Antifungal activity of natural extract isolated from fungal *Xylaria* sp. BCC1067 against drug resistance

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Abstract

Fungal disease such as Candidiasis is one of life-threatening infection among immunocompromised patients. Up-regulation of some genes involved in ergosterol biosynthesis (*ERG11*) or drug efflux transporter (*PDR5*, *CDR1*, *CDR2* or *MDR1*) are important mechanisms to resist with clinically antifungal drugs. Currently, effective antifungal agents from natural source have been used to fight with drug-resistance. In this study, natural antifungal extract isolated from fungal *Xylaria* sp. BCC1067 was investigated against mutant yeast *Saccharomyces cerevisiae*. The mutant yeast strains were overexpressed with genes involved in drug-resistant mechanisms such as *ScPDR5*, *CaCDR1*, *CaMDR1* or *CaERG11*. The result found that all the overexpressed yeast strains showed resistance to clinical antifungal drug fluconazole. The yeast strains overexpressing *ScPDR5* or *CaCDR1* also showed resistance to *Xylaria* sp. BCC1067 extract suggesting some compounds present in the fungal extract are substrate of drug efflux pump Pdr5 or Cdr1. Interestingly, yeast strain overexpressing *CaMDR1* showed sensitivity to the fungal extract suggesting the fungal extract might interfere function of Mdr1 transporter. In contrast, yeast overexpressing *CaERG11* did not show sensitivity to the fungal extract suggesting irrelevant function in resistance to the extract. In addition, antifungal activity of the fungal extract against mutant yeast strains lacking genes in ergosterol biosynthesis pathway, including *HMG1*, *ERG4*, *ERG5*, *ERG6* or *ERG28*, and transcription regulators involved in ergosterol, such as *UPC2* or *SUT1*, were investigated. There was only the yeast strain lacking *ERG6* gene showed sensitivity to the fungal extract suggesting Erg6 might be an important protein that confers resistance to the fungal extract.

Introduction

Candida albicans is one of life-threatening infection that causes high mortality rate among immunocompromised patients[1]. Nowadays, the clinical antifungal drugs are limited to some classes. For example, Polyenes (amphotericin B) binds to ergosterol molecules and generate a pore through the yeast cell membrane[2]. The other class is azoles, for example fluconazole, itraconazole, isavuconazole, posaconazole and voriconazole, which are the most commonly used in clinical. The azoles target sterol 14α-demethylase or Erg11 enzyme in ergosterol biosynthesis. The important problem of failure treatment is from repetitive use of antifungal drugs that allows fungi to develop drug resistance, especially azoles which are commonly the first choice for antifungal treatment[3]. Upregulation of *ERG11* gene encoding azole target enzyme leads to increase azole resistance in several *C. albicans* isolates[4]. The other azoles resistant mechanisms is up-regulation of genes that encode efflux transporters which reduce the accumulation of drugs in the cells[5]. One of the important group of drug efflux pumps

is ATP-binding cassette (ABC) transporter such as ScPdr5p and CaCdr1p which utilize energy from ATP to translocate substrates across membrane[6]. The other group is the Major Facilitator Superfamily (MFS) transporters. CaMdr1p from pathogenic fungus *C. albicans* is the first identified and characterized MFS transporter. The MFS transporter confers primary resistance to fluconazole[7]. To solve drug resistance problem, attempt on a search for new and effective antifungal agents have initiated.

Currently, natural products still the most potential resource for novel and effective drugs. Several of natural products are a potential source to produce important compounds with a pharmacological properties[8]. In some filamentous fungi such as *Xylaria* sp. produce important bioactive compounds with biological properties such as antimicrobial and anticancer[9,10]. *Xylaria* sp. BIOTEC culture collection (BCC) 1067 extract has been reported antifungal activity against wild-type *Saccharomyces cerevisiae*. The extract shows synergistic effect when combined with an antifungal drug ketoconazole[11].

S. cerevisiae is the budding yeast commonly used as a model organism to investigate the regulation of drug resistance genes. *S. cerevisiae* is the first eukaryotic organism that its genome was sequenced completely. It can grow rapidly and easy to manipulate in laboratory[12]. Many regulatory mechanisms have been discovered and elucidated. Importantly, several useful molecular tools are available for experiments and data analysis. On the other hand, the homolog sequences of *S. cerevisiae* genes have been shown closed relationship with other pathogenic yeasts and fungi[13].

