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Fungicidal Effect of Resveratrol on Human Infectious Fungi

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Resveratrol, a phenolic antioxidant found in grapes, has been known to mediate various biological activities on the human body. In the present study, we tested the antifungal activity of resveratrol against human pathogenic fungi before carrying out further studies to elucidate the antifungal mechanism(s) of resveratrol. Resveratrol displayed potent antifungal activity against human pathogenic fungi at concentration levels of 10-20 μg/mL. Furthermore, time-kill curve exhibited fungicidal effect of resveratrol on *C. albicans*, but the compound had no hemolytic activity against human erythrocytes. The destruction of *C. albicans* cells by resveratrol was confirmed by scanning electron microscopy. These results suggest that resveratrol could be employed as a therapeutic agent to treat fungal infections of humans.

Key words: Resveratrol, *trans*-3,5,4'-Trihydroxystilbene, Phenolic antioxidant, Fungicidal activity, Hemolytic effect

INTRODUCTION

Resveratrol (*trans*-3,5,4'-trihydroxystilbene), one of the natural phytoalexins found in grapes and spermatophytes, possess cancer chemopreventive activity that inhibits the process of carcinogenesis (Jang *et al.*, 1997). Recently, resveratrol was found to exhibit cancer preventive activity in several animal models. Especially, *in vitro* studies on the effects of resveratrol have proven that resveratrol inhibits proliferations of HL60 leukemia cells (Clement *et al.*, 1998), oral cancer cells (ElAttar *et al.*, 1999), and prostate cancer cell lines (Hsieh *et al.*, 1999). Because of the presence of significant amount of resveratrol in red wine and based on the studies on human and animal models, it has been established that consumption of red wine is beneficial; as resveratrol possess anti-inflammatory activity (Rotondo *et al.*, 1998) and brings about inhibition of platelet aggregation (Pace-Asciak *et al.*, 1995). Moreover, a recent study that revealed a direct correlation of reduced mortality to breast cancer and heart disease (Gronbaek *et al.*, 1995) also supported the contention that consumption of red wine containing high levels of resveratrol is beneficial.

Recently, based on the anticarcinogenic functions of resveratrol, the molecular mechanism(s) by which resveratrol displays various biological effects (Yoon *et al.*, 2002) and antibacterial activities on human pathogenic bacteria (Daroch *et al.*, 2001; Tegos *et al.*, 2002) have also been postulated to some extent. However, though antibacterial activities and various biological activities of resveratrol are known, the effects and mechanism of resveratrol on human infectious fungi that is another cause of human diseases still remains to be mostly unknown.

In this paper, we suggest that resveratrol has a potential to serve as an antifungal agent against fungal infections in humans prior to any further studies on the mechanism(s) by which resveratrol exerts its antifungal effects.

MATERIALS AND METHODS

Materials preparation and fungal strains

Resveratrol (R5010) and amphotericin B (A4888) were purchased from Sigma. Stock solutions of resveratrol and amphotericin B were prepared in dimethyl sulfoxide (DMSO) and stored at -20°C. *Aspergillus flavus* (KCTC 1375), *Saccharomyces cerevisiae* (KCTC 7296) and *Trichosporon beigelii* (KCTC 7707) were obtained from the Korean Collection for Type Cultures (KCTC), at the

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Korea Research Institute of Bioscience Biotechnology (KRIBB), Taejon, Korea. *Candida albicans* (TIMM 1768) was obtained from the Center for Academic Societies, Osaka, Japan.

Antifungal activity assay

The fungal cells were seeded on 96-well microtiter plates at a density of 2×10^3 cells per well in 100 μL of YPD (Dextrose 2%, Peptone 1%, Yeast extract 0.5%) media. Ten microliters of the serially-diluted resveratrol or amphotericin B (used as a positive control) were added to each well and incubated at 28°C for 18 h. After incubation of cell suspension, 5 μL of 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2*H*-tetrazolium bromide (MTT) solution [5 mg/mL MTT in phosphate-buffered saline (PBS), pH 7.4] was added to each well and the plates were further incubated at 37°C for 3 h. The absorbance of each well was measured at 580 nm by a microtiter ELISA plate reader (Molecular Devices Emax, California, USA) (Jahn *et al.*, 1995).

Antifungal time-kill assay

Time-kill plots of the effect of resveratrol on the fungal survival were evaluated with *C. albicans*. Fungal cells (2×10^3 CFU/mL) were incubated with 20 μg of resveratrol and 5 μg of amphotericin B, which was used as a positive control. Aliquots obtained at fixed time intervals were appropriately diluted and spread on YPD agar plate, and then colony counts were made after incubating the plates at 28°C for 18 h (Klepser *et al.*, 1998).

Hemolytic activity

The hemolytic activity of resveratrol was evaluated by a microtiter ELISA plate reader by determining the release of hemoglobin (at 414 nm) from 4% suspension of fresh human erythrocytes. The percentage of hemolysis was calculated by employing the equation: % hemolysis = [($Abs_{414\ nm}$ in the resveratrol solution – $Abs_{414\ nm}$ in PBS) / ($Abs_{414\ nm}$ in 0.1% Triton-X100 – $Abs_{414\ nm}$ in PBS)] × 100.

