclusion:** Pdr-mediated detoxification is pivotal to protect *S. cerevisiae* cells from *Xylaria* sp. BCC 1067 extract as an interesting and potentially useful new source of antifungals for future combinatorial therapy against azole resistance.

Keywords: Antifungal activity, Xylaria sp. BCC 1067, Oxidative stress, PDR5, PDR1, YAP1

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Researcher

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List of Acronyms

ATP	=	Adenosine triphosphate
ERG5	=	ERGosterol biosynthesis
HPLC	=	High Performance Liquid Chromatography
MEA	=	Mlt extract agar
MEB	=	Malt extract broth
Mdr1	=	Mac1-Dependent Regulator
MIC	=	Minimal Inhibitory Concentration
ml	=	Milligram
msn2	=	Multicopy suppressor of SNF1 mutation
Oaf1	=	Oleate-Activated transcription Factor
PCR	=	Polymerase chain reaction
PDR1	=	Pleiotropic Drug Resistance
Pip2	=	Peroxisome Induction Pathway
ROS	=	Reactive oxygen species
S. cerevisiae	=	*Saccharomyces cerevisiae*
Snq2	=	Sensitivity to 4-NitroQuinoline-N-oxide
sp.	=	Species
yap1	=	Yeast AP-1
Yrr1	=	Yeast Reveromycin-A Resistant
YPD	=	Yeast extract Peptone Dextrose
μg	=	Microgram

Chapter 1

Introduction

Currently, the number of available antimicrobial drugs is limited and, the existing ones are becoming less effective for therapeutic treatments of infection. This phenomenon is in contrast to the antimicrobial resistance that has continuously risen over the years, following repeated drug exposures and microbial evolution [1, 2]. Thus, it makes the discovery of agents with antimicrobial/antifungal potential, along with rapid diagnosis and new therapeutic options, the urgent and utmost important global public health issues. To restrict the emergence of drug-resistant strains, new rapidly fungicidal drugs are needed [3].

Although antifungal drugs with fungicidal activity are more effective for treatments of fungal infections and prevention of drug resistance as compared to the fungistatic ones, there are a few available. The modes of action of currently antifungal agents include inhibition of ergosterol biosynthesis (azoles), alteration of fungal plasma membrane (polyenes) and cell wall integrity (echinocandins), as well as blocking of DNA synthesis and repair (flucytosine) [4]. The most notorious fungicidal drug is amphotericin B of the polyenes that binds to ergosterol in the plasma membrane of pathogen, resulting in pore formation with leakage of cellular contents and membrane instability [5]. Amphotericin B has also been shown to cause formation of lethal ROS, contributing to cell death [5, 6]. Thus, it is considered a gold-standard drug commonly administered to treat invasive fungal infections such as *candidiasis* and *aspergillosis* [5, 7], despite being quite toxic to patients [8].

Improved understanding of resistance mechanisms and their clinical impact are also essential for design of new antifungal drugs [9]. Changes in the expression of other genes or in protein

abundance may also follow antifungal drug exposure, including those associated with oxidative stress [10-13]. To cope with oxidative stress, cells possesses antioxidant defense systems, involving several reactive oxygen species (ROS) scavenging enzymes as well as non-enzymatic mechanisms which rely on antioxidant molecules such as glutathione and thioredoxin for detoxification of excessive harmful ROS [6, 14, 15]. In the yeast *S. cerevisiae*, the oxidative stress response is induced by different transcription regulators [16, 17]. These include the Yap1 regulator whose gene deletion results in hypersensitivity to various ROS [18]. Its paralog Yap2 responds to cellular stress involving metal resistance, and regulates genes involved in stabilizing proteins [19]. Msn2/4 are transcriptional regulators for general stress response against environmental stress and metabolic cues such as hydrogen peroxide (H_2O_2) exposure [16]. In addition, there are newly characterized regulators of oxidative stress tolerance including Tog1, Znf1 and Asg1, that play roles in H_2O_2 and/or weak acid tolerance [20-22]. Other transcription factors can affect stress responses including the pleiotropic drug resistance (Pdr) transcription factors Pdr1 and Pdr3 [23, 24]. Pdr1 is the key transcriptional regulator of PDR and regulates *PDR* genes by direct binding to PDR elements, located within promoters of genes encoding efflux transporters, including *PDR5*, *SNQ2*, and *YOR1* [25]. Pdr5 is an important multi-drug efflux transporter of *S. cerevisiae* and is homologous to Cdr1/2 from pathogenic *Candida* yeast species [26]. Pdr3 exhibits roles in the responses to drugs, organic solvents and retrograde signaling [27, 28]. In addition, the Stb5 regulator also functions in Pdr and oxidative stress protection through controlling the regeneration of NADPH [29].

Microorganism are a valuable source of antibiotics, including fungal-derived Penicillin which revolutionized healthcare worldwide [30]. *Xylariaceae* fungi, namely *Xylaria* spp., are commonly present as natural decomposers in tropical forests, are rich producers of bioactive compounds

with good biological properties [31], and represent a promising resource for drug discovery [31-36]. For example, the polypropionates xylarinic acids A and B with antifungal activity were isolated from *X. polymorpha* and the antimicrobial 7-amino-4-methylcoumarin was isolated from *Xylaria* sp. YX-28 [37]. *Xylaria* sp. BCC 1067 has been reported to produce several polyketides, namely depudecin, 19, 20-epoxycytochalasin Q and xyrrolin, with anticancer, antiprotozoal and antimalarial activities [31, 33, 38].

Here, we investigated the antifungal potential of an extract of *Xylaria* sp. BCC 1067 and addressed whether the generation of ROS contributes to its antifungal effect, using a panel of *S. cerevisiae* strains in which genes involved in the defense against ROS-mediated toxicity, or drug efflux, were deleted.

Objectives

1 To identify new sources of natural products with good antifungal active against various yeast in comparison with existing antifungal agents.

2 To examine the synergistic and additive effects of combined antifungal agents.

3 To characterize the mechanisms if action for these natural antifungal products and targets via biochemical analysis and gene expression study.

Scope

1. Extract/obtain natural bioactive products from filamentous fungi and test for antifungal activities via clear zone of inhibition, spot, MIC, MFC assays

2. Determine and compare the antifungal activity of natural bioactive extracts/products and combined effect with clinically used antifungal drugs via cross-check assay.

3. Identify the mechanism of antifungal actions of natural bioactive products via biochemical assays and apply yeast knockout deletion system to screen for antifungal drug targets with a focus on some key metabolic enzyme, drug transporters, cell wall proteins and oxidative enzyme.

Schedule for the entire project and expected outputs

Table 1 plan proceeds

activity	Month			
	1-6	7-12	13-18	19-24
1. Yeast strains and media	↔			
2. Xylaria culture and extraction	↔			
3. Assays of antifungal activity	↔			
4. Combinatorial study of *Xylaria* sp. BCC 1067 extract and ketoconazole	↔	↔		

5. Measurement of reactive oxygen species (ROS) formation		←	→	
6. Human cell cytotoxicity bioassays			↔	
7. Statistical analysis				↔

Expected Benefits

1. The new knowledge on gene related to synthesis of bioactive and antifungal extract could publish in international journals and presented at national and international conferences as well as patented in the future.

2. Transferred knowledge obtained from this study and technology related to gene and yeast genetics and molecular biology to students in order to train new researchers for human development and to some pharmaceutical industries.

Transmission of Technology Plan or Research Outcomes for a Target Group

Researcher has a plan to transmit knowledge, research methodology to a target group of students at School of Biotechnology and Technology King Mongkut's University of Technology Thonburi. We also plan to collaborate research project with other research laboratories both in and outside the country. We also will start cooperation with the interested government and private sectors for continued production and application of the antifungal extract/compounds.

