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# Candicidal Action of Resveratrol Isolated from Grapes on Human Pathogenic Yeast *C. albicans*

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**Abstract** Resveratrol (3,5,4'-trihydroxystilbene) is a naturally occurring, multi-biofunctional chemical existing in grapes and various other plants as a polyphenol type, and it is one of the best known natural anticancer and antiatherosclerosis reagents. In this study, we investigated the antifungal action by resveratrol in *Candida albicans*, which is a human infectious fungi as an agent of candidiasis. Resveratrol displayed potent fungicidal activity in an energy-dependent manner, without any hemolytic effects against human erythrocytes. It was found that the serum-induced mycelial forms, which play a crucial role in the pathogenesis of *C. albicans* during host tissue invasion, were disrupted by resveratrol. To understand the correlation between lethal effects and resveratrol action, we examined the physiological changes of *C. albicans*. A significant accumulation of intracellular trehalose was induced by stress responses to resveratrol action, and a remarkable arrest of cell-cycle processes at the S-phase in *C. albicans* occured. Therefore, the fungicidal effects of resveratrol demonstrate that this compound is a potential candidate as an antifungal agent in treating infectious diseases by candidal infections.

**Keywords:** Resveratrol, *trans*-3,5,4'-trihydroxystilbene, phenolic antioxidant, fungicidal activity, anticandidal activity, *Candida albicans*

Resveratrol (3,5,4'-trihydroxystilbene) is the parent compound of polymers, existing in *trans* configurations predominantly in a narrow range of spermatophytes of which grapevines, peanuts, and pines are the prime representatives [9, 31]. Resveratrol is contained in high concentration levels of about 0.1–2 mg/kg in red grapes and is more predominant in red wines than in white wines because it exists mainly in the grape skin [18]. It explains the benefits of red wine consumption that have been attributed to resveratrol in human and animal models, such benefits including anti-inflammatory activity [27] and the inhibition of platelet aggregation [25]. Moreover, recent studies have revealed a direct correlation between the consumption of red wine and a reduction in mortality due to breast cancer and heart disease causes [10]. In addition to its anticarcinogenic functions, the molecular mechanism(s) by which resveratrol displays various biological effects have also emerged [33, 34].

Resveratrol has been identified as a main bioactive component in plant extracts, which have been used for centuries in traditional medicine in treating human diseases. Recently, many studies have reported on cancer-prevention activity in animal cancer models [14], and it is expected that more studies are in progress. In particular, *in vitro* studies regarding the biological effects of resveratrol show that it inhibits the proliferation of HL60 leukemia cells [4], oral cancer cells [8], and prostate cancer cell lines [12]. Although there are some studies regarding the antifungal activities of resveratrol against phytopathogenic fungi [30, 35], the action of resveratrol toward candidal infection remains mostly unknown.

Candidiasis is the fourth most common cause of hospital-acquired infections and is the cause of significant morbidity and mortality in immunocompromised patients. There are several *Candida* species that cause candidiasis, but the majority of candidiasis is still caused by *C. albicans* [32]. *Candida albicans* is a dimorphic fungus that causes severe opportunistic infections in commensal animals including humans, and it colonizes on the mucosal surface of the oral and vaginal cavities and in the digestive tract [24]. The various properties of *C. albicans*, including those above, make it a major model of pathogenic yeast.

In this paper, we suggest that resveratrol has potential as an antifungal agent regarding candidal infections in humans. This will be demonstrated through an *in vitro* study, in which it exerts its antifungal effects against *C. albicans*.

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## MATERIALS AND METHODS

### Material Preparation and Fungal Strains

Amphotericin B (A4888) was purchased from Sigma, and stock solutions of amphotericin B were prepared in dimethyl sulfoxide (DMSO) and kept at −20°C. *Candida albicans* (TIMM 1768) was obtained from the Center for Academic Societies, Osaka, Japan.