Morphological changes observed by scanning electron microscopy after resveratrol treatment

Subcultured *C. albicans* cells were incubated in YPD media at 28°C for 4 h in the presence of 20 μg/mL resveratrol. Negative control was run in the absence of resveratrol. The cells were fixed with an equal volume of 4% glutaraldehyde and 1% paraformaldehyde in 0.05 M cacodylate buffer (pH 7.2). The samples were dehydrated with a graded ethanol series (50%, 70%, 90%, 95%, and 100% ethanol). After lyophilization and gold coating, the samples were examined on a HITACHI S-2400 (Tokyo, Japan) (Lee *et al.*, 2002).

RESULTS AND DISCUSSION

Resveratrol, a phenolic substance present in grapes and other spermatophytes and which also functions as a natural chemopreventive agent, exist in nature as two geometrical isomers, *trans* and *cis*. Usually, *trans*-resveratrol (Fig. 1) is more predominant than *cis*-form (Filip *et al.*, 2003). Resveratrol was detected as a main bioactive component in plant extracts that have been used for centuries in traditional medicine to treat human diseases. Although there are many studies on biological activities of resveratrol, the effects and mechanisms of resveratrol on human infectious fungi remain mostly unknown.

In the present study, to investigate the antifungal effect of resveratrol against human infectious fungi, the antifungal activity was measured by MTT assay. The antifungal activity of resveratrol in comparison with amphotericin B (used as a positive control), are epitomized in Table I. For nearly 50 years, amphotericin B has been employed as a potent fungicidal agent to treat serious systemic infections. However, the use of amphotericin B is limited because of high toxicity to patient such as in bringing about hemolytic effect (Adams *et al.*, 2003). Amphotericin B showed antifungal activity at 5-10 μg/mL, and resveratrol mediated antifungal activity at 10-20 μg/mL against tested fungal strains. Resveratrol was potent to the same extent as amphotericin B against *S. cerevisiae* and *T. beigelii* but less potent than amphotericin B against *C. albicans*. Thus, antifungal effect of resveratrol suggests that resveratrol has a potential to function as an antifungal agent against

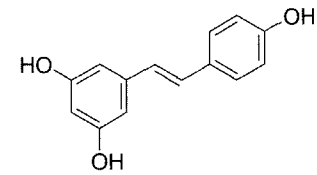


Fig. 1. Chemical structure of resveratrol (*trans*-3,5,4'-trihydroxystilbene)

Table I. Antifungal activities of resveratrol against various fungi

	MIC (μg/mL)		
	C. albicans	*S. cerevisiae*	*T. beigelii*
Resveratrol	20	10-20	10
Amphotericin B	5	10	5-10

The fungal strains were grown at 28°C in YPD medium. The fungal cells were seeded on the wells of a 96-microtiter plate of YPD media at a density of 2×10^3 cells (100 mL per well). The serially diluted compound solutions of resveratrol and amphotericin B were added to each well, and the cell suspension was incubated at 28°C for 18 h. Five μL of MTT solution was added to each well, and the plates were incubated at 37°C for 3 h. The absorbance of each well was measured at 580 nm using a microtiter ELISA reader (Molecular Devices Emax).

systemic infections with similar functions as amphotericin B. In addition, we also performed agar hole assay (Lee *et al.*, 2001) to confirm the antifungal effect of resveratrol on filamentous fungi (Fig. 2). *A. flavus*, one of causes of aspergillosis, was treated with resveratrol on YPD agar plate. Resveratrol was found to bring about inhibition of filament growth at the circle of center, rather than at the rim of the plate. This result demonstrates that resveratrol also has a possibility to serve as an antifungal agent on human infectious molds.

Moreover, resveratrol showed potent fungicidal activity as confirmed by antifungal time-kill assay against *C. albicans*, which is a significant opportunistic pathogen of humans. As in the case of amphotericin B, colony forming units (CFU) of *C. albicans* cells decreased rapidly within 8 h of treatment with resveratrol. The result showed that resveratrol affected the growth of fungal cells by fungicidal actions, and not by fungistatic actions.

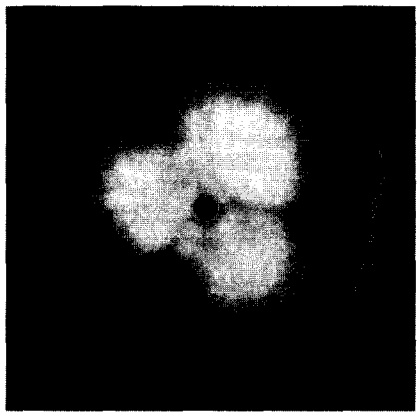


Fig. 2. Agar hole assay for antifungal effect of resveratrol against *A. flavus*. Resveratrol (0.5 mg/50 μL) was located at the center of a *A. flavus* mycelium disk on the 1.5% YPD agar plate. The plate was incubated at 28°C for 4 days.