Chapter 2

Materials and Method

1. Yeast strains and media

The *S. cerevisiae* wild-type BY4742 (MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0) and the strains listed in Table 1 were used in this study [39]. A yeast extract–peptone–dextrose (YPD) culture media was utilized for routine growth of yeast cells (Himedia Laboratories, Mumbai, India) [40]. Double and triple deletion strains were generated using plasmids pLJ247 (*YAP1* disruption) and pLJ513 (*STB5* disruption). *YAP1* was deleted in the *pdr5*Δ::*kanMX4* strain resulting in LJ458 (*yap1*Δ::*URA3*, *pdr5*Δ::*kanMX4*). Deletion of *STB5* was performed in both *pdr3*Δ::*kanMX4* and WC081 resulting in strains LJ456 (*stb5*Δ::*LEU2*, *pdr3*Δ::*kanMX4*), and LJ457 (*stb5*Δ::*LEU2*, *pdr1*Δ::*HIS3*, *pdr3*Δ::*kanMX4*). Strain WC081 (*pdr1*Δ::*HIS3*, *pdr3*Δ::*kanMX4*) has been described previously [41]. Strains had genomic deletion of *YAP1* sequences -35 to +2125, *PDR1* sequences -14 to +2495, and *STB5* sequences -14 to +2260. Gene deletions were verified by *in vivo* PCR using flanking primers [42].

2. *Xylaria* culture and extraction

The *Xylaria* sp. BCC 1067 was obtained from the BIOTEC Culture Collection (BCC culture 6200032292; National Science and Technology Development Agency, Bangkok, Thailand). Cultivation of *Xylaria* sp. BCC 1067 was modified from the method of Phonghanpot et al [33]. Each *Xylaria* culture was filtered to separate the mycelia and broth. The broth was then extracted

with a two-fold volume of ethyl acetate (EtOAc). The dried crude extract was kept at 4 °C and freshly dissolved with methanol before use.

Table 2 Strains used in this study.

Strain	Genotype	Ref.
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	[39]
Δ*pdr1*	BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) Δ*pdr1::kanMX4*	[39]
Δ*pdr3*	BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) Δ*pdr3::kanMX4*	[39]
Δ*pdr5*	BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) Δ*pdr5::kanMX4*	[39]
Δ*stb5*	BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) Δ*stb5::kanMX4*	[39]
Δ*abp1*	BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) Δ*abp1::kanMX4*	[39]
Δ*glr1*	BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) Δ*glr1::kanMX4*	[39]
Δ*gpx1*	BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) Δ*gpx1::kanMX4*	[39]

Strain	Genotype	Ref.
Δ*gpx2*	BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) Δ*gpx2::kanMX4*	[39]
Δ*grx1*	BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) Δ*grx1::kanMX4*	[39]
Δ*gsh1*	BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) Δ*gsh1::kanMX4*	[39]
Δ*gsh2*	BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) Δ*gsh2::kanMX4*	[39]
Δ*prx1*	BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) Δ*prx1::kanMX4*	[39]
Δ*sod2*	BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) Δ*sod2::kanMX4*	[39]
Δ*trr2*	BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) Δ*trr2::kanMX4*	[39]
Δ*tsa1*	BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) Δ*tsa1::kanMX4*	[39]
Δ*tsa2*	BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) Δ*tsa2::kanMX4*	[39]
Δ*yap1*	BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) Δ*yap1::kanMX4*	[39]

Strain	Genotype	Ref.
Δ*yap2*	BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) Δ*yap2::kanMX4*	[39]
Δ*pdr1*Δ*pdr3*	BY4742 *(MATα leu2Δ0, lys2Δ0, ura3Δ0, his3Δ1)* Δ*pdr3::kanMX4,* Δ*pdr1::HIS3*	[41]
Δ*pdr1*Δ*stb5*	BY4742 *(MATα leu2Δ0, lys2Δ0, ura3Δ0, his3Δ1)* Δ*pdr1::HIS3,* Δ*stb5::kanMX4*	This study
Δ*pdr3*Δ*stb5*	BY4742 *(MATα leu2Δ0, lys2Δ0, ura3Δ0, his3Δ1)* Δ*pdr3::kanMX4,* Δ*stb5::LEU2*	This study
Δ*yap1*Δ*pdr5*	BY4742 *(MATα leu2Δ0, lys2Δ0, ura3Δ0, his3Δ1)* Δ*pdr5::kanMX4,* Δ*yap1::URA3*	This study
Δ*pdr1*Δ*pdr3*Δ*stb5*	BY4742 *(MATα leu2Δ0, lys2Δ0, ura3Δ0, his3Δ1)* Δ*pdr3::kanMX4,* Δ*pdr1::HIS3, stb5Δ::LEU2*	This study
AD124567	MATα *PDR1–3 ura3 his1* Δ*yor1::hisG* Δ*snq2::hisG* Δ*pdr10::hisG* Δ*pdr11::hisG* Δ*ycf1::hisG* Δ*pdr3::hisG*	[26] ADD REF
ADΔ	AD1-8u in FULL, Δ*ura3*	[26]
AD/ScPDR5	AD1-8u^{-} Δ*pdr5*::pABC3-ScPDR5	[26]

3. Assays of antifungal activity

The MICs of the *Xylaria* sp. BCC 1067 extract against *S. cerevisiae* strains were determined using the microdilution reference method of the National Committee for Clinical Laboratory Standards (NCCLS) [43]. An automated microplate reader (M965+; Metertech, Taipei, Taiwan) was used to determine OD_{600} values. MFCs for the *Xylaria* sp. BCC 1067 extract were determined by sampling onto YPD agar plates 30 µl from each well of the MIC determination plates which showed no growth. The plates were incubated at 30 $^{\circ}$C for 48 h before determining the colony count. The antifungal activity against different *S. cerevisiae* strains was also examined, using spot assays. After 24 h exposure to *Xylaria* sp. BCC 1067 extract, cells were taken from the wells for ten-fold serial dilutions. 3 µl of cell dilution was spotted onto YPD agar plates. Cell growth was monitored after incubation at 30°C for 48 h.

4. Combinatorial study of *Xylaria* sp. BCC 1067 extract and ketoconazole

The combined effect of *Xylaria* sp. BCC 1067 extract with the antifungal drug ketoconazole was studied to determine the antifungal interaction activity, using a two-dimensional checkerboard micro dilution technique as described by Zhou, Y. *et al* [44]. The *Xylaria* sp. BCC 1067 extract was used in a concentration range from 0–2000 mg/l. Ketoconazole was similarly prepared with 16 mg/l as the highest concentration used. 100 µl of yeast cell culture (at OD_{600} of 0.01) was added to each well of the 96-well plates, containing indicated concentrations of the extract and drug. Cells were then incubated at 30°C, at 150 rpm for 24 h. The antifungal interaction activity was evaluated by calculating the fractional inhibitory concentration index (FICI) as shown [45]. FIC of ketoconazole = (MIC of drug in combination with extract) / (MIC of drug alone) and FIC of

extract = (MIC of extract in combination with drug) / (MIC of extract alone). FICI=FIC of drug + FIC of extract. The synergistic activity is defined as FICI ≤ 0.5, FICI between 0.5 and 4.0 is defined as no interaction between agents, and FICI > 4.0 is defined as antagonism activity [45].

5. Measurement of reactive oxygen species (ROS) formation

Endogenous ROS levels of *S. cerevisiae* cells were measured by a fluorometric assay using 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich) as a ROS indicator, as previously described by Wu X-Z et al [46]. Cultures of *S. cerevisiae* were treated with different concentrations of *Xylaria* sp. BCC 1067 extract for 2 h and/or 4 h and incubated with 10 mM DCFH-DA for an additional 30 min for ROS determination. To confirm the ROS determination assay, the antioxidant thiourea (50 mM) or ascorbic acid (10 mM) was also included and added prior to the addition of the extract for 30 min [47]. Supernatants were collected and fluorescence intensity was monitored at an excitation wavelength of 490 nm and an emission wavelength of 524 nm using a multi-mode microplate reader (Spectramax®; Molecular Devices, Sunnyvale, CA, USA). Protein content of samples were measured using the Bradford method [48] and the ratio of fluorescence intensity/total protein level was determined.

