

Novel Antifungal Mechanism of Resveratrol: Apoptosis Inducer in *Candida albicans*

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Abstract Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) is a well-known natural polyphenolic compound that has garnered considerable interest because of its bioavailability and pharmacokinetics in humans. Although the antimicrobial activity of resveratrol has recently been focused, however, the antifungal activity and its mechanism are still largely unknown. Here, we report for the first time the potential of resveratrol as an apoptosis inducer in the human pathogenic fungus *Candida albicans*. The results showed that resveratrol exerted its effects from the early to the late stages of apoptosis and involved the activity of reactive oxygen species, particularly hydroxyl radicals ($\cdot\text{OH}$). DiOC₆(3) and JC-1 staining indicated that loss of mitochondrial membrane potential ($\Delta\Psi_m$) is a key event in resveratrol-induced apoptosis. Finally, we investigated metacaspase activation resulting from mitochondrial dysfunction. The result showed that resveratrol strongly activated metacaspase and promoted cytochrome *c* release. In summary, resveratrol induces fungal apoptosis through a caspase-dependent mitochondrial pathway and is a potential agent for treating human fungal diseases.

Introduction

Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) is a type of natural phenolic compounds found in grapes, peanuts, and

berries [4, 28, 30]. It was first derived from the dried powdered roots of Itadori tea, *Polygonum cuspidatum*, which is traditionally used in Asia, especially Japan and China, as an active ingredient for the treatment of human fungal, inflammatory, hypertensive, allergic, and lipid diseases [2, 17, 18, 26]. Furthermore, resveratrol has been extensively investigated for its biological activities, such as antioxidant, anticancer, anti-inflammatory and antimicrobial activities. Jang et al. suggested that resveratrol exerts potent cancer chemopreventive effects in the major stages of carcinogenesis: (1) anti-initiation activity, (2) anti-promotion activity, and (3) anti-progression activity [15]. Resveratrol also showed protective effects against oxidative stress and inflammation by substantially reducing reactive oxygen species (ROS) generation. This finding was confirmed by the decreased expression of p47phox, NF κ B, JNK-1, PTP-1B and SOCS-3 in peripheral blood mononuclear cells (PBMC) [11]. Furthermore, resveratrol inhibited the expression of TNF- α , IL-6 and C-reactive protein [11]. Other studies showed that resveratrol had antimicrobial activities against both bacterial strains, such as *Staphylococcus aureus*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa*, and fungal strains, such as *Trichophyton mentagrophytes*, *Trichophyton tonsurans*, *Trichophyton rubrum*, *Epidermophyton floccosum*, and *Microsporium gypseum* [5].

Candida albicans is a dimorphic fungal pathogen in humans, causing both superficial and life-threatening infections, especially in immunocompromised patients [3, 10]. *C. albicans* can cause infections in humans by (a) epithelial adhesion in the yeast form, (b) yeast-hyphae transition and epithelial penetration, and (c) dissemination and infection [22, 23]. *Candida* species may also cause the biofilm formation in medical devices and implants of patients [6]. Furthermore, *Candida* species has been

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reported to show azole resistance through upregulation of efflux [31], mutations in yeast transcription factor PDR1 [31], mutations or overexpression of ERG11 [21], and respiratory deficiency in mutants [9]. In terms of the activity of resveratrol against *C. albicans*, there is still some controversy. Weber et al. showed resveratrol did not show any significant effects on several *Candida* species [32]. However, the latest report suggested that resveratrol derivatives could affect on *Candida* species [13]. Therefore, to thoroughly examine its antifungal effect, we focused on not only the activity, but also the intracellular mechanism of resveratrol in *C. albicans*. Finally, we suggested the apoptosis-inducing mechanism of the compound.