Many antifungal agents have been limited clinically, as they bring about various toxicities to patients like hemolytic effect on human erythrocytes. We tested the effect of resveratrol on human red blood cells (RBCs) by determining the percentage hemolysis of RBCs. Though amphotericin B showed potent hemolytic activity, resveratrol exhibited no hemolytic activity against human erythrocytes (Fig. 4). These results further suggested that resveratrol has potent fungicidal activity against human pathogenic fungi with no drastic effect on human erythrocytes.

In order to confirm that fungicidal effect of resveratrol does brings about morphological changes in fungal cells, we examined the morphology of *C. albicans* cells in the presence of resveratrol by scanning electron microscopy (SEM). As shown in Fig. 5, *C. albicans* cells in the absence of resveratrol had regular and sleek shape, same as the natural shape of yeast (Fig. 5A). In contrast, after 4 h of treatment with resveratrol, the shape of *C. albicans* cells changed ruggedly and was destroyed gradually (Fig. 5B). The SEM observations provide the sufficient morphological evidence that resveratrol possess fungicidal activity against *C. albicans* cells.

In conclusion, resveratrol is an excellent candidate to be used as a potential antifungal agent against fungal

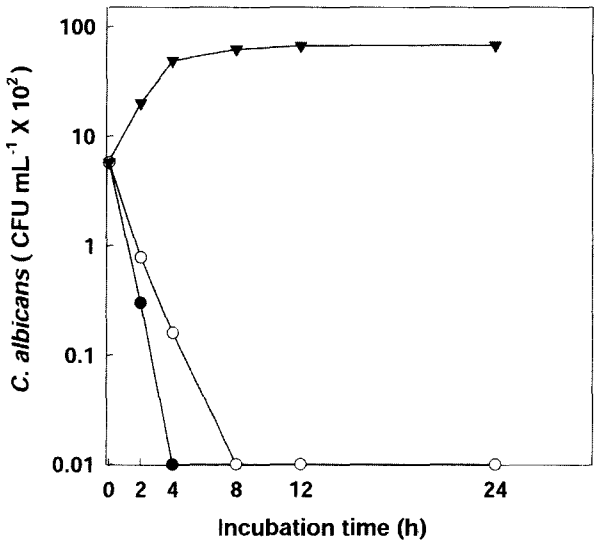


Fig. 3. Time killing plots for *C. albicans* by resveratrol. The kinetics of fungal killing of resveratrol was evaluated by using *C. albicans* as the host fungi. Log-phase fungal cells (2×10^3 CFU/mL) were incubated with 20 μg of resveratrol and 5 μg of amphotericin B (positive control). Symbols denote: ▼; not treated, ○; treated with resveratrol, ●; treated with amphotericin B.

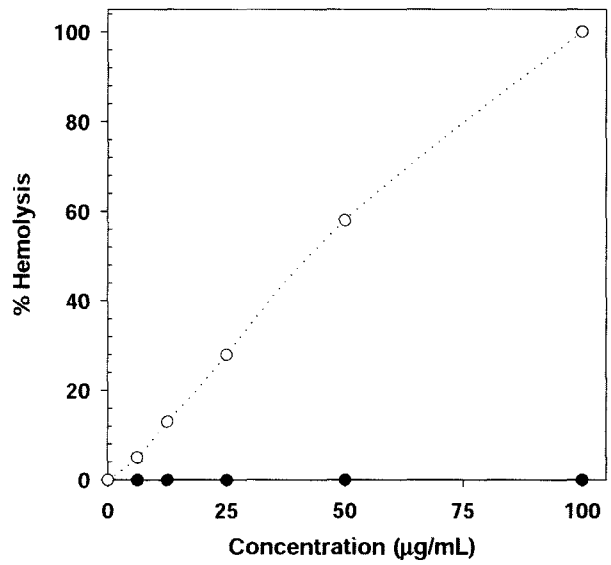
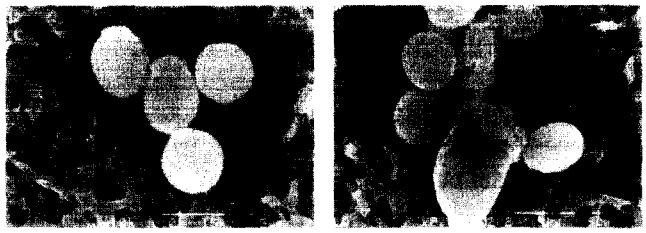


Fig. 4. Hemolytic activity of resveratrol. Hemolytic activity of resveratrol was evaluated by determining the hemoglobin release of 4% suspensions of fresh human erythrocytes at 414 nm. Symbols denote: ●; in the presence of resveratrol, ○; in the presence of amphotericin B.



A B

Fig. 5. Scanning electron microscopic pictures of: (A) untreated *C. albicans* and after treatment at 28°C for 4 h, (B) with 20 μg of resveratrol.

systemic infections, without bringing about hemolysis of human erythrocytes, and also possess other biological activities as it is derived from grapes.

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