### The Isolation of *Trans*-Resveratrol from Grapes

About 600 g of grape skins were detached from 2.0 kg of UV-irradiated grapes. The separated grape skins were extracted with ethyl acetate twice, and the extracts were evaporated to dryness in a vacuum, at a temperature lower than 30°C, in order to yield 4.5 g of reddish crude powder with hygroscopicity. This crude product was chromatographed on to a silica gel column, using hexane:EtOAc (3:1→1:1) as an elution solvent, to provide 12 mg of resveratrol (Fig. 1) as a white powder. $^1$H-NMR ($CDCl_3$) δ 6.15 (1H, t, J=2.4 Hz), 6.44 (2H, d, J=2.4 Hz), 6.75 (2H, d, J=8.7 Hz), 6.78 (1H, d, J=16.2 Hz), 6.95 (1H, d, J=16.2 Hz), 7.34 (2H, d, J=8.7 Hz). $^1$H-NMR data were identical to those reported in the literature [5].

### Antifungal Time-Kill Assay

The time-kill plots of the antifungal effects of resveratrol were evaluated with *C. albicans*. Fungal cells were seeded on to 96-well microtiter plates, at a density of $2\times10^3$ cells per well, in 100 μl of a YPD (Dextrose 2%, Peptone 1%, Yeast extract 0.5%) medium. Ten μl of the serially diluted compound solution were added to each well and incubated for 18 h at 28°C. After incubation, an appropriately diluted aliquot was spread on to a 1.5% YPD agar plate at 2 h intervals, and CFUs (colony forming units) were counted following incubation for 18 h at 28°C [19, 33].

### Effects of Resveratrol on Dimorphic Transition

*C. albicans* was maintained by periodic subculturing in a liquid YPD medium. Yeast-cell cultures (blastoconidia) were maintained in a liquid YPD medium at 28°C. To induce hyphal formation, cultures were directly supplemented with a 20% fetal bovine serum. The dimorphic transition in *C. albicans* was investigated from cultures containing various concentration levels of resveratrol, incubated for 48 h at 28°C. The dimorphic transition to hyphal forms was detected by phase-contrast light microscopy (NIKON, ECLIPSETE300, Japan).

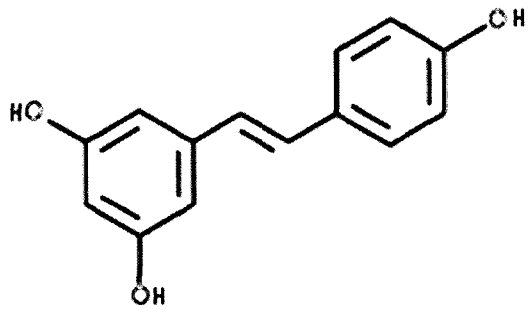


**Fig. 1.** Chemical structure of resveratrol (trans-3,5,4'-trihydroxystilbene).

### Effects of Sodium Azide ($NaN_3$) on Antifungal Activity

To examine the effects of $NaN_3$ on the antifungal activity of resveratrol, cultured *C. albicans* cells, subcultured in a YPD medium, were incubated with 10 μg of resveratrol and serially diluted $NaN_3$ (0.01–0.08%). Antifungal activity, in the presence of $NaN_3$, was described by survival percentage evaluated by a MTT assay [13, 15].

### Hemolytic Activity

The hemolytic activity of resveratrol was evaluated by a microtiter ELISA plate reader. It was done by determining the hemoglobin release from a 4% suspension of fresh human erythrocytes at 414 nm [17]. The percentage of hemolysis was calculated employing the following equation: % hemolysis=[($Abs_{414\,nm}$ in the compound solution−$Abs_{414\,nm}$ in PBS)/($Abs_{414\,nm}$ in 0.1% Triton-X100−$Abs_{414\,nm}$ in PBS)]×100.