This study aimed to determine bioactive compound profile of fungal extract, isolated from fungal *Xylaria* sp. BCC1067. Second, the fungal extract was determined the antifungal activity against the model yeast, overexpressing gene in drug resistant system in comparative with clinically used antifungal drug fluconazole. Last, the fungal extract was determined the antifungal activity against mutant yeast strains lacking genes in ergosterol biosynthesis in comparative with fluconazole drug.

Methodology

Yeast strains

Table 1 Genotype or description of *S. cerevisiae* strains in this study

Strain	Genotype
AD124567	MATα *PDR1–3 ura3 his1 Δyor1::hisG Δsnq2::hisG Δpdr10::hisG Δpdr11::hisG Δycf1::hisG Δpdr3::hisG*
AD1-8u	AD124567 *Δpdr5::hisG Δpdr15::hisG*
ADΔ	AD1-8u, Δura3
AD/CaERG11A	AD1-8U- ,*Δpdr5*::pABC3-CaERG11A
ADΔ/ScPDR5	ADΔ, *Δpdr5*::pABC3-ScPDR5
ADΔ/CaCDR1A	ADΔ, Δ*pdr5*::pABC3-CaCDR1A
AD/CaMDR1A	AD1-8U- ,*Δpdr5*::pABC3-CaMDR1A
BY4742	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0
Δ*upc2*	BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0ura3Δ0*) *Δupc2::kanMX4*
Δ*sut1*	BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0ura3Δ0*) *Δsut1::kanMX4*
Δ*hmg1*	BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0ura3Δ0*) *Δhmg1::kanMX4*
Δ*erg4*	BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0ura3Δ0*) *Δerg4::kanMX4*
Δ*erg5*	BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0ura3Δ0*) *Δerg5::kanMX4*
Δ*erg6*	BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0ura3Δ0*) *Δerg6::kanMX4*
Δ*erg28*	BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0ura3Δ0*) *Δerg28::kanMX4*

Xylaria culture and extraction

The *Xylaria* sp. BCC1067 was obtained from the BIOTECH Culture Collection (BCC culture 6200032292); National Science and Technology Development Agency, Bangkok, Thailand) and originally isolated from a single stroma grown on the petiole of a leaf in Nam Nao National Park in Northeastern Thailand. Cultivation of *Xylaria* sp. BCC 1067 was modified from the method of Phonghanpot et al[10]. The *Xylaria* sp. BCC 1067 was grown on solid media containing 1.5% (w/v) of malt extract broth (MEB, OXOID, Oxoid Ltd., UK) and 2% (w/v) of agar (Himedia, India). After that, *Xylaria* sp. BCC 1067 were cut into small pieces and transferred to fresh liquid MEB and allowed to grow for 1 month at 25°C without shaking. After incubation period, the culture was separated by filter paper (Whatman No.1 paper) The liquid fraction was extracted with a two volumes of ethyl acetate (EtOAc, QREC, New Zealand). The dried crude extract was kept at 4°C and freshly dissolved with methanol prior to use.

High Performance Liquid Chromatography

The fungal extract obtained from *Xylaria* sp. BCC 1067 was chromatographed by using HPLC coupled with PDA detector with various wavelength including 210, 254, 280, 310 and 360 nm. The mobile phase was the combination of purified water (Milli-Q® Integral Water Purification System, USA) and acetonitrile, pumped in gradient manner starting at 95:5, 0:100, 95:5 and ends at 0:100). Each injection required 10 µl of sample. The mobile phase was pumped through the C18 column (VertiSepTM UPS C18 150 HPLC, 4.6×250 mm, 5 µm, Vertical Chromatography Co., Ltd., Thailand). Sample and mobile phase was filtrated through 0.22 µm membrane prior to be used.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of Xylaria sp. BCC 1067 extract and antifungal drugs

A standard culture media YPD for baker's yeast was used for MIC and MFC determinations. Briefly, yeast strains were grown overnight in YPD media at 30°C at 150 rpm. Culture was adjusted to OD_{600} of 0.001. The fungal extract and antifungal drugs were prepared as two-fold serial dilution in flat bottom 96-well microtiter plate (Corning® Costar®, Sigma-Aldrich, China). Cells were incubated at 30°C, 150 rpm for 24 hours. An automated microplate reader (M965+; Metertech, Taipei, Taiwan) was used to determine OD_{600} values. Finally, 3 µl/ml in each well was spotted in agar plate then incubated for 2 days and observed the colony growth on the agar plate.

