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Antifungal activity of *Xylaria* sp. extract and synergistic effect in combination with antifungal drug caspofungin againsts model yeast *Saccharomyces cerevisiae*

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Abstract

Fungal drug resistance is a major concern in treatment of fungal infection disease which occurred mostly in immunocompromised patients. One of the most important drug resistance mechanism is occurred from overexpression of drug transporter that reduce the accumulation drug amount in fungal cell. ATP-binding cassette (ABC) transporters, such as the human P-glycoprotein (P-glycoprotein, Mdr1 or ABCB1) and common opportunistic pathogenic fungi *Candida albicans* Cdr1 are homologues of *Saccharomyces cerevisiae* Pdr5. They play major role in drug resistance due to broad substrates. Therefore, new potential treatments for fungal infections are needed. In this study, the antifungal activity of *Xylaria* sp. extract in combination with antifungal drug caspofungin (CSP) against *S. cerevisiae* BY4742 and mutant strain lacking *PDR5* were evaluated using broth microdilution checkerboard method. The fractional inhibitory concentration index (FICI) was used to interpret drug interactions. Extract of *Xylaria* sp. enhanced the antifungal activity of CSP against BY4742 and Δ*pdr5* strain with additive effect (FICI > 0.5). Furthermore, 19,20-epoxycytochalasin Q, an isolated compound from *Xylaria* sp. extract, exhibited synergistic effects against BY4742 in combination with CSP (FICI = 0.266-0.5). No antagonistic interaction was observed. The results demonstrate that synergistic combination between cytochalasin and CSP may be affected by efflux-mediated resistance. Our study suggests the cytochalasin creating new potential combination therapies to combat fungal infection.

Introduction

Increasing number of death caused by fungal infection resulting to a major global health problem.[1] Invasive fungal infections are mostly serious condition found in immunocompromised patients with AIDS, cancer, organ transplantation, and corticosteroid therapies. Major deaths of these patients are resulting from infection with *Cryptococcus*, *Candida*, and *Aspergillus* species.[2] Currently, fungal infection therapy is treated by limited classes of antifungal drugs, including polyenes, azoles, allylamines and echinocandins. Treatment with azoles and echinocandins are rapidly caused drug resistance, while the resistance to polyene drug such as amphotericin B is rare. Several drug resistant mechanisms were found in fungi. First of all, antifungal drug resistant strains can deduce the efficacy of antifungals by over production of drug targets. Although drug with highly selective target is required it is easily for fungi to develop drug resistance by alteration of drug target. Protection of cell by prevention entering of drug is another way to resist effect of drug. Moreover, fungi can reduce concentration of intracellular drug accumulation by function of drug transporters identified as ATP-binding cassette (ABC) transporter family and Major Facilitator Superfamily

(MFS) efflux pump.[3] There are many drug transporters that implicated in antifungal drug resistance, for example Pdr5 which confers resistance to various chemicals, drug, and hormone.[4]

In order to decreased public health problem from lack of new drugs and development of drug resistance urgently call for new pharmaceutical strategies. Drug combination with synergistic activity is a strategy to apply drugs with novel mode of actions. It provides benefits for fungal therapeutic, for instance reduction dose of single drug usage with increased drug-efficacy, and subsequently lower the drug toxicity. In addition, use of drug combination can postpone the development of drug resistance due to multi-target strategy.[5] Several studies have been reported antifungal activity of drugs that enhanced by combined drugs or compounds, for example, beauvericin, a marine natural product, has potent synergistic activity when combined with several azole drugs such as ketoconazole, miconazole against *C. albicans* by inhibiting the ABC transporters.[6] Berberine is another natural product that can enhance antifungal activity of azoles and provides synergistic effect against *C. albicans* by reverse the efflux function of a major facilitator transporter Mdr1.[7]