### Determination of Accumulated Intracellular Trehalose

*C. albicans* cell suspensions, containing resveratrol or amphotericin B, were incubated for 1 h at 28°C. Fungal cells were settled by centrifugation (8,000 rpm for 20 min), and harvested cells were dried. Three mg (dry weight) of dried fungal cells were destroyed in boiling 0.025 mM potassium-phosphate buffer (pH 6.6), and crude neutral trehalose-containing fractions were extracted by removing the cell debris. Trehalase (Sigma, T8778) stock solution, containing 0.05 units of trehalase, was added to the aliquots containing trehalose. After allowing an enzymatic reaction for 30 min at 37°C, the reaction suspension was mixed with $H_2O$ and a 16% DNS reagent (3,5-dinitrosalicylic acid 1%, NaOH 2%, sodium potassium tartrate 20%) [29]. For a reaction between the glucose and DNS reagent, the mixture was boiled for 5 min and cooled. Color formation was measured at 525 nm.

### Cell-Cycle Analysis

Log-phased cells of *C. albicans* ($1\times10^9$ cells) cultured in a YPD medium were harvested and resuspended with 1 ml of a fresh YPD medium containing 0.1 mg of resveratrol. After incubation for 6 h, the cells were washed with PBS and fixed with 70% ethanol (in PBS) at 4°C overnight [26]. Following cell fixation, the cells were harvested and washed with PBS, and 0.5 ml of a PBS containing 200 μg of RNase A was mixed with the cells and left to react for 2 h at 37°C in a water bath. After the reaction, 0.5 ml of a

PBS containing 50 μg of propidium iodide was added and the samples were incubated for 4 h at 4°C in the dark [7, 22]. Some 15,000 cells were scored.

## RESULTS AND DISCUSSION

### Fungicidal Activity and the Hemolytic Effects on Human Erythrocytes

We have reported the antifungal activity of resveratrol, with that of amphotericin B, determined by a MTT assay to investigate the antifungal effects of resveratrol (Fig. 1) toward human infectious fungi [16]. Resveratrol showed potent antifungal activity at final concentration levels of 10–20 μg/ml against tested fungal strains, and it was a little less potent than amphotericin B having MIC values between 5 and 10 μg/ml. Amphotericin B was used as a positive control and amphipathic polyene macrolide was derived from *Streptomyces nodosus*, a potent fungicidal agent widely used to treat serious systemic infections. In this study, resveratrol showed potent candicidal activity by antifungal time-kill assay against *C. albicans*, which is a significant opportunistic pathogen of humans. As in the case of amphotericin B, the colony forming units (CFU) of *C. albicans* cells, treated with resveratrol, decreased rapidly within 8 h. The results demonstrate that resveratrol affected fungal cell growth *via* fungicidal action in *C. albicans* cells.

On the other hand, many antifungal agents were limited clinically to various toxins that are harmful to humans including the hemolytic effects on human erythrocytes. The use of amphotericin B is also limited because of its high toxicity to humans *via* the hemolytic effect [1]. For this reason, we tested the hemolytic activity of resveratrol on human erythrocytes by determining the hemolysis percentage of human red blood cells (hRBCs) (Table 1). Whereas amphotericin B showed potent hemolytic effects, resveratrol exhibited no hemolytic effects to hRBCs up to 100 μg/ml of concentration. With several studies reporting various biological effects in humans, these results suggest that resveratrol has potential as a fungicidal agent toward systemic infections of *C. albicans*.

**Table 1.** Hemolytic activity of resveratrol against human erythrocyte cells.

| | % Hemolysis (μg/ml) | | | | |
|---|---|---|---|---|---|
| | 100 | 50 | 25 | 12.5 | 6.25 |
| Resveratrol | 0 | 0 | 0 | 0 | 0 |
| Amphotericin B | 100 | 58 | 28 | 13 | 5 |

The hemolytic activity of resveratrol was evaluated by determining the hemoglobin release of 4% suspensions of fresh human erythrocytes at 414 nm. The hemolysis percentage was calculated using the following equation: % hemolysis=[($Abs_{414\,nm}$ in the compound solution−$Abs_{414\,nm}$ in PBS)/($Abs_{414\,nm}$ in 0.1% Triton X-100−$Abs_{414\,nm}$ in PBS)]×100.