Materials and Methods

Analysis of Apoptotic Markers

The apoptosis-inducing activity of resveratrol in *C. albicans* was investigated by examining representative apoptotic markers, such as phosphatidylserine exposure, nuclear fragmentation, and DNA fragmentation using annexin V-FITC/PI, DAPI, and TUNEL assays, respectively. Amphotericin B (Sigma-Aldrich, A4888) was used as a positive control. For annexin V-FITC/PI assay, the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen) was used and propidium iodide (PI) was simultaneously stained. To obtain viable protoplasts of *C. albicans* (ATCC 90028), cells were washed in 0.1 M potassium phosphate buffer (pH 6.0), resuspended in buffer containing 1 M sorbitol, and then digested in 20 mg/ml lysing enzyme. The protoplasts (2×10^4 cells/ml) were treated with 20 μ M resveratrol or 10 μ M amphotericin B for 2 h at 28 °C. The cells were washed and resuspended in annexin binding buffer and incubated with 5 μ l/ml annexin V-FITC and PI for 15 min. The cells stained were immediately analyzed with a FACSCalibur flow cytometer (Becton–Dickinson, USA) [19].

Nuclear and DNA fragmentation were examined using DAPI (4',6-diamidino-2-phenylindole) assay and TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) assay, respectively. *C. albicans* cells (2×10^4 cells/ml) were treated with 20 μ M resveratrol or 10 μ M amphotericin B for 3 h at 28 °C. For DAPI staining, the cells were washed, resuspended in PBS and then stained with 1 μ g/ml DAPI for 10 min. For TUNEL assay, the cells were washed with PBS containing 1 M sorbitol and stained with an *In Situ* Cell Death Detection Kit, Fluorescein (Roche Applied Science). The cells were examined under a fluorescence microscope (Nikon ECLIPSE Ti–S, Japan) [19].

ROS and Hydroxyl Radical Assessment

ROS production and hydroxyl radical (OH) accumulation were assessed using DHR 123 (dihydrorhodamine 123) and HPF (2-[6-(4'-hydroxy)phenoxy-3H-xanthen-3-on-9-yl] benzoic acid), respectively. For ROS detection, *C. albicans* (ATCC 90028) cells (2×10^4 cells/ml) were treated with 20 μ M resveratrol or 10 μ M amphotericin B for 2 h at 28 °C. The cells were washed with PBS (pH 7.4) and stained with 5 μ g/ml DHR 123. For OH detection, *C. albicans* (ATCC 90028) cells (2×10^4 cells/ml) were treated with 20 μ M resveratrol or 10 μ M amphotericin B for 2 h at 28 °C, and then incubated in PBS containing 5 μ M HPF. The cells were washed and analyzed using a FACSCalibur flow cytometer (Becton–Dickinson, USA) [19].

Mitochondrial Membrane Potential ($\Delta\Psi_m$) Assay

To examine the changes of mitochondrial membrane potential ($\Delta\Psi_m$) after resveratrol treatment, the cells were stained two mitochondrial dyes, DiOC₆(3) (3,3'-dihexyloxacarbocyanine iodide) and JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolyl carbocyanine chloride) were used. *C. albicans* (ATCC 90028) cells (2×10^4 cells/ml) were treated with 20 μ M resveratrol or 10 μ M amphotericin B for 2 h at 28 °C. The cells were washed in PBS and incubated in staining solution containing 1 μ M DiOC₆(3) or 2 μ g/ml JC-1 for 20 min. The cells were washed in PBS and analyzed using a FACSCalibur flow cytometer (Becton–Dickinson, USA) [19].

Mitochondrial Ca²⁺, Metacaspase Activation and Cytochrome *c* Release Assay

To assess mitochondrial Ca²⁺, *C. albicans* (ATCC 90028) cells (2×10^4 cells/ml) were treated with 20 μ M resveratrol or 10 μ M amphotericin B for 2 h at 28 °C. The cells were washed with Krebs buffer (pH 7.2) and treated with 0.01 % Pluronic F127 (Molecular Probes) and 1 % BSA. Then, the cells were treated with 10 μ M Rhod-2AM (Molecular Probes) and incubated at 28 °C for 30 min. The cells were washed in calcium-free Krebs buffer (pH 7.2). Finally, the fluorescence intensity of Rhod-2AM was detected with a spectrofluorophotometer (Shimadzu, RF-5301PC) [1].