Since the combinations of different antifungal drugs or compounds with different mechanisms might improve clinical results, reduce doses and dose-related toxicity, we further investigated the interactions of caspofungin with crude extract of *Xylaria* sp. BCC 1067 and isolated compound, namely19,20-epoxycytochalasin Q. Caspofungin belongs to drug class echinocandin which inhibit beta-(1 3)-D-glucan synthesis and consequently disrupting the fungal cell wall integrity.[8] Cell wall present in fungi and absent in human, therefore, it is effective target for caspofungin which cause low toxicity. Caspofungin is actively inhibit azole resistant yeasts such as *C. glabrata* and *C. krusei*, as well as to some *Candida* biofilms. Unfortunately, echinocandin resistance occurs in these *Candida* spp. with azole-resistant background leading to multidrug resistance which is an unfavorable outcome.

Present study, caspofungin was combined with crude extract of *Xylaria* sp. BCC 1067 which is member of the Xylariaceae in the order of Xylariales. *Xylaria* spp. are commonly found in tropical forest such as forest in Thailand. They are powerful sources of bioactive compounds that provide antimicrobial, antioxidant, anticancer, and antiplasmodial activity. For example, terpeniods, cytochalasins, mullein, alkaloids, polyketides, aromatic compounds and organic compounds.[9] Cytochalasins are mycotoxin produced by most of fungi including *Xylaria* spp.[10-13] Cytochalasins inhibit actin polymerization by binding at fast growing plus end of microfilament leading to stop both the polymerize and depolymerize of actin filament resulting to defect of cellular morphology, inhibit cell division and cause apoptosis.[10, 14-15] Furthermore, some of cytochalasins exhibit other activity such as cytochalasin B that inhibit glucose uptake.[16] Cytochalasin H can regulate plant growth, cytochalasin D can inhibit protein synthesis and cytochalasin E can prevent angiogenesis.[10] In this study, 19,20-epoxycytochalasin Q was isolated from crude extract of *Xylaria* sp. BCC 1067 and was investigated synergistic activity when combined with caspofungin against *Saccharomyces cerevisiae* which could be useful therapeutically to reduce antifungal resistance.

Methodology

Yeast strains and media

The *S . cerevisiae* wild-type BY4742)*MATα his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0* ([17] and Δ*pdr5* strain were obtained from Open Biosystems (Dharmacon, Inc, Lafayette, CO, USA) . Yeast extract–peptone–dextrose)YPD (culture media was utilized for routine growth of yeast cells) Himedia Laboratories, Mumbai, India(.[18] Caspofungin was purchased from Sigma (Sigma, St. Louis, USA).

Xylaria culture, extraction and purification

Xylaria sp. BCC 1067 was purchased from the BIOTEC Culture Collection (BCC culture 6200032292; National Science and Technology Development Agency, Bangkok, Thailand) was cultivated and extracted as previously described.[19] The dried crude extract was stored at 4°C and freshly dissolved with methanol prior to use.

The dried crude extract was purified by silica gel column chromatography. The fractions were chromatographed by using HPLC (C-10ADVP, Shimadzu, Japan) couple with PDA detector (SPD-M10A, Shimadzu, Japan) with various wavelength including 210, 254, 280, 310 and 360 nm. The mobile phase was pumped through the C18 column (VertiSep™ UPS C18 HPLC, 4.6×250 mm, 5 μm, Vertical Chromatography Co., Ltd., Thailand).