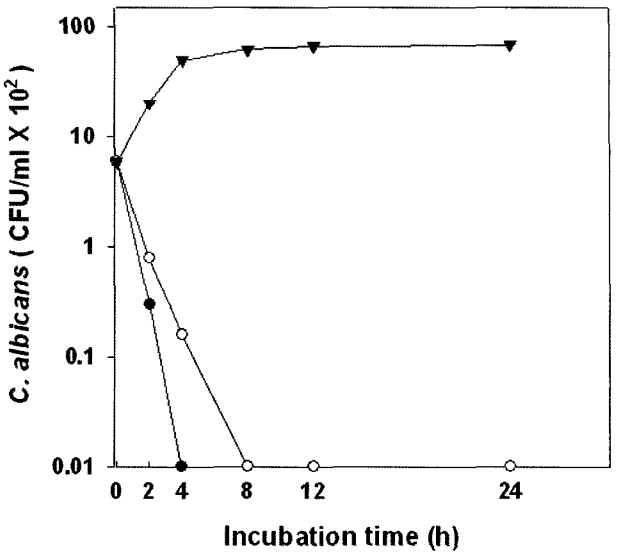


**Fig. 2.** Time-killing plots for *C. albicans* by resveratrol.
The kinetics of fungal killing of resveratrol was evaluated using *C. albicans*. Log-phase fungal cells ($2\times10^3$ CFU/ml) were incubated with 20 μg/ml resveratrol or 5 μg/ml amphotericin B. Symbols: ▼, not treated; ○, resveratrol; ●, amphotericin B.

### Effects of Resveratrol on the Dimorphic Transition of *C. albicans*

The ability of some fungal strain(s) in undergoing a morphological transition between unicellular forms and hyphae structures may be considered as a simple model of cellular development. *C. albicans* is a prototypic dimorphic yeast and this diploid pathogen is of increasing importance in human medicine. In *C. albicans*, dimorphism plays a crucial role in pathogenesis, with mycelial shapes being predominantly found during host tissue invasion [23]. A variety of protocols designed to induce dimorphic transition in *C. albicans* have been reported [6, 2]. To induce filamentation, cultures were directly supplemented with a fetal bovine serum (FBS) [21]. To investigate the effects of the compound on the dimorphic transition of *C. albicans*, this transition was examined in cultures containing the MIC concentration of resveratrol, which was incubated with FBS for 48 h at 37°C. Fig. 3 shows that resveratrol destroyed the hyphal forms of the MIC concentration, indicating that the compound could cause a disruption of the serum-induced filamentous form of *C. albicans*.

### Effects of Sodium Azide on Fungicidal Activity

$NaN_3$ has been known to inhibit the ATP synthesis of mitochondria and ATP-dependent transport systems such as endocytosis [11, 28]. To determine whether the fungicidal activity was dependent on the cellular metabolic ability of *C. albicans* cells, the fungicidal activity in the presence of sodium azide ($NaN_3$) was examined with *C. albicans*. By increasing the $NaN_3$ concentration, the fungicidal activity