6. Human cell cytotoxicity bioassays

A microassay for cytotoxicity in the Wi-38 (lung fibroblast) and the CCD (skin fibroblast, Human) cell lines was performed using the Microculture Tetrazolium Assay (MTT) method [49]. The adherent cells were incubated for 24 h to allow cell attachment. The extract was added to the cell culture, and the cells were incubated for 3 days. The MTT solution was added 4 h before the

end of the incubation time. Cell survival was evaluated with a multi- well scanning spectrophotometer at 540 nm and IC_{50} values calculated.

7. Statistical analysis

Results are expressed as mean ± SD. Comparisons between groups were made by one- or two-way ANOVA, followed by Tukey's pairwise comparison. Statistical analysis was carried out using the SPSS Statistics 17.0 software (IBM, NY, USA) with a P value of <0.05 or <0.01 which were considered as significant different. All experiments were performed at least three independent experiments with three to five replicates.

Chapter 3

Results and Discussion

Results

The antifungal potential of *Xylaria* sp. BCC 1067 extract against *S. cerevisiae*

The antifungal activity of *Xylaria* sp. BCC 1067 extract against wild-type *S. cerevisiae* BY4742 was determined in parallel with the cytotoxicity using the Wi-38 lung fibroblast and the CCD human skin fibroblast cell lines (Table 2). 75 and 500 mg/l of the extract was found to inhibit growth of *S. cerevisiae* wild-type by 50% and 100%, respectively (Table 2). For the value of MFC, 1000 mg/l of extract was required to kill (Table 2 and 4). A ratio of MFC/MIC, an indication of whether a drug is fungicidal or fungistatic, was calculated. An agent is considered fungicidal if the MFC/MIC ratio is $\leq$ 4 as in the case for amphotericin B and, fungistatic if the ratio is > 4 as reported for fluconazole with the ratio of MFC/MIC of 64 [50]. The *Xylaria* sp. BCC 1067 extract had the MFC/MIC ratio of 2 and was considered to have a fungicidal effect (Table 2). The IC_{50} were found to be 110 and 470 mg/l, respectively (Table 2). The fungicidal effect of *Xylaria* sp. BCC 1067 extract occurs at much higher concentrations than in case of ketoconazole or other conventional antifungal drugs. However, the crude *Xylaria* extract is most likely composed from several compounds and identification of effective component could decrease MIC and MFC values or eliminate cytotoxicity. Analogously, various *Xylaria* extract effects on yeast cells can be ascribed to different components of the extract. Given the interesting fungicidal property with some cytotoxicity to some normal cell lines tested (Table 2), further purification of pure antifungal compounds is in progress and required prior to potential uses as antifungal drug, topical agent or nutricosmetic products.

Table 3. The antifungal and cytotoxicity effects of *Xylaria* sp. BCC 1067 extract against the model yeast BY4742 strain of *S. Cerevisiae* and fibroblast cell lines[1]

Antifungal activity				Cytotoxicity (IC_{50})	
MIC_{50}	MIC_{100}	MFC	MFC/MIC	Wi-38	CCD
75	500	1000	2	110	470

[1] MIC, MFC and IC_{50} were determined in mg/l of *Xylaria* sp. BCC 1067 extract

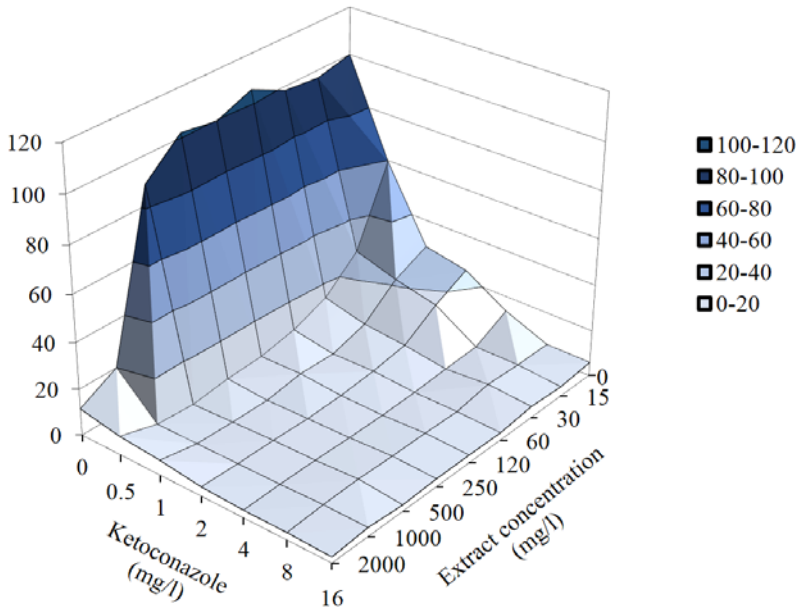

Figure 1 Antifungal effects of the combination between the *Xylaria* sp. BCC 1067 extract and ketoconazole on growth of *S. cerevisiae* wild-type strain BY4742 strain.

Table 4. Potential synergistic combinations between the *Xylaria* sp. BCC 1067 extract and ketoconazole as determined by checkerboard assay that was set up as described in Fig. 1. The concentrations of ketoconazole (KTC) tested was 0.5-16 mg/l and of the extract was 15-2000 mg/l. FIC, FICI and definition of synergy were described in the Materials and Methods.

MIC_{80} (mg/l)		FIC		FICI	activity
KTC	Extract	KTC	Extract		
4	0				
2	15	0.5	0.015	0.515	no synergy
1	30	0.25	0.03	0.280	synergy
0.5	60	0.125	0.06	0.185	synergy
0.5	120	0.125	0.12	0.245	synergy
0	1000				

Synergistic activity between the *Xylaria* sp. BCC 1067 extract and ketoconazole

Resistance to fungistatic drugs such as azoles are problematic in treatments of fungal infection. Currently, drug combination/cocktail formulations are commonly administered to patients in clinical settings to achieve better antifungal efficacy and therapeutic outcomes [51]. The combinatorial effect of the *Xylaria* sp. BCC 1067 extract with ketoconazole known to inhibit 14-α demethylase, a cytochrome P-450 enzyme [4]was examined, using a two-dimensional checkerboard micro-dilution technique [44] to investigate its potential application in combinatorial therapy. The concentrations in which MIC_{80} values were obtained were subsequently used for calculation of FICI values. The best FICI value of 0.2 was found for the combination of 60.0 mg/l of *Xylaria* sp.

BCC 1067 and 0.5 mg/l of ketoconazole which indicated synergistic effect against the wild-type model yeast *S. cerevisiae* BY4742 (Fig. 1 and Table 3).

Exposure to the *Xylaria* sp. BCC 1067 extract induced ROS generation in some deletion mutants

Fungicidal mode of action of amphotericin B is thought to kill yeast cells via plasma membrane pore formation [52] and ROS-mediated lethality [47]. We asked whether the exposure of *Xylaria* sp. BCC 1067 extract generate the ROS surge as shown for amphotericin B. This was determined for the wild-type strain *S. cerevisiae* and for a number of deletion mutants (Table 1) lacking key enzymes in the anti-ROS system and redox homeostasis [14]. Yeast cells were exposed to different of the extract concentrations, ranging from 0-1000 mg/l. The wild-type strain showed no increased ROS level in the presence of the highest concentration of extract tested, 1000 mg/l, after 2-h (Fig. 2a) and even after 4-h exposure (data not shown). In contrast, the Δ*yap1* showed enhanced level of relative ROS of 24-fold while the Δ*yap2* showed moderated increase in the relative ROS level of 9-fold at 1000 mg/l of the extract for 2-h exposure (Fig. 2a and b) and the ROS levels were doubled after 4-h exposure (data not shown). A threshold effect of ROS induction is clearly observed in the Δ*yap1* strain, showing a sharp increase at 1000 mg/l of extract concentration; however, treatment with 50 mM of the antioxidant thiourea reduced ROS to basal levels, confirming the presence of ROS (Fig. 2a).