To examine cytochrome *c* release, mitochondria were isolated from *C. albicans* (ATCC 90028) cells [25] and treated with 20 μ M resveratrol or 10 μ M amphotericin B. The cells were then lysed in lysis buffer [150 mM sodium chloride, 1 % Triton X-100, 1 mM EDTA, 1 mM EGTA, and 50 mM Tris (pH 8.0)] and centrifuged at 2,000 \times g for 10 min. The supernatant was further centrifuged at 40,000 \times g for 1 h. The protein concentration was examined

using a NanoVue Plus spectrophotometer (GE Healthcare). Finally, 12 % SDS-PAGE and western blot were performed using rabbit polyclonal anti-yeast cytochrome *c* and HRP-linked goat anti-rabbit immunoglobulin G [19].

Metacaspase activation in *C. albicans* (ATCC 90028) cells was investigated using the CaspACETM FITC-VAD-FMK *In Situ* Marker (Promega). Cells (2×10^4 cells/ml) were treated with 20 μ M resveratrol or 10 μ M amphotericin B for 2 h at 28 °C. The cells were washed with PBS, resuspended in 200 μ l of staining solution containing 10 μ M CaspACETM FITC-VAD-FMK *In Situ* Marker, and incubated for 30 min in the dark. The cells were washed with PBS and fluorescence was detected using fluorescence microscope (Nikon ECLIPSE Ti-S, Japan) [19].

Results and Discussion

Resveratrol Induces Apoptosis in *C. albicans*

Recent research on antifungal drugs has focused on the induction of endogenous programmed cell death of pathogenic fungal strains [14, 19, 27]. For example, a well-known anti-cancer compound amentoflavone, isolated from a traditional herb *Selaginella tamariscina*, showed antifungal

activity through hydroxyl radical generation and mitochondrial dysfunction, followed by caspase activation [14]. Our present study investigated the antifungal activity of resveratrol against *C. albicans*, focusing its apoptotic mechanism. Previously, resveratrol was shown to suppress the proliferation of *C. albicans* cells through cell cycle arrest at the S-phase [16]. However, the exact mechanism was not fully understood. We hypothesized that resveratrol causes apoptosis in *C. albicans* because apoptosis shares some molecular features with cell cycle arrest. In this study, we used amphotericin B, a conventional antifungal drug for systemic diseases, as a positive control. Phillips et al. established the physiology of cell death in *C. albicans* induced by environmental stresses using acetic acid or hydrogen peroxide, and amphotericin B as an antifungal agent [27]. On the basis of their research, we considered amphotericin B to be appropriate for the model of apoptosis used in our study.

Resveratrol showed an MIC of 20 μ M, whereas amphotericin B showed an MIC of 10 μ M (data not shown). We then examined the apoptotic features of the treated cells. The result showed that the protoplasts of *C. albicans*, treated with resveratrol or amphotericin B, displayed an annexin V(+)/PI(−) phenotype (resveratrol, 17.39 %; amphotericin B, 27.26 %) as an early marker of apoptosis (Fig. 1a). Interestingly, the number of annexin