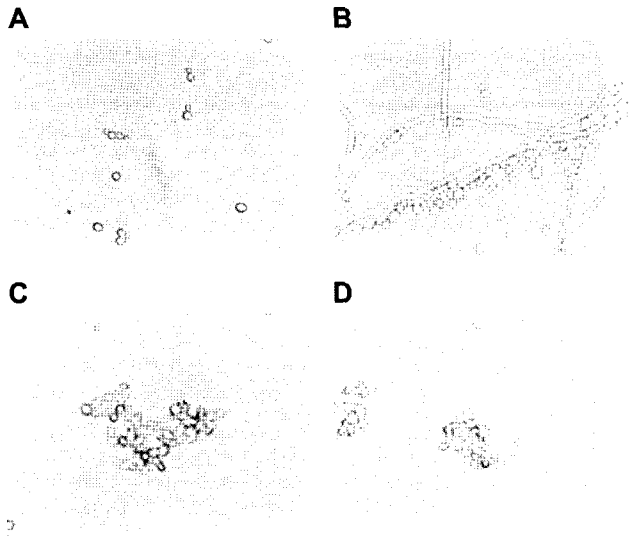


**Fig. 3.** The effect of resveratrol on the dimorphic transition in *C. albicans*.
Each culture was incubated with various concentrations (20, 40 μg/ml) of resveratrol for 48 h in YPD medium with 20% FBS: (**A**) yeast control with no 20% FBS and resveratrol, (**B**) hyphae control with no treated resveratrol, (**C**) with 20 μg of resveratrol, and (**D**) with 40 μg of resveratrol.

of resveratrol was reduced, and consequently these factors were related to an increase in the survival percentage of *C. albicans* cells. The results demonstrate that the fungicidal effects of resveratrol on *C. albicans* cells are in an energy-dependent manner that was inhibited by $NaN_3$ as a metabolic inhibitor (Fig. 4). Moreover, it is believed that the antifungal activity of resveratrol correlates with certain ATP-dependent processes, such as transport systems or enzymatic processes.

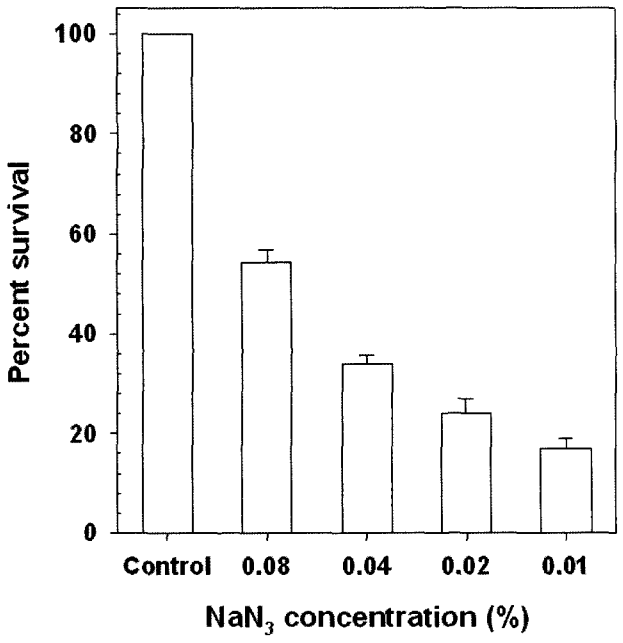


**Fig. 4.** The effect of sodium azide ($NaN_3$) on resveratrol.
Resveratrol was added a final concentration of 10 μg/ml, and the serially diluted $NaN_3$ solutions as a metabolic inhibitor were added for the energy-dependent test.

### Effects of Resveratrol as a Drug Stress Factor on *C. albicans*

In yeast and bacteria, trehalose (α-D-glucopyranosyl-1, 1-α-D-glucopyranoside) is accumulated owing to environmental stresses such as heat, desiccation, freezing temperatures, and toxic agents [3]. To determine the amounts of intracellular trehalose accumulated by resveratrol, the amount of glucose degraded by trehalase was estimated by a dinitrosalicylic acid (DNS) reagent. The glucose concentration significantly increased in *C. albicans* cells incubated with resveratrol when compared with the negative control cells (Fig. 5). An increase in glucose concentration was thought to reveal an increase of intracellular trehalose by resveratrol, as a toxic agent on *C. albicans* cells. These results indicate that resveratrol has an effect on *C. albicans* cells as a drug stress factor, which induces the accumulation of intracellular trehalose.