Interactions of Drugs

Drug interactions were assessed by a checkerboard microdilution method that also included the determination of the MIC of each drug alone in the same plate using the guidelines presented in National Committee for Clinical Laboratory Standards (NCCLS) with some modification.[19] Caspofungin was placed in columns of trays to perform possible combinations, with concentration from 1.6 to 0.03 mg/l for BY4742 or 0.4 to 0.006 mg/l for Δ*pdr5*. Crude extract and 19,20-epoxycytochalasin Q were placed in the rows of the trays with concentrations from 1000 to 1.95 mg/l. For all combinations, MIC was determined after 24 h of incubation at 30°C with the endpoint criterion that was defined as the lowest concentration resulting in 80% inhibition of visible fungal growth. Triplicate testing was performed. The fractional inhibitory concentration index (FICI) was used to classify drug interaction. The FICI is the sum of the FIC of each combination between caspofungin, crude extract, and 19,10-epoxycytochalasin Q. FIC is defined as the individual MIC when used in combinations divided by the individual MIC when used alone. The FICI was defined as the following: synergic if the FICI was ≤ 0.5; neither synergistic nor antagonistic if the FICI was > 0.5 and ≥ 4.0; and antagonistic if FICI was > 4.0.

Results and Discussion

The rising number of antifungal drug resistance fungi is of concern. Therefore, we examined whether crude extract of *Xylaria* sp. BCC 1067 acted synergistically with caspofungin (CSP), a cell wall targeting antifungal drug, against *S. cerevisiae*. Combinations provided additive effect and no antagonistic effect was observed (Fig. 1a and Table 1). Combination of 0.025 mg/l CSP with 1.935-500 mg/l crude extract exhibited additive effect in BY4742, while 0.006-0.4 mg/l CSP combined with 500 mg/l crude extract showed additive effect in Δ*pdr5*, which lack of *PDR5* transporter gene. Crude extract contains many compounds that may be insufficient to enhance antifungal activity of CSP. Therefore, 19,20-epoxycytochalasin Q, a compound isolated from crude extract of *Xylaria* sp. BCC 1067, was used to investigate synergistic effect with CSP. Cytochalasin inhibits actin polymerization resulting inhibition of cell proliferation and exerts many biological activities such as anticancer, antimalarial, antimicrobial.[10, 20] Rarely resistance to cytochalasins are found because actin is elementary structure in every organism.[10] It has to be noted that less selective target of cytochalasin can cause undesirable effect during treatment.[10] Therefore, combination therapeutic is a better opportunity to avoid toxic and improve antifungal activity of treatment by cytochalasin. In this study, combination of 19,20-epoxycytochalasin Q and CSP caused synergistic effect against *S. cerevisiae* BY4742 (Fig. 1b and Table 2). 0.25 mg/l CSP combined with 15.625-250 mg/l of 19,20-epoxycytochalasin Q provided FICI value of 0.266-0.5 (Table 2) suggested for synergistic effect. It could enhance antifungal activity of CSP for 4-fold. However, combination of 19,20-epoxycytochalasin Q and CSP could not cause synergistic effect in the Δ*pdr5* strain (Fig. 1b and Table 2). Additive effect was observed in combination of 0.006-0.4 mg/l of CSP and 62.5-12.5 mg/l of 19,20-epoxycytochalasin Q (Table 2). Importantly, there was no antagonistic effect observed.

Pdr5 is one of an importance ABC transporter involved in drug resistance. The Δ*pdr5* strain which lack *PDR5* transporter gene showed better sensitivity to both crude extract and 19,20-epoxycytochalasin Q than wild-type BY4742 (Figure 1 and Table 1,2) suggesting significance role of Pdr5 transporter in resistance to those compounds. This can be supported by study of Berger and co-workers about P-glycoprotein (P-gp) which is human ATP-binding-cassette transporter protein.[21] Pdr5 transporter belongs to ABC family which is same family with P-gp transporter found in mammalian cell.[4] They proposed that eukaryotic cells activates an adaptive defense mechanism against cytochalasin by P- gp transporter.[21] Our results suggesting that 19,20-epoxycytochalasin Q could be one of substrate of Pdr5.

19,20-epoxycytochalasin Q acted synergistically with caspofungin but not in combination with crude extract (Fig. 1 and Table 2,3). This suggested that the efficacy of caspofungin against its target was a prerequisite for observing synergy with 19,20-epoxycytochalasin Q. Moreover, it indicated that antifungal activity of 19,20-epoxycytochalasin Q did not affected by present of Pdr5 drug efflux transporter, when combined with CSP.