To identify the target enzymes that were responsible for ROS removal following the extract exposure, sixteen deletion strains (Table 1) lacking enzymes of the antioxidative stress and redox homeostasis [53] were included. Among others, three strains with a deletion in the *SOD2* (Mn-superoxide dismutase 2), *GPX2* (glutathione peroxidase 2) or *GSH2* (glutathione synthetase 2),

genes necessary for ROS removal, exhibited significant increased ROS levels of 6-, 9- and 7-fold, respectively, after 2-h Of extract exposure (Fig. 2b). Deletions of other enzymatic genes had no significant effect on differences in ROS levels as compared to that of the wild-type strain (a P-value $\geq$ 0.05) (Fig. 2b). In summary, there were three different types of responses; the Δ*yap1* showed highly elevated level of ROS generation (as indicated by group I with a P-value $<$ 0.01), group II showed moderate increase between 6.0- and 8.7-fold (with a P-value $<$ 0.1 and 0.05, respectively), group III was no significant change in ROS as compared to the wild-type BY4742 cells (Fig. 2b).

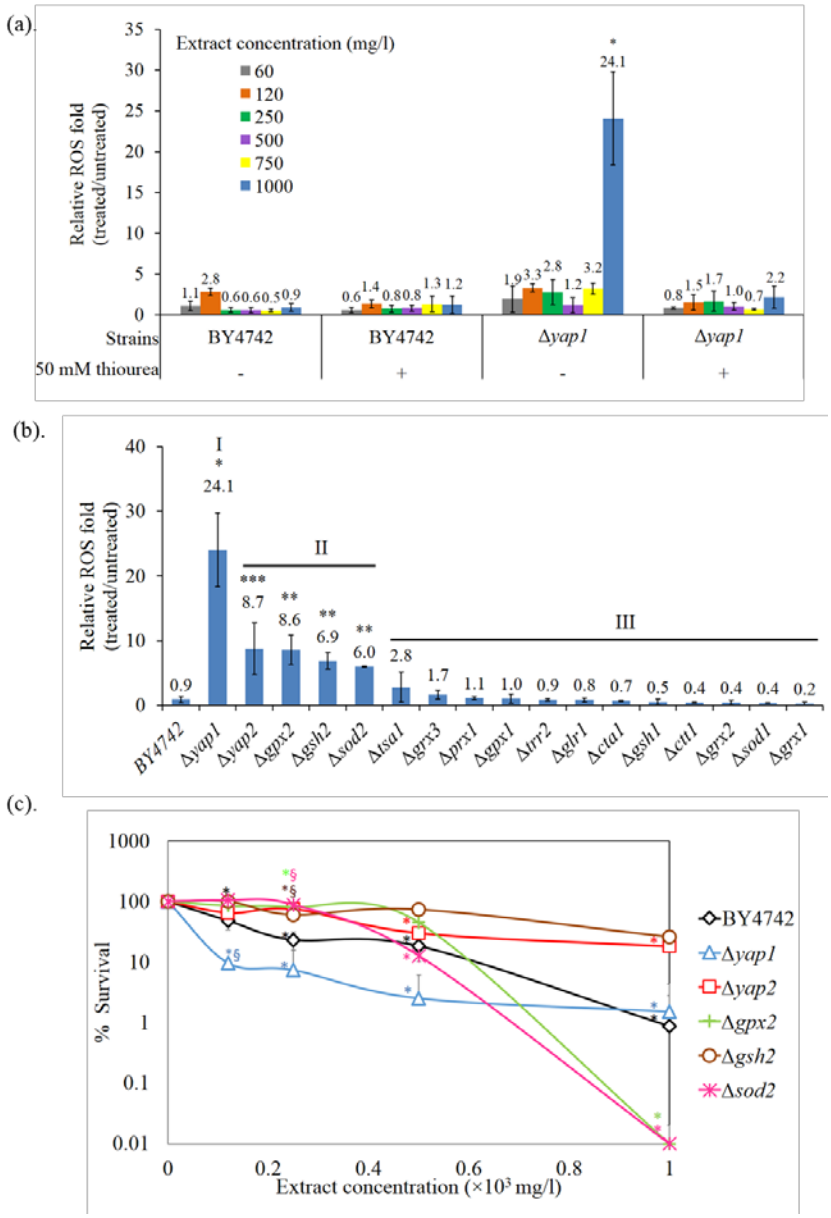

Figure 2. Relative ROS levels of different *S. cerevisiae* deletion strains and cell survival following the *Xylaria* sp. BCC 1067 extract treatment. (a) Relative ROS levels of the wild-type and the Δ*yap1* strains treated with different concentrations of the extract, ranging from 0- 1000 mg/l for 2-h (b) Relative ROS levels generated from wild type *S. cerevisiae* BY4742 cells and the deletion mutants of the anti-oxidative stress response by exposing cells with the extract of *Xylaria* sp. BCC 1067 at 1000 mg/l for 2-h The *, ** and *** signs indicated the ROS levels of the deletion

mutants that were significantly affected by the extract as compared to the wild-type strain. (c) the percentage survival of cells after incubation with the extract at various concentrations, ranging from 0 to 1000 mg/l for 24-h. Cell viability were determined using the MFC assay. Colonies of viable cells were counted and data was expressed as percentage (%) survival, relative to a no-extract control culture. Comparison between groups were made by one-way ANOVA, followed by Tukey's pairwise comparison. *,**,***,§

* The mean difference is significant at P <0.01 compared with untreated.

** The mean difference is significant at P <0.05 compared with untreated.

*** The mean difference is significant at P <0.1 compared with untreated.

§ The mean difference is significant at P <0.01 compared with wild-type strain at individual concentration of *Xylaria* sp. BCC 1067 extract.

Table 5 MICs and MFCs of the *Xylaria* sp. BCC 1067 extract against different *S. cerevisiae* deletion strains lacking genes of the oxidative stress response or the PDR system. The MFC values were determined via colony count assay and given when less than 1% survival was observed.

Involved pathway	Strain	MIC_{50} (mg/l)	MFCs (mg/l)
Anti-oxidative stress	BY4742	75	1000
	$\Delta yap1$	40	>1000
	$\Delta yap2$	320	> 1000

	Δ*gpx2*	720	1000
	Δ*gsh2*	650	> 1000
	Δ*sod2*	550	1000
Pleiotropic drug resistance	Δ*pdr1*	60	1000
	Δ*pdr3*	40	500
	Δ*stb5*	30	>1000
	Δ*pdr5*	50	250
	Δ*pdr1*Δ*stb5*	20	250
	Δ*pdr3*Δ*stb5*	30	250
	Δ*pdr1*Δ*pdr3*	40	120
	Δ*pdr1*Δ*pdr3*Δ*stb5*	20	120
	Δ*yap1*Δ*pdr5*	40	120

The oxidative stress-prone mutant strains showed individual sensitivity to the extract.