Results and Discussion

The fungal extract obtained from *Xylaria* sp. BCC 1067 was chromatographed by using HPLC compared with methanol which used as a solvent. The result found that, there were six peaks presented in the extract indicated as A – F (**Figure 1**). The fungal extract showed high concentration of peak C suggested that the peak C might be a major compound of the extract.

(a)

(b)

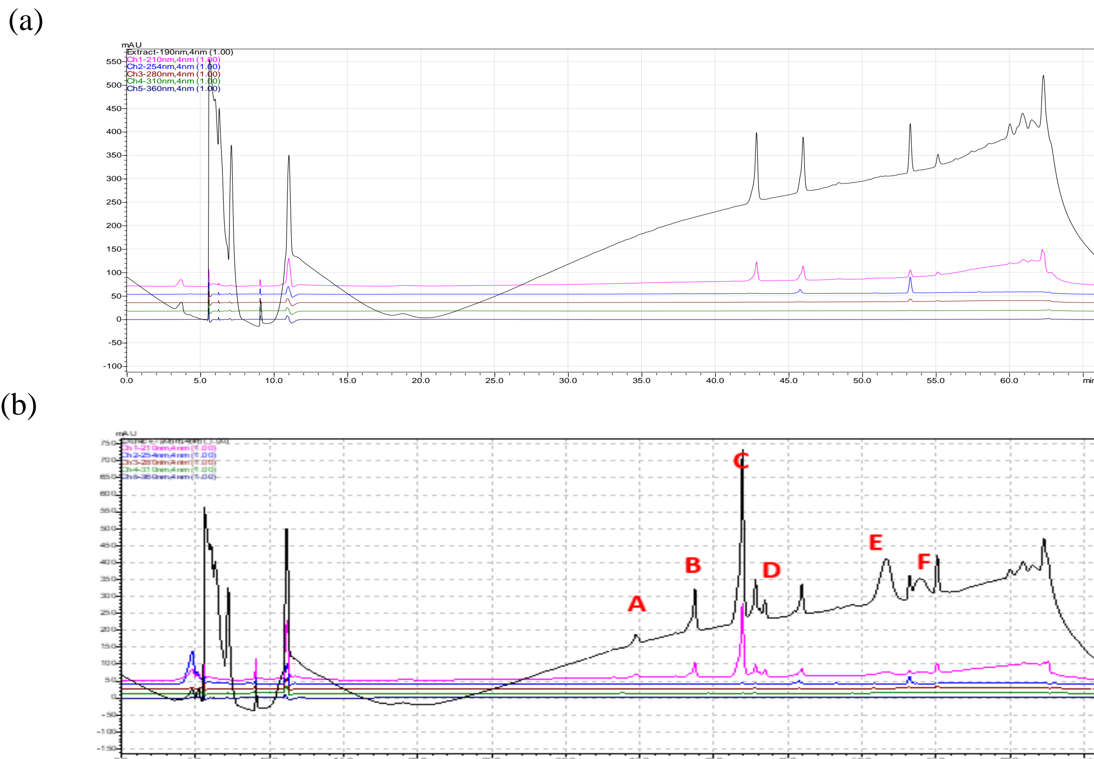
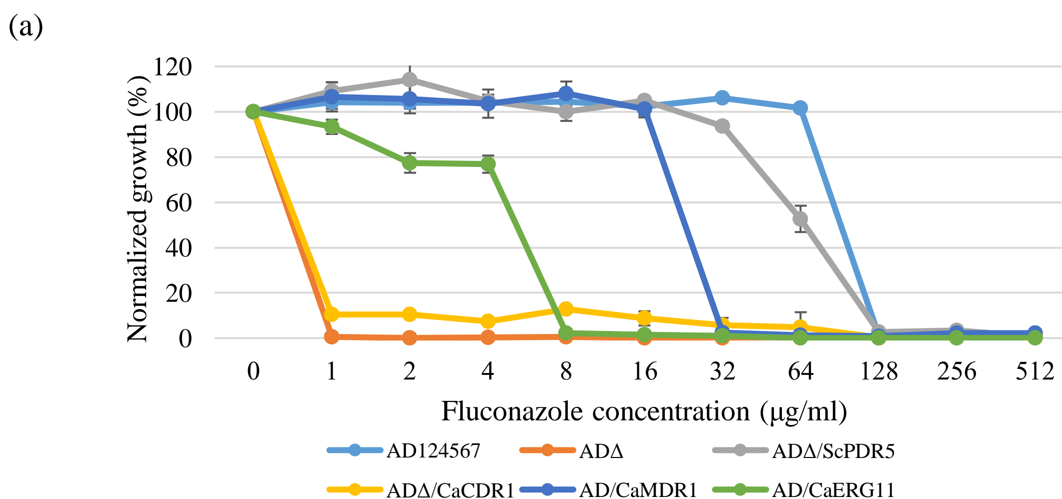