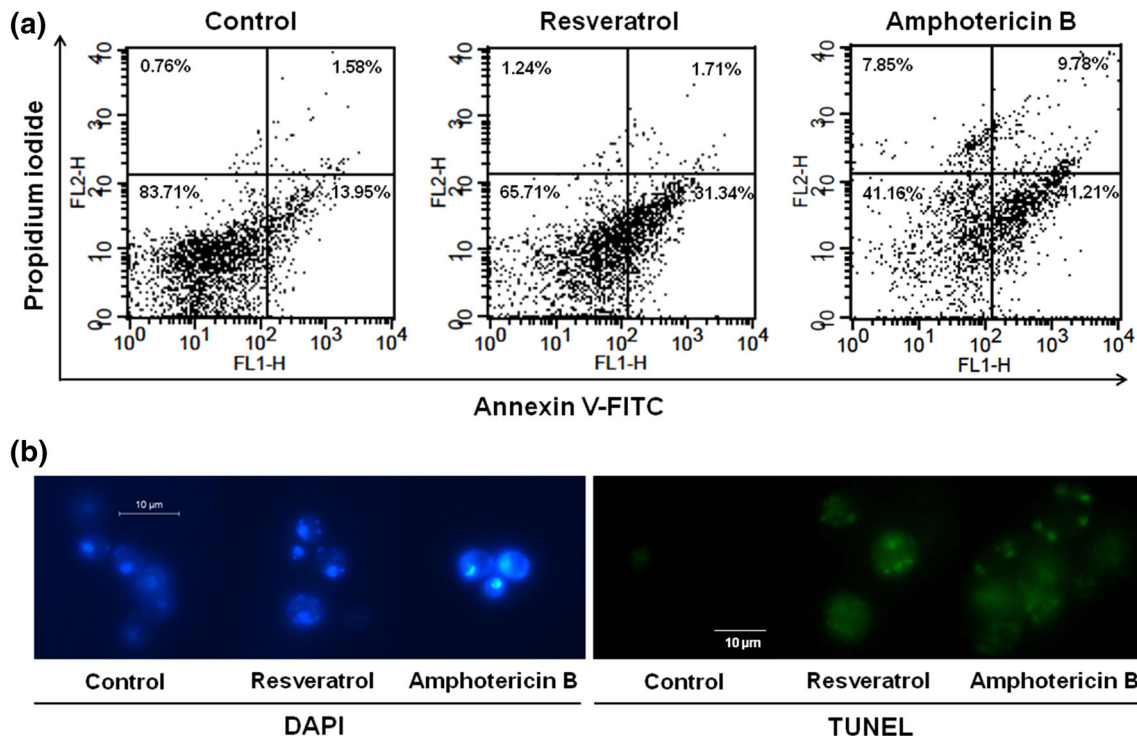


Fig. 1 Phosphatidylserine externalization (a), nuclear and DNA fragmentation (b) in *C. albicans*, induced by resveratrol and amphotericin B. Cells treated with 20 μ M resveratrol or 10 μ M

amphotericin B were incubated for 2 h (annexin V-FITC/PI assay) or 3 h (DAPI and TUNEL assays) at 28 °C

V(-)/PI(+) cells (a necrosis marker) simultaneously increased in amphotericin B-treated cells (7.09 %), whereas this phenomenon was rarely observed in resveratrol-treated cells (0.48 %). This result strongly indicated that resveratrol-induced cell death occurred through apoptosis rather than necrosis. DAPI and TUNEL assays showed nuclear and DNA fragmentation as a late marker of apoptosis (Fig. 1b). Taken together, the results suggested that resveratrol is involved in the entire process of apoptosis in *C. albicans*.

Cells Treated with Resveratrol Produce ROS and Hydroxyl Radicals

ROS are suggested to be the intracellular messengers of apoptosis in *Saccharomyces cerevisiae* [20, 24]. In *Aspergillus nidulans*, intracellular ROS induces cell apoptosis [7]. We therefore assessed whether resveratrol triggers the production of ROS, a key factor in apoptosis, in *C. albicans*. We used DHR 123 because ROS can oxidize it to rhodamine 123, resulting in increased fluorescence intensity [18]. The results showed that resveratrol (31.74 %) as well as amphotericin B (62.08 %) significantly induced endogenous ROS in *C. albicans*, compared to negative control (Fig. 2, upper panels). We further

examined the involvement of $\cdot\text{OH}$, which is a highly active ROS and the most significant mediator among ROS [19]. HPF staining showed that resveratrol treatment induced $\cdot\text{OH}$ production in *C. albicans* (20.53 %) compared to the control, similar to the result obtained with amphotericin B treatment (28.46 %) (Fig. 2, lower panels). These results suggest that resveratrol-induced $\cdot\text{OH}$ production causes an oxidative stress that stimulates mitochondrial dysfunction leading to apoptosis.

Resveratrol Causes Mitochondrial Dysfunction in *C. albicans*

Mitochondria play an important role in the regulation of apoptosis in mammalian cells, such as the release of cytochrome *c* for activating caspase, the disruption of electron transport, the decrease of mitochondrial membrane potential, and the regulation of Bcl-2 [12]. Among the key events in mitochondrial apoptosis, we focused on mitochondrial membrane permeability ($\Delta\Psi_m$) to investigate mitochondrial dysfunction induced by resveratrol. We used two mitochondrial dyes, DiOC₆(3) and JC-1. DiOC₆(3) is a positively charged dye that is selective for normal mitochondria at low concentrations. JC-1 is used specifically to