Susceptibilities to the *Xylaria* sp. BCC 1067 extract of the deletion mutants that demonstrated significant ROS generation were however complicated (Fig. 2c). The Δ*yap1* strain was more sensitive than the wild-type strain, while the Δ*yap2* strain were more resistant than the wild-type strain and the Δ*gsh2* strain was unaffected at the higher concentration of the extract (Fig. 2c). More complicatedly, at lower concentrations of the extract, the Δ*gpx2* and the Δ*sod2* strains with moderate levels of relative ROS exhibited better growth than the wild-type strain (Fig. 2c),

suggesting for protective effect at low extract concentrations. Nevertheless, at higher concentrations of the extract, they became more sensitive and death was observed at the MFC value of 1000 mg/l (Fig. 2c). MIC and MFC values of the extract to the wild-type and the oxidative stress-prone mutant strains were demonstrated in Table 4. The Δ*yap1* strain showed lower MIC_{50} and MFC than the wild-type strain, while other antioxidant strains with moderate increased ROS levels, including the Δ*yap2*, the Δ*gpx2*, the Δ*gsh2* and the Δ*sod2* strains, were found to have higher MIC values than the wild-type. Among those, the Δ*yap2* and the Δ*gsh2* strain showed higher MFCs than the wild-type. The results suggested an individual effect and unequal contribution of antioxidant regulators and enzymes to the cell sensitivity to the *Xylaria* sp. BCC 1067 extract.

Pdr1/3 and Stb5 regulators of Pdr5 transporter and involvement to the *Xylaria* sp. BCC 1067 extract tolerance

To investigate the involvement of the Pdr system, including the Pdr5 multi-drug transporter and transcriptional regulators of *PDR5*, namely Pdr1, Pdr3 and Stb5, in the detoxification of the *Xylaria* sp. BCC 1067 extract, ROS and viability assays were again performed. Interestingly, the Δ*pdr5* strain showed an elevated increase in the relative ROS levels to approximately 20- and 38-fold ($P \leq 0.001$), respectively, following 2 h. or 4 h. of exposure to 1000 mg/l of the *Xylaria* sp. BCC 1067 extract (Fig. 3a). The Δ*pdr1*, the Δ*pdr3* and the Δ*stb5* strains showed moderate increases in relative ROS (approximately 3- to 7-fold while the wild-type strain showed less than a 2-fold increase even after 4 h. exposure (Fig. 3a). There were no correlation between the MIC_{50} and the ROS levels of the single deletion mutants of Pdr strains (Table 4 and Fig. 3a). The double deletion strains of the Δ*pdr1*Δ*stb5,* the Δ*pdr3*Δ*stb5* and the Δ*pdr1*Δ*pdr3* strains displayed lower

MIC values (Table 4). Furthermore, the triple deletion strain Δ*pdr1*Δ*pdr3*Δ*stb5* exhibited MIC_{50} value of approximately 4 fold lower than that of the wild-type strain (Table 4). According to the MFC assay, the correlation between the relative ROS levels and MFC was better observed for the Δ*pdr5* strain (Table 4).

Regarding cell survival, it was clear that the tested strains could be categorized into two major groups according to the percentage survival curve following the extract exposure as shown in Fig. 3b. The first group included the Δ*pdr1*, the Δ*pdr3* and the Δ*stb5* strains with MFC values similar to the wild-type strain or lower at any concentrations up to 1000 mg/l for the Δ*pdr5* strain (Fig. 3b). The second group, with sharp declining in the percentage survival, included the double and triple deletion mutants of PDR transcriptional regulators (Fig. 3b).

In addition, strains in which *PDR5* and other drug transporters were deleted (ADΔ strain; Table 1) and showed enhanced sensitivity as cells barely grew at 30 mg/l of the *Xylaria* sp. BCC 1067 extract, implying the involvement of these PDR transporters (Fig. 3c). In contrast, expression of the *PDR5* gene (strains AD124567 which *PDR5* gene is intact and AD/ScPDR5 strain which *PDR5* gene is put back under its own natural promoter) resulted in enhanced cell survival in the presence of the antifungal extract *Xylaria* sp. BCC 1067 (Fig. 3c). Thus, it is clear that Pdr5 transporter contributes to the detoxification of the antifungal *Xylaria* sp. BCC 1067 extract which may contain Pdr5 new substrates from *S. cerevisiae* cells.

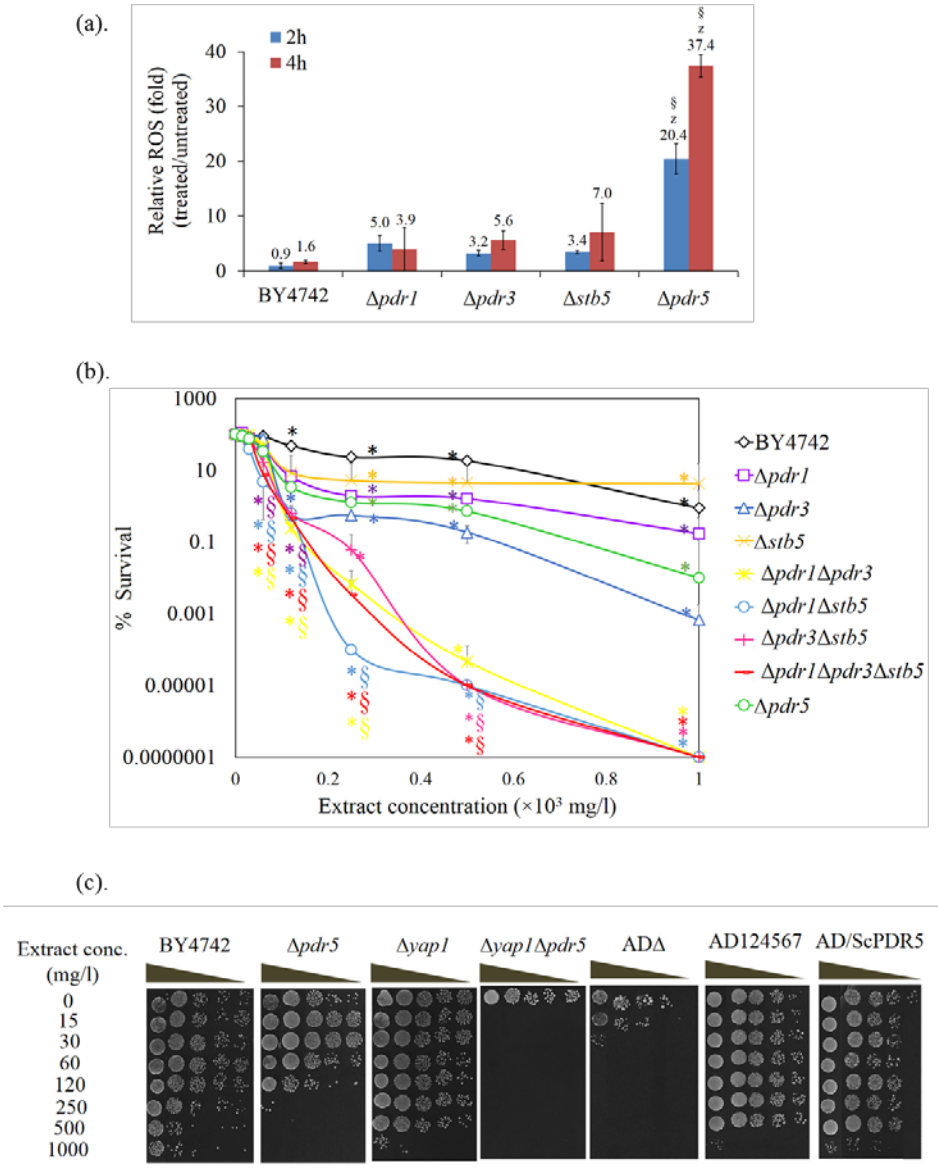

Figure 3. ***PDR1*, *PDR3* and *STB5* transcriptional regulators and *PDR5* transporter are involved in ROS induction and the detoxification of a *Xylaria* sp. BCC 1067 extract.** Relative ROS induction (fold) at 2 and 4 h[†] following 1000 mg/l exposure of extract (a) as well as the percentage cell survival(b), and spot assays (c) of different PDR deletion strains of *S. cerevisiae*, after the exposure to the *Xylaria* sp. BCC 1067 extract at various concentrations as indicated. Comparisons between groups were made by one-way ANOVA, followed by Tukey's pairwise comparison[*,§]

† Survival was expressed as the percentage (%)of cell survival relative to that found in the absence of extract condition .ROS levels were evaluated by a fluorescence microplate reader, using 10 mM of 2′,7′-dichlorofluorescein diacetate as a fluorescent probe .The data show the relative ROS of treated versus untreated cells.