Figure 1. HPLC chromatogram of (a) methanol and (b) fungal extract obtained from *Xylaria* sp. BCC 1067

The *S. cerevisiae* strain ADΔ which was engineered by deleting ABC transporter genes including PDR5 gene was used as a negative control showed susceptibility to antifungal drug amphotericin B and fluconazole when compared with positive control, AD124567, which was engineered by deleting ABC transporter genes except for *PDR5* gene. The model yeast *S. cerevisiae* strain overexpressing gene involved drug resistant mechanisms such as AD/CaERG11A. *Erg11* gene encodes sterol 14α-demethylase or Erg11 enzyme which known as target of azoles. The result found that the model yeast AD/CaERG11A showed resistance to fluconazole according to increasing to fluconazole target. Moreover, the model yeast strain ADΔ/ScPDR5, ADΔ/CaCDR1 and AD/CaMDR1 showed resistance to fluconazole drug (**Figure 2a** and **b**) which known as substrate of drug efflux pumps Pdr5p, Cdr1p and Mdr1p transporter[14,15,16].

(a)



(b)

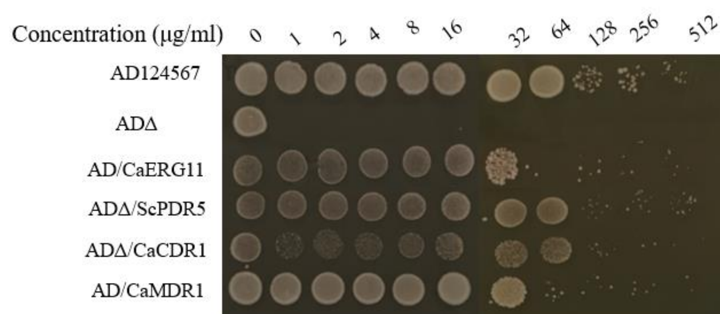
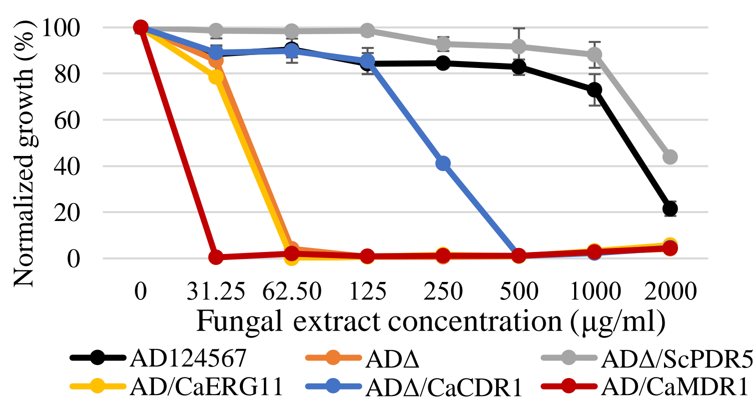


Figure 2 Susceptibility of *S. cerevisiae* strains overexpressing genes involved drug resistance system in treatment with fluconazole (a) normalized growth and (b) survival on YPD agar. Normalized growth of cells treated with fluconazole or cultural media extract were calculated by comparing growth with untreated condition. Cultures were directly spotted onto YPD agar, incubated for 48 h, and observed survival

(a)



(b)