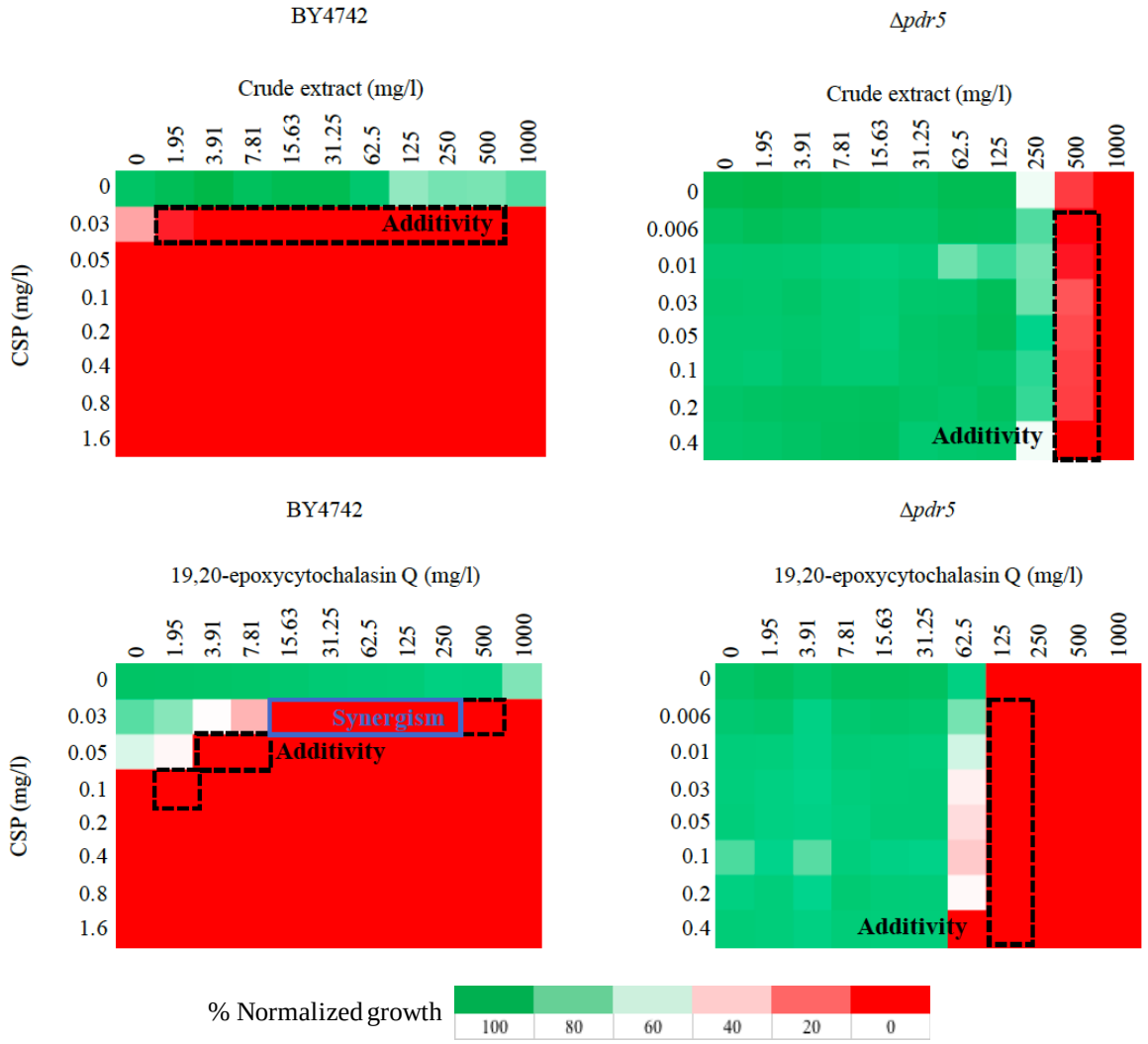

Figure 1. Combined antifungal activity of crude extract of *Xylaria* sp. BCC 1067 (a) and 19,20-epoxycytochalasin Q (b) with caspofungin (CSP) against BY4742 and Δ*pdr5*, represented by heat maps plots of results of checkerboard assay. The color-coding indicated that the closer to red referring the more effective combination.