* The mean difference is significant at the P <0.01 comparing with untreated.

§ The mean difference is significant at the P <0.01 comparing with wild-type strain at individual concentration of *Xylaria* sp .BCC 1067 extract.

YAP1 and _PDR5_ double deletion increased extract-induced ROS level and enhanced cell killing

Given that the crude *Xylaria* sp. BCC 1067 extract is a complex mixture of compounds, there may be different mechanisms at work causing changes in ROS accumulation and cell survival. Additional experiments were performed to examine a genetic interaction between the *YAP1* of the antioxidative stress response and *PDR5* of the Pdr system. Treatment with 1000 mg/l of the *Xylaria* sp. BCC 1067 extract for 2 h. induced ROS levels by 24, 20 and 31-folds for the Δ*yap1*, the Δ*pdr5* and the Δ*yap1*Δ*pdr5* strains, respectively (Fig. 4a). Treatments with the ROS-quenchers thiourea [54] or ascorbic acid [55] abolished the observed ROS surges, caused by the antifungal *Xylaria* extract for all strains tested (Fig. 4a). Thiourea or ascorbic treatment alone had no effect on the ROS level (data not shown).

The inhibitory assays showed that the Δ*yap1*Δ*pdr5* strain was sensitive to the extract with less MIC_{50} than that of the wild-type strain but similar to those of the corresponding single deletion strains although the MFC of the Δ*yap1*Δ*pdr5* was lower than the wild-type and the single deletion strains (Table 4). However, cell survival analysis clearly demonstrated the combined effect of the

PDR5 and *YAP1* genes on the oxidant protection against the *Xylaria* sp. BCC 1067. As shown, the Δ*yap1*Δ*pdr5* strain was killed at significantly lower concentration of the extract than the wild-type or the single deletion strains (Fig. 4b), demonstrating the combined effect of double deletion that was also confirmed by the spot assay (Fig. 4c).

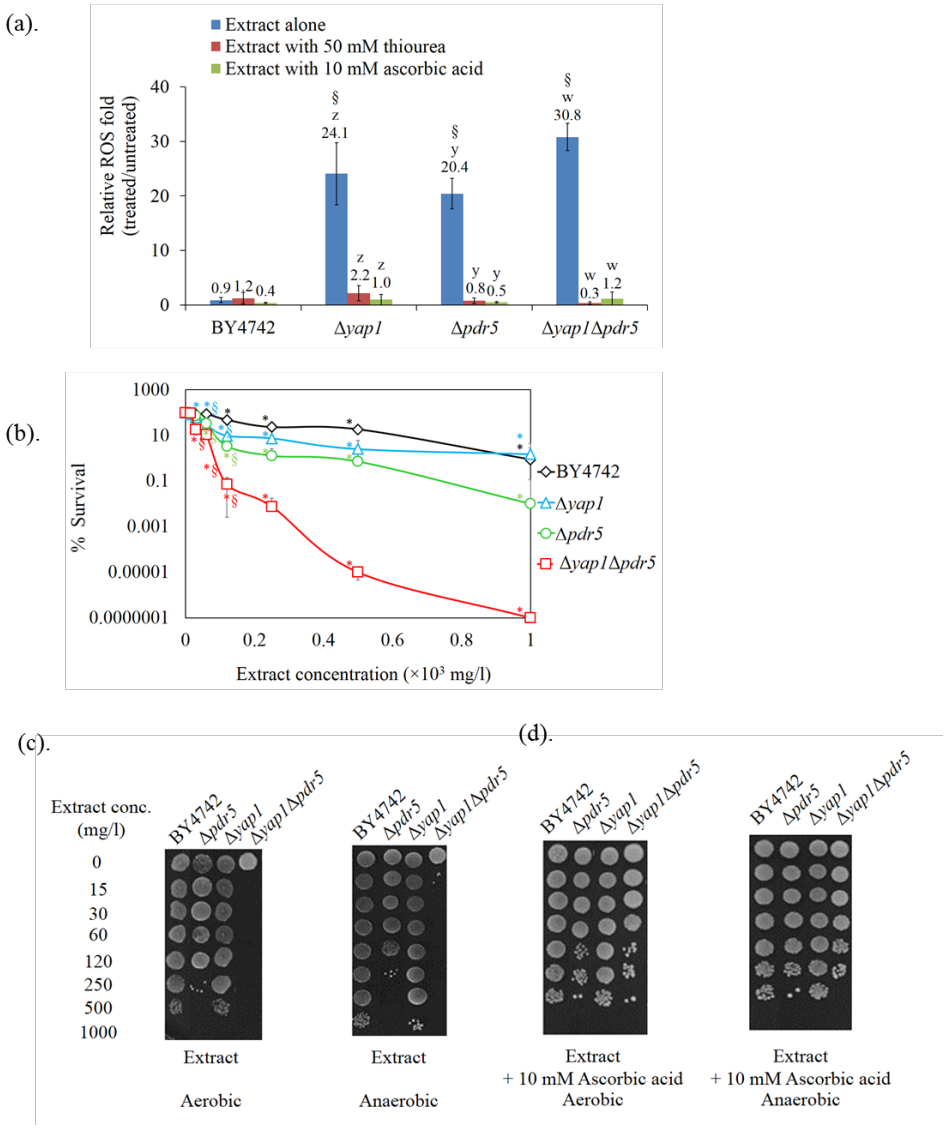

Figure 4. Contribution of the transcription regulators of oxidative stress response Yap1 and the multi-drug transporter Pdr5 in cellular defense against the ROS-generated *Xylaria* sp. BCC 1067 extract with antifungal property. Relative ROS levels at 2 and 4 h with/without the thiourea or ascorbic treatment (a), percentage survival (b) spotting assays under aerobic and

anaerobic conditions (c) and with/without antioxidant ascorbic acid (10 mM) (d) after the *Xylaria* sp. BCC 1067 extract exposure.[†] Comparisons between groups were made by one-way ANOVA, followed by Tukey's pairwise comparison [*,§]

[†] Survival was expressed as the percentage (%)of cell survival relative to that found in the absence of extract condition .ROS levels were evaluated by a fluorescence microplate reader, using 10 mM of 2′,7′-dichlorofluorescein diacetate as a fluorescent probe .The data show the relative ROS of treated versus untreated cells.

[*] The mean difference is significant at $P < 0.01$ compared with untreated.

[§] The mean difference is significant at $P < 0.01$ compared with wild-type strain at individual concentration of *Xylaria* sp. BCC 1067 extract.

Alternatively, cells were treated the *Xylaria* sp. BCC 1067 extract for 24 h. and grown under anaerobic condition or in the presence of 10 mM ascorbic acid to prevent ROS formation. Growth was slightly better in the absence of oxygen for the wild-type and the Δ*yap1* strains (Fig. 4c). Despite, the extract exposure, growth was significantly better in the presence of ascorbic acid for all strains especially in the Δ*yap1*Δ*pdr5* strain and was best when grown under anaerobic condition with ascorbic acid (Fig. 4d). This suggested that ROS is partly responsible for cell killing and, in addition, there may be existing lethal mechanisms that operate, independent of ROS activity.