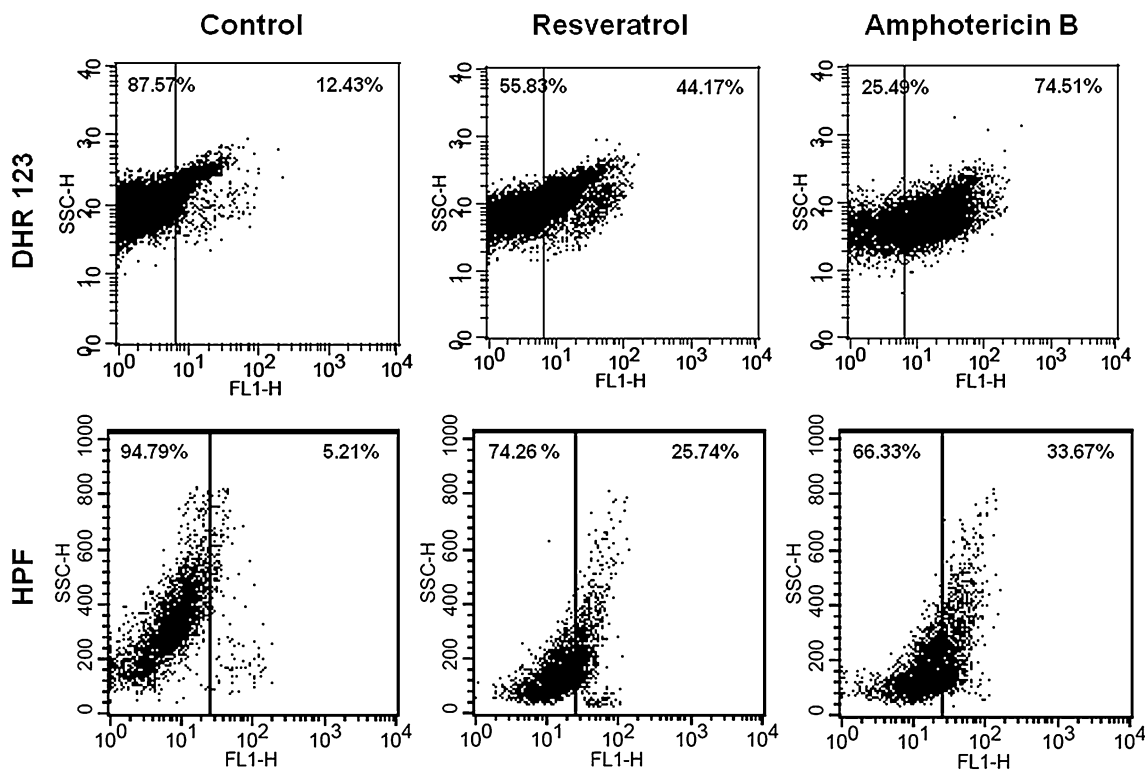


Fig. 2 DHR 123 staining for ROS production (upper panels) and HPF staining for $\cdot\text{OH}$ generation (lower panels) in *C. albicans*, induced by resveratrol and amphotericin B. Cells treated with 20 μM

resveratrol or 10 μM amphotericin B were incubated at 28 $^{\circ}\text{C}$ for 2 h. For staining, 5 $\mu\text{g/ml}$ DHR 123 or 5 μM HPF were used