### Arrest of the *C. albicans* Cell Cycle by Resveratrol

To further investigate the effects of resveratrol on physiological conditions, the distribution of the cell cycle in each phase was analyzed by determining the DNA content with flow cytometry (Fig. 6). In determining the DNA content of *C. albicans* cells, DNA was stained with propidium iodide (PI), which is a DNA-intercalating dye [20]. Whereas the number of fungal cells in the G1- and G2/M-phase was significantly reduced by about 13% and 12%,

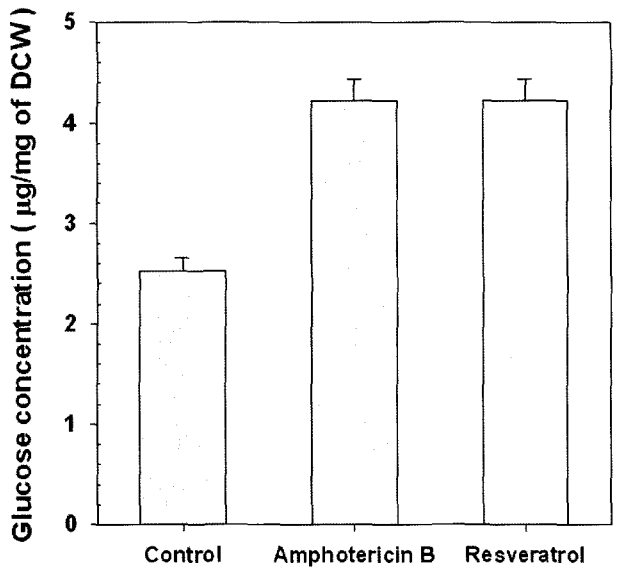


**Fig. 5.** Trehalose assay after addition of resveratrol and amphotericin B used as a positive control.
Subcultured *C. albicans* cells treated with 200 μg of resveratrol or 50 μg of amphotericin B were incubated at 28°C for 1 h.

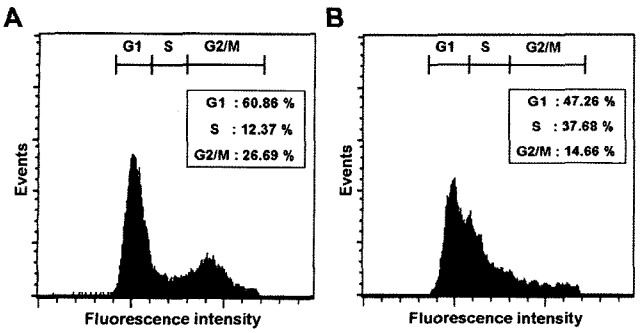


**Fig. 6.** The effect of resveratrol on the cell-cycle process of *C. albicans*.

Subcultured fungal cells ($1\times10^9$ cells) were treated with resveratrol and incubated for 6 h. After washing the cells with PBS, the cells were fixed in 70% ethanol (in PBS, v/v) for 12 h, and then stained with 50 μg of propidium iodide. (**A**) Not treated, (**B**) treated with 100 μg of resveratrol after 6 h.

respectively, when comparing with control cells (G1= 60.86%, S=12.37%, G2/M=26.69%), the number of fungal cells in the S-phase increased by about 25% after incubating with 100 μg of resveratrol for 6 h. This cell-cycle arrest resulted from a metabolic inhibition, which was induced by the activity of resveratrol that could lead to fungal cell death. It is thought that the cell-cycle arrest in S-phase by application of resveratrol is related to the effects on regular cellular metabolism.

Resveratrol has been extensively studied as an anticancer material that can inhibit various human cancer cells. In the previous study, we reported the antifungal effect of resveratrol against human pathogenic fungi. To understand the antifungal action of resveratrol, we isolated resveratrol from the ethyl acetate extracts of grape skins, and investigated their antifungal action toward *C. albicans*, which is a significant opportunistic pathogen causing candidiasis. Resveratrol showed potent candicidal activity that was mediated by the cell entry through the transporting system that needs ATP consumption. The action of resveratrol as a toxic agent to the fungal cell induced some intracellular physiological changes, which were exhibited by trehalose accumulation and cell-cycle arrest. To overcome a threat from various microbial pathogens emerging recently, we need a novel antimicrobial agent that is harmless to the human body, to protect against these pathogens. Therefore, it is thought that resveratrol may be an excellent agent in preventing or treating fungal infections in the human body.

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