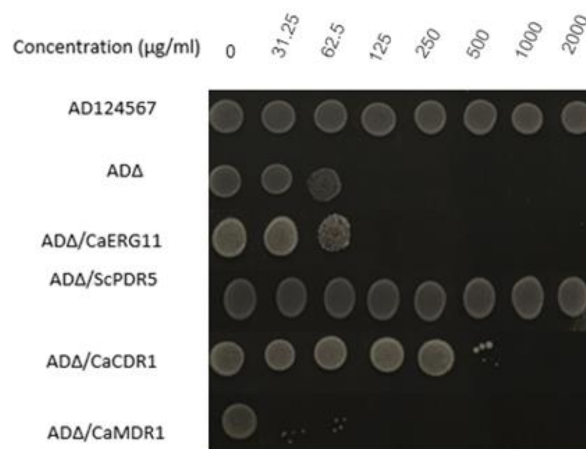
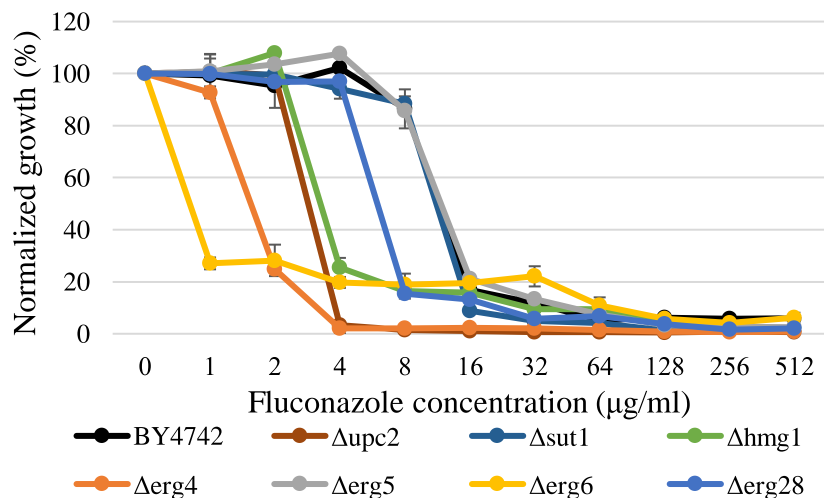


Figure 3 Susceptibility of *S. cerevisiae* strains expressing genes involved drug resistance system in treatment with fungal extract isolated from *Xylaria* sp. BCC 1067 (a) normalized growth and (b) survival on YPD agar.

The fungal extract isolated from *Xylaria* sp. BCC 1067 was investigated antifungal activity against the model yeast overexpressing genes involved in drug resistant system. The result found that the yeast strains overexpressing *ScPDR5* or *CaCDR1* showed resistance to the fungal extract as well as fluconazole. We suggested that there are some compounds present in the fungal extract are substrate of drug efflux pump Pdr5 and Cdr1 (**Figure 3a** and **b**).

Interestingly, the yeast strain overexpressing *CaMDR1* showed resistance to fluconazole, while sensitivity to the fungal extract. We suggested that the fungal extract might interfere function of Mdr1 transporter. Moreover, the yeast strain overexpressing *CaERG11* did not show sensitivity to the fungal extract suggesting irrelevant function in resistance to the fungal extract. While Somboon et al.[11] reported that the fungal extract isolated from *Xylaria* sp. BCC1067 resulted in plasma membrane lesion indicating effects on the structure of the plasma membrane. The antifungal susceptibility test of the extract was performed against mutant strains lacking of genes and transcriptional regulators in ergosterol biosynthesis pathway. The genes and transcription regulators were used in this study, for example *UPC2* which encoded transcription activator that regulate of ergosterol biosynthesis[17], *SUT1* which encoded transcription factor that regulated sterol uptake genes[18], *HMG1* which encoded HMG-CoA reductase[19], *ERG28* which encoded endoplasmic reticulum (ER) transmembrane protein (Erg28p)[20] and also *ERG4*, *ERG5* and *ERG6* which encoded enzymes for the late steps of ergosterol biosynthesis[21].The fluconazole drug acts as inhibitor of ergosterol synthesis was used to determine the susceptibility . All mutant strains, except *Δerg5* strain, showed sensitivity to fluconazole when compared with the wild type strain BY4742 (**Fig.4a** and **4b**). For the susceptibility of the fungal extract against the mutant yeast strain, there was the Δ*erg6* strain showed sensitivity to the extract when compared with the wild type strain (**Fig.5a** and **5b**). We suggested that Erg6p might be an important protein that confers resistance to the fungal extract.