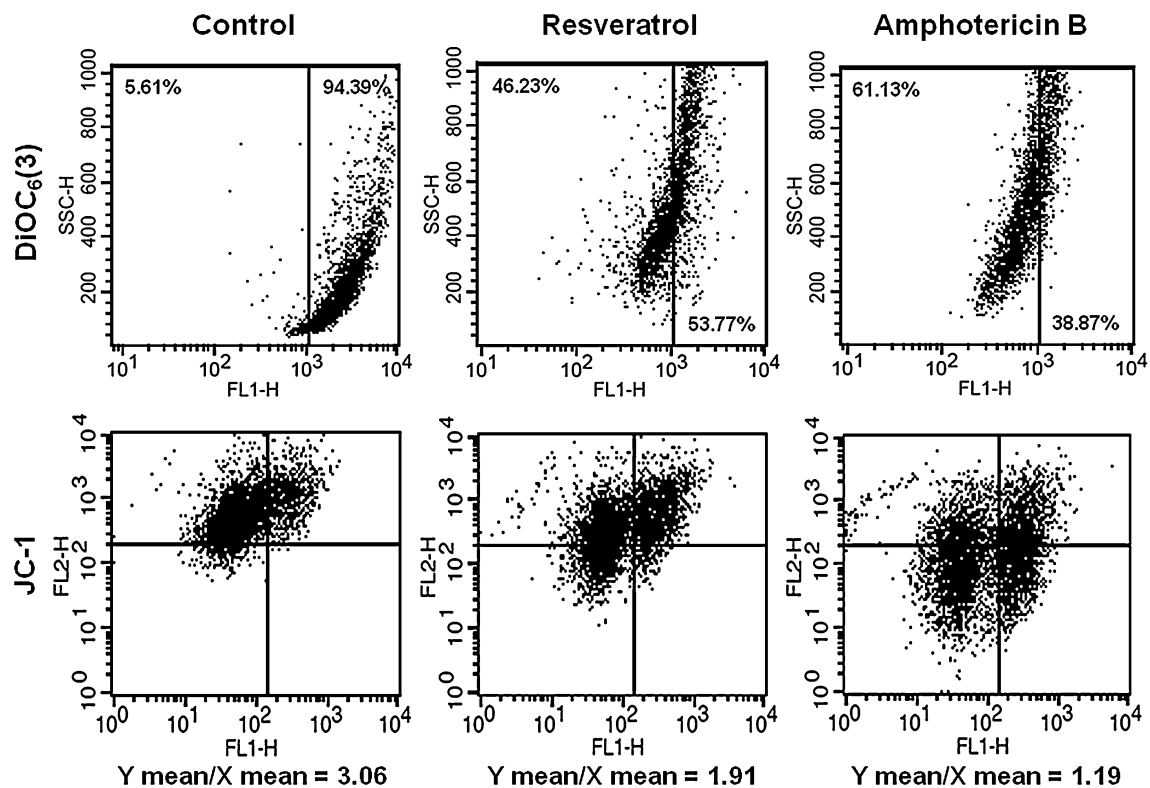


Fig. 3 DiOC₆(3) and JC-1 staining for detection of mitochondrial membrane potential ($\Delta\Psi_m$) changes in *C. albicans*, induced by resveratrol and amphotericin B. Cells treated with 20 μ M resveratrol

or 10 μ M amphotericin B were incubated at 28 $^{\circ}$ C for 2 h. For staining, 1 μ M DiOC₆(3) or 2 μ g/ml JC-1 were used

determine the $\Delta\Psi_m$ [29]; therefore, this dye was additionally used for the characterization of resveratrol-induced mitochondrial damage. The result showed that the uptake of DiOC₆(3) decreased in both resveratrol-treated cells (40.62 %) and amphotericin B-treated cells (55.52 %), compared to the control (5.61 %) (Fig. 3, upper panels). In JC-1 staining, the result further showed that the value of Y mean/X mean was reduced in both resveratrol-treated cells (1.91) and amphotericin B-treated cells (1.19), compared to the control (3.06) (Fig. 3, lower panels). These results indicate that resveratrol causes mitochondrial dysfunction in *C. albicans* by decreasing the $\Delta\Psi_m$.

Resveratrol Induces Apoptosis Through the Caspase-Dependent Pathway

Mitochondrial dysfunction or caspase activation is not universal to apoptosis. In this study, we found that resveratrol-induced fungal apoptosis involving mitochondrial pathways. To further confirm the mitochondrial apoptotic features, we examined the changes in mitochondrial Ca²⁺, using the Rhod-2AM dye. The result showed mitochondrial Ca²⁺ overload in both resveratrol- or

amphotericin B-treated cells confirming the involvement of mitochondria (Fig. 4a). Next, we investigated the caspase dependence of resveratrol's apoptotic effects and examined cytochrome *c* release as an indicator of caspase activation. Using western blot analysis, we found that resveratrol treatment of *C. albicans* induced the release of cytochrome *c* from mitochondria to cytosol (Fig. 4b). Further, treatment with resveratrol strongly induced the activation of metacaspase (green fluorescence) similar to the effect observed with amphotericin B treatment (Fig. 4c).