Discussion

Synergistic antifungal activity of the *Xylaria* sp. BCC 1067 extract and ketoconazole

The growing incidence of antifungal drug resistance deserved special attention and, new approaches for better treatment is an emergent health issue [56]. The combination of known antifungal agents is an established therapeutic tactic in control of infection to increase spectrum of activity [51]. Drug combination not only reduces drug resistance but also requiring doses, cost, and toxic side effects. As shown, the *Xylaria* sp. BCC 1067 extract has the antifungal property with fungicidal effect (Table 2) and a potential application in the combinatorial treatment with ketoconazole (Fig. 1 and Table 3). While ketoconazole disrupts the membrane integrity via inhibition of the Erg11 enzyme of the ergosterol biosynthesis [4], the *Xylaria* sp. BCC 1067 extract causes intracellular ROS accumulation that may possibly affect membrane integrity via ROS-generated plasma membrane/lipid damage, providing a plausible explanation for their synergy. This is analogy to the synergism obtained from combinations of azoles and amphotericin B [57, 58]. In support, Wu *et al* has reported that the cyclo-(-NMePhe-Pro-Leu-Ile-Val-) isolated compound from another species of *Xylaria* sp. (75-1-3-1) also shows synergistic antifungal activity against *Candida albicans* at 6.25 mg/l with 0.004 mg/l ketoconazole (FICI < 0.3) [59]. Thus, *Xylaria* sp. BCC 1067 extract is a new and promising source of antifungal compounds with synergistic antifungal activity even in crude form.

Proposed the antifungal action of *Xylaria* sp. BCC 1067 extract against *S. cerevisiae*

The antifungal action of *Xylaria* sp. BCC 1067 extract is proposed (Fig. 5). One possibility is that the extract may cause the damage in the membrane. The extract can have antifungal compounds of organic nature or protein origin and this could define the way the antifungal drug acts.

Alternatively, the *Xylaria* sp. BCC 1067 extract may enter the *S. cerevisiae* cell and attack unknown molecular targets (route 1) and, at the same time, generate ROS. This may elicit various cellular responses, including the antioxidative stress and Pdr systems. Alternatively, via route 2, mixtures of cytotoxic compounds including antifungals present in the extract may themselves generate ROS-derivatives. Either way, production of toxic ROS may damage various macromolecules, resulting in DNA fragmentation, inactivation of enzymes, lipid peroxidation, or membrane/organelle damages. This would disturb cellular redox balance/homeostasis and lead to additional ROS-generation known as the oxidative burst, cell dysfunction and death as shown for some antimicrobials [60]. Cells may respond to the toxicity of this antifungal extract and counteract through the activation of Yap1-oxidative stress response through enhanced expression/activity of antioxidant enzymes such as Sod2 and Gpx2 to decrease intracellular ROS accumulation. Alternatively, Yap1 may be involved in the removal of ROS through regulation of the expression of the vacuolar pump encoded by *YCF* [61]. It is possible that Ycf1 may eliminate ROS toxicity by sequestering the *Xylaria* sp. BCC 1067 extract inside the vacuoles since growth of the ADΔ strain with deletions in *YCF1* and other Pdr transporter genes is severely impaired in the presence of extract (Fig. 3c).

Moreover, the Pdr system is clearly involved in mediating the *Xylaria* sp. BCC 1067 extract resistance through the involvement of the transcriptional regulators Pdr1, Pdr3, and Stb5 that may up-regulate *PDR5* expression and possibly of other Pdr transporter genes as observed previously for other cytotoxic substances [24], thereby reducing the extract cytotoxicity and preventing cellular damages and death (Fig. 5). This is supported by the low MIC and MFC values of the PDR deletion mutants, in particular the triple deletion strain of PDR regulatory genes *PDR1*/*PDR3*/*STB5* (Table 4 and Fig. 3b). In addition, the AD124567 strain with hyperactive

PDR1-3 allele and intact *PDR5* gene shows robust tolerance to the antifungal *Xylaria* sp. BCC 1067 extract in contrast to the ADΔ strain with poor growth, (Fig. 3c).

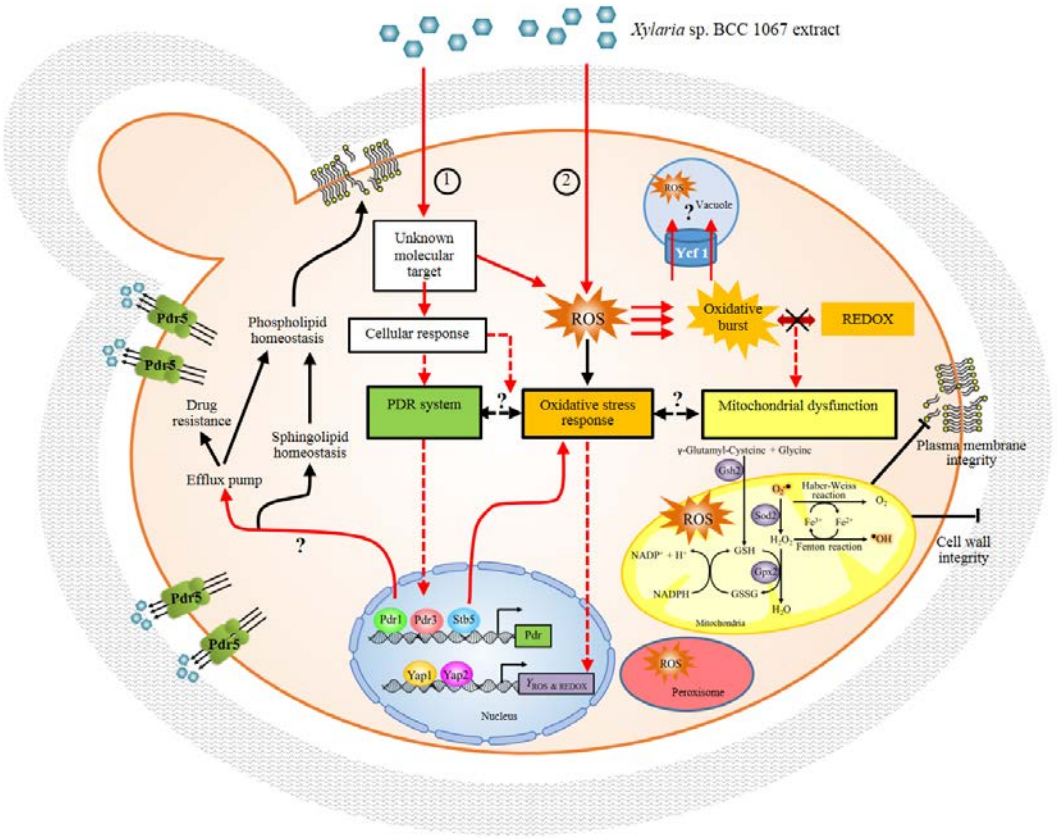

Figure 5. Schematic overview of the proposed mechanism of action of the fungicidal *Xylaria* sp. BCC 1067 extract and the cellular responses involving and Yap1- dependent anti-oxidative stress response and activation of the Pdr5 transporter.

***S. cerevisiae* ROS responses to the antifungal extract *Xylaria* sp. BCC 1067 extract**

Generation of ROS may augment the fungicidal activity of some antifungal agents providing an additional killing mechanism as shown for some antifungals amphotericin B, miconazole and ciclopirox [62], antibacterial drugs [63-65] as well as insecticides [66]. Some support for the ROS-mediated cellular damage in response to the *Xylaria* sp. BCC 1067 extract treatment are provided,

using mutant strains of anti-oxidant system (Fig. 2-4). Many points of observation were found through detail examination of each deletion strain. Despite some overlapping involvement, antioxidant regulators Yap1 and Yap2 confer differential response to a wide variety of extracellular stimuli [19, 67, 68], including the *Xylaria* extract sp. BCC 1067 (Fig. 2 and Table 4). Treatments with high extract concentrations suggest a distinct response, primarily controlled by the Yap1-dependent pathway with a functional compensation for the Yap2 regulator (Fig. 2c). Yap1 typically has a stronger modulating effect than Yap2 [68].

