Oxyresveratrol-induced DNA cleavage triggers apoptotic response in Candida albicans

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Abstract

Oxyresveratrol is a naturally occurring phytoalexin produced by plants in response to infection. Biological activities of oxyresveratrol have been studied such as antioxidant, anticancer and anti-inflammatory. However, further antimicrobial activity and its mechanism need to be investigated. This study exhibited growth inhibition against pathogenic fungi and investigated its mode of action. Oxyresveratrol inflicted cleavage on DNA, leading to G₂/M phase arrest. DNA damage by oxyresveratrol was not the result of oxidative stress but it was triggered by direct binding to DNA. Oxyresveratrol-treated cells showed an apoptotic pathway characterized by phosphatidylserine exposure, apoptotic volume decrease and mitocaspase activation. Mitochondria-associated apoptotic features also appeared. Oxyresveratrol-induced Ca²⁺ overload led to mitochondrial membrane depolarization and release of cytochrome c from mitochondria to cytosol. In conclusion, oxyresveratrol with DNA-binding affinity induces DNA cleavage, and eventually leads to mitochondria-mediated apoptosis in Candida albicans.

INTRODUCTION

Candidiasis is a fungal infection caused by yeasts belonging to the genus Candida [1]. The majority of these are caused by Candida albicans [2]. This fungal strain exists widely in yeast form and can be a part of the ordinary human saprophyte as commensal growth [3, 4]. However, C. albicans gains pathogenic potential, facilitated by the reduced immune competence or imbalance of the competing bacterial microflora in humans. These pathogenic fungi cause superficial infections (oral thrush or vaginitis), which are the most common types of candidiasis [5]. The fungi enter the blood stream and colonize major organs, resulting in life-threatening systemic candidiasis [6]. Patients receiving long-term antifungal therapy show a marked increase in the incidence of treatment failures [7]. The indiscriminate use of many conventional drugs causes several problems, such as undesirable side effects, being ineffective against new or re-emerging fungi, and the development of resistance, which markedly affects human health. These threats to human health continuously demand new anti-infectives [8].

Natural compounds are potential candidates for the development of anti-infectives [9]. Plants exposed to various pathogenic micro-organisms adapt to survive in their natural environment [10]. As a defense mechanism, plants produce secondary metabolites called phytoalexins in response to abiotic and biotic stresses [11]. Phytoalexins accumulate at infection sites and inhibit growth in vitro. Therefore, phytoalexins are considered possible defense compounds against diseases [12]. Different types of phytoalexins, such as resveratrol, piceatannol and pinosylvin are present in nature. These compounds affect human