Previously, Collado-González et al. suggested that resveratrol did not possess critical antifungal and killing effects against *C. albicans* even in high concentration [8]. However, in this study, we focused on the intracellular mechanism of resveratrol in *C. albicans*. As a high dose could not necessarily ensure higher effects, our results suggested that resveratrol of low concentration could penetrate into the cell without fatal cell membrane damage and induce apoptosis in *C. albicans*. Finally, our results suggest the potential of resveratrol as a novel antifungal agent and its use as a positive control in the study of pro-apoptotic compound such as amphotericin B.

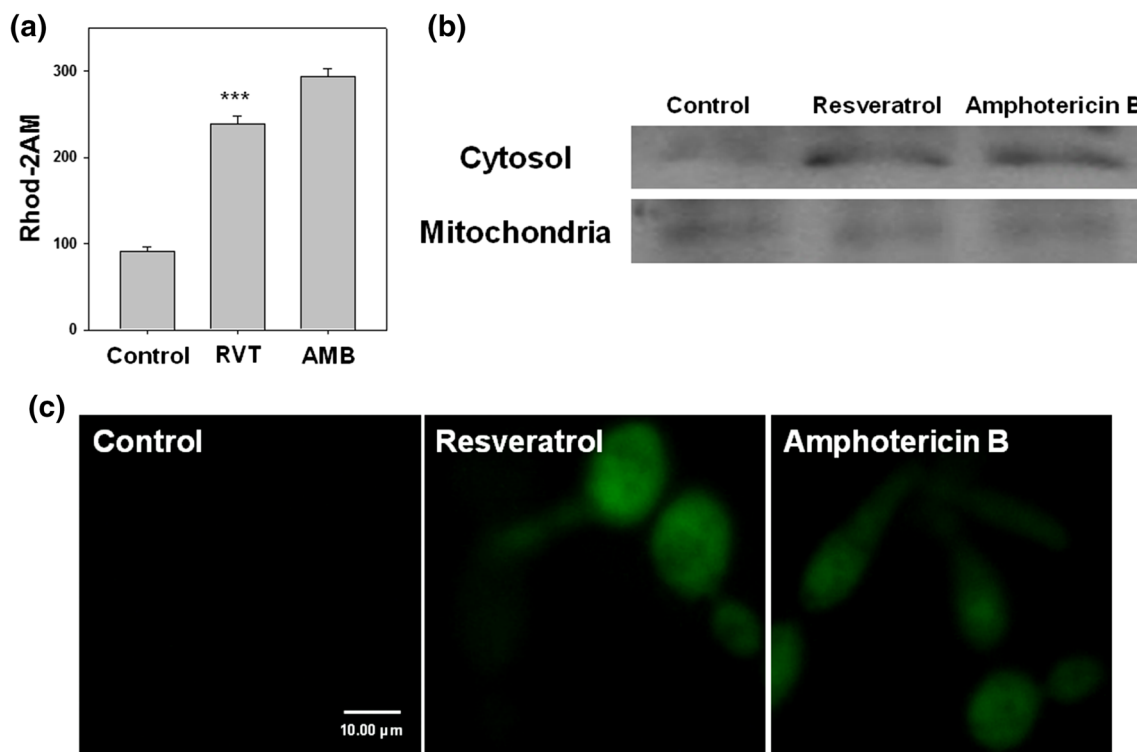


Fig. 4 **a** Mitochondrial Ca^{2+} levels in *C. albicans* after treatment of 20 μM resveratrol (RVT) and 10 μM amphotericin B for 2 h at 28 $^{\circ}\text{C}$, using 10 μM Rhod-2AM dye. Data are presented from three independent experiments using the mean \pm SD. *** $P < 0.001$

(Student's *t* test). **b** Cytochrome *c* release using Western blotting. **c** Metacaspase activation in *C. albicans* after treatment of 20 μM resveratrol and 10 μM amphotericin B for 2 h at 28 $^{\circ}\text{C}$, using 10 μM CaspACETM FITC-VAD-FMK *In Situ* Marker

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