(a)



(b)

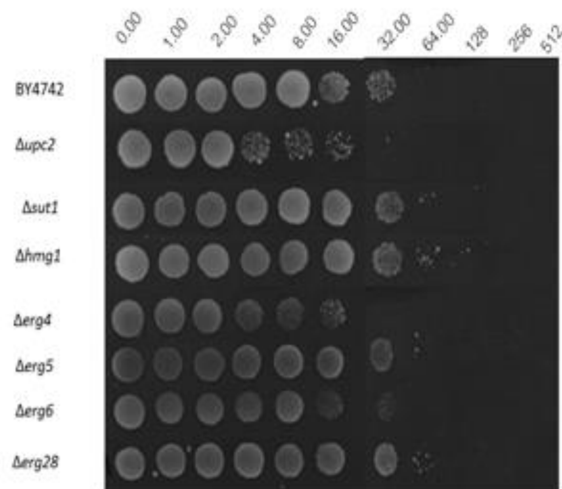
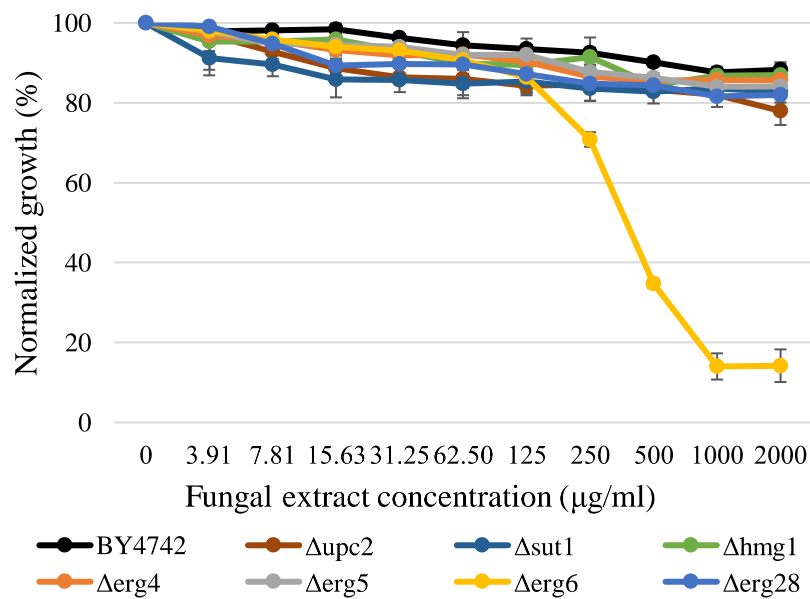


Figure 4. Susceptibility of *S. cerevisiae* strains lacking genes involved in ergosterol biosynthesis pathway in treatment with fluconazole (a) normalized growth and (b) survival on YPD agar.

(a)



(b)

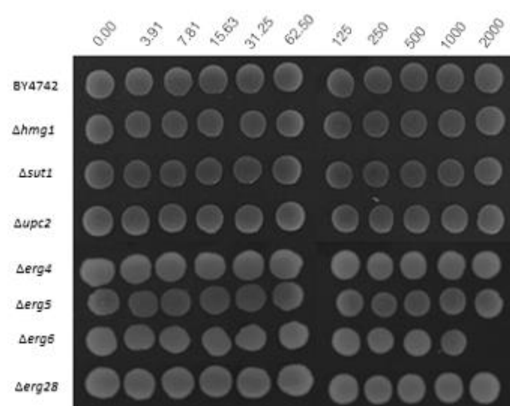


Figure 5. Susceptibility of *S. cerevisiae* strains lacking genes involved in ergosterol biosynthesis pathway in treatment with fungal extract isolated from *Xylaria* sp. BCC 1067 (a) normalized growth and (b) survival on YPD agar.

Conclusion

The model yeast strain overexpressing *ScPDR5* and *CaCDR1* genes show resistance to antifungal drug fluconazole and the fungal extract isolated from *Xylaria* sp. BCC1067. The model yeast strain overexpressing *CaMDR1* gene show sensitivity to the fungal extract while resistance to the tested antifungal drug. Moreover, the fungal extract is irrelevant function in the model yeast overexpressing *CaERG11* while relevant function in the yeast strain lacking *ERG6* gene.

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