Table 1. Combined antifungal effect of crude extract of *Xylaria* sp. BCC 1067 with caspofungin (CSP) against *S. cerevisiae* evaluated by FICI model.

Strain	MIC (mg/l)		FIC		FICI	Activity
	CSP	Crude extract	CSP	Crude extract		
BY4742						
Alone	0.05±0.01	>1000±0.02				
Combined	0.025±0.02	1.953±0.05	0.5	0.002	0.502	Add
Δ*pdr5*						
Alone	>0.4±0.09	500±0.07				
Combined	0.006±0.08	500±0.04	0.016	1	1.016	Add

Table 2. Combined antifungal effect of 19,20-epoxycytochalasin Q with caspofungin (CSP) against *S. cerevisiae* evaluated by FICI model.

Strain	MIC (mg/l)		FIC		FICI	Activity
	CSP	19,20-epoxy cytochalasin Q	CSP	19,20-epoxy cytochalasin Q		
BY4742						
Alone	0.1±0.06	>1000±0.01				
Combined	0.025±0.09	15.625±0.10	0.25	0.016	0.266	Syn
	0.5±0.06	3.906±0.12	5.0	0.004	5.004	Add
Δ*pdr5*						
Alone	>0.4±0.02	125±0.05				
Combined	0.4±0.08	62.5±0.03	1	0.5	1.500	Add

According to mode of action of CSP and crude extract as well as 19,20-epoxycytochalasin Q in combination condition, the synergistic effect can be categorized into several groups, including antifungal drugs and drug efflux pump inhibitors, antifungal drugs and drug resistant efflux pump reversers which can increase the entrance and accumulation of antifungal drugs, and antifungal drugs and cell wall or cell membrane disrupting agents which enhance antifungal drugs penetrating the cell barriers. Our previous study reported that cell treated with crude extract of *Xylaria* sp. BCC 1067 show cell membrane damage which involved in cell death.[19] Suggesting that combination of crude extract can enhance the CSP penetrating into cell leading to higher amount of CSP accumulation inside cell and cause antagonistic effect. Moreover, ROS produced by crude extract may cause serious damage to cell which also has stress created cell wall damage from action of CSP.[19, 22] We have shown that 19,20-epoxycytochalasin Q is synergistic with CSP in the wild-type strain and mechanisms of enhancement of CSP activity has not been investigated here. However, it can be proposed that 19,20-epoxycytochalasin Q easily penetrate into cell through perturbation of the cell wall induced by CSP and it generated ROS which lead to cell stress and may decrease cell membrane integrity, rendering it more susceptible to CSP, or might facilitate membrane insertion of CSP. It should be noted that not all membrane-targeting drugs are synergistic with echinocandins. For instance, azoles and polyenes are not generally regarded as synergistic with echinocandins in *Candida* species, although some examples of synergy have been reported.[23-25] It is suggesting that synergistic effect observed in wild-type may interfere function of Pdr5

transporter whose substrate is 19,20-epoxycytochalasin Q leading to accumulation of this compound resulting to inhibition of actin polymerization.

Conclusion

A bioactive compound 19,20-epoxycytochalasin Q isolated from *Xylaria* sp. BCC 1067 showed synergy with caspofungin against *S. cerevisiae* provide synergistic effect which also provide new mechanism of action for invasive fungal infection. Further study based on these findings could produce a broad range of anti-fungal agents suitable for clinical use.

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