Next, strains lacking *SOD2*, *GPX2* or *GSH2* also grow better than the wild-type strain at low extract concentrations (Fig. 2c). This implies that low concentrations of the extract could protect the cells. Similarly, following the exposure to superoxide, *SOD* deletion protects *Escherichia coli* from lethality by bleomycin, a ROS-induced cancer drug [69, 70] and for some oxidants when used at small concentrations [71] although the protective mechanism of *SOD*-related adaptive response remains unknown. However, support for the mechanism in which *Xylaria* sp. BCC 1067 extract induced-ROS levels are associated with killing of *S. cerevisiae* cells is exemplified by the lethality of *SOD2* or *GPX2* deletion at high extract concentrations after 24 h. incubation (Fig. 2c). The enhanced ROS-generated during antifungal extract treatment is likely additive with ROS produced through other cellular processes in the absence of these two critical ROS eradicating enzymes.

Compromised mitochondrial damages following ROS induction by the antifungal extract

Antioxidant enzymes Sod2 and Gpx2 act to scavenge or reduce ROS within the mitochondria, a principle source of intracellular ROS generation and central to many cellular processes, energy production and maintenance of general homeostasis, including membrane lipids and heme [72].

The increased accumulation of excess ROS in these deletion strains may further damage mitochondrial components/ integrity, leading to mitochondrial dysfunction and lethality at high doses of extract or ROS levels. Interestingly, the antifungal amphotericin B has been shown to induce a signaling and metabolic cascade that is accompanied by increased mitochondrial activity, respiration and changes in intracellular activity, including increased ATP consumption, leading to ROS production and cell death [47]. Thus, in part, the killing of the antifungal *Xylaria* sp. BCC 1067 extract may result from a similar mechanism of cell death.

A reverse correlation between cell survival and high intracellular ROS level has been shown here (Figs. 2-4) and previously for some antifungal drugs with ROS mediated-killing [62]. High intracellular ROS level is a correlated factor but not necessary a death indicator if the excessed toxic ROS is removed prior to irreversible mitochondrial or cell damages. As shown here, the lethal effect of the *Xylaria* sp. BCC 1067 extract is found only in certain deletion strains (Fig. 2-4). The results suggest that the ROS-mediated killing do not depend solely on the extract concentrations. Instead, cell survival appears to be largely determined by the cell ability to tolerate oxidative stress, following extract induced-cell injury. In support of individual effect, death is not observed with *GSH2* deleted cells, despite similar level of ROS produced as found for those of the *GPX2* or *SOD2* deleted cells (Fig. 2). Death could be prevented if alternative anti-oxidant mechanism operates to compensate for the loss of *GSH2* function, for examples, the presence of intact Gsh1 for glutathione synthesis or of alternate antioxidative molecules such as thioredoxins or NADPH, thereby preventing ROS toxicity.

The DCF fluorescence limitation for ROS detection should also be noted. As describes in the literature [73], this dye is more suitable to investigate changes in intracellular iron signaling, associated mitochondrial damages and indicator of mitochondria generation of oxidants and

peroxynitrite formation. These points agree well with our assumption that the *Xylaria* extract may induce ROS-related mitochondrial damage and should be further investigated in detail. Importantly, mitochondria dysfunction has been identified as a key contributor to the drug tolerance in *S. cerevisiae* and many human fungal pathogens [72]. Specific mitochondrial mutations are shown to alter tolerance to antifungal drugs such as azoles, polyenes, and echinocandins [72]. Mitochondrial dysfunction or loss of the mitochondrial genome (mtDNA) is considered a key activating mutation for drug resistance in *S. cerevisiae* and other pathogenic yeasts [72, 74].

Lastly, examination of the PDR system provides many important clues to cellular adaptation to the antifungal *Xylaria* extract and some connection to mitochondrial dysfunction. A comprehensive review of Pdr system and compromised mitochondrial function relationship is well-described [72]. Briefly, a change in protein and lipid structure of mitochondrial membrane has been proposed to trigger the activation of drug resistance despite unknown mechanism [72]. The altered localization of the enzyme called Psd1 is proposed to create a signal for activation of PDR-regulated transcription [75]. Disturbed membrane lipid homeostasis is affected by mitochondrial dysfunction, resulting in the activation of sphingo- and phospho-lipid homeostasis, and the Pdr system function to couple lipid homeostasis with the activation of drug-resistance genes or transporters in compensation for this loss [76]. The activation of transcription factors of multi-drug resistance Pdr3 of *S. cerevisiae* and Pdr1 of *C. glabrata* has been shown to subsequently act to up-regulate respective target genes encoding efflux pumps, such as ScPdr5 or CgCdr1/Cdr2 [59], providing a link between lipid homeostasis and drug resistance.

Chapter 4

Conclusion and Future perspective

The *Xylaria* sp. BCC 1067 extract has a promising antifungal potential with fungicidal effect, particularly when used in the combination with the antifungal drug ketoconazole. The extract augments intracellular ROS accumulation that may disrupt cellular redox homeostasis, contributing to cell lethality. Its primary mechanism of killing is to be determined; however, a possible link between the plasma membrane rupture and severe mitochondrial damages as a result of ROS-mediated cell destruction is proposed. Importantly, the overall results indicate that both the anti-oxidative stress response and the multi-drug resistance system are vital for cellular protection/detoxification of the new antifungal *Xylaria* sp. BCC 1067 extract.

Future perspective

This study first establishes a potential application of the *Xylaria* sp. BCC 1067 extract as a new and effective natural source of antifungal agents with ROS-mediated killing mechanism in reminiscent to amphotericin B or phagocytic cells. The discovery of this extract isolated from the *Xylaria* fungal species provides new hope to overcome a global health challenge of antifungal drug resistance, commonly observed in current treatments of fungal infections with existing drugs. Paradoxically, *fungal* extract is potentially the *novel antifungal* agent of the future. Although the extract shows some toxicity to human cells, separation of the potential antifungal molecules from within the *Xylaria* extract has potential for development of new drugs as well as application in combinatorial therapy as demonstrated by its robust synergy with ketoconazole. Furthermore, it

could present an alternative choice to combination between the azoles and amphotericin B treatment or when other combinations fail to be effective, and especially, when the development pipeline for new antifungals or antibiotics is low or for patients who develop resistance to current antifungal drugs. Indeed, in some countries, *Xylaria* species are typically consumed as edible food and considered safe for use in folk medicine [77]. The antifungal extract could provide other applications as food supplements or healthcare products.

Importantly, this study of natural extract of fungal origin also suggests many prime targets for future antifungal drug development. As demonstrated here and elsewhere, many transcription factors of anti-oxidative stress response and the multi-drug resistance as well as corresponding enzymes or drug transporters act as important defense line against antifungal drug [72], they could as well be exploited as valid antifungal targets. Mitochondrial dysfunction is reported to affect azole sensitivity/resistance in both *S. cerevisiae* and *Candida* species [72]. Targeting specific mitochondrial proteins has been suggested as an alternative way to fight fungal infection [78]. Here, the Yap1 regulator and the mitochondrial-associated antioxidant enzymes, for examples Sod2 and Gpx2, may be promising candidates for drug targets especially when used as combined drugs with those having an additive ROS effect such as amphotericin B or as novel antifungal agent. Alternatively, targeting the transcriptional activators of multi-drug efflux transporters such as Pdr1, Pdr3 and Stb5 with the highly conserved zinc cluster motif that unique to fungi or Pdr5 multi-drug efflux transporter could be effective drug design for therapeutic purpose in the combat against *PDR* resistant strains.

To this end, despite many remaining questions for further exploration of this highly complex phenomenon, this study exemplifies how cells employ various molecular components, particularly transcriptional regulators and targets, to evolutionally adjust and refine their responses during the

exposure to the antifungal. Determination of the molecular mechanism behind increased susceptibility to azoles (or other drugs) in cells with dysfunctional mitochondria is key to better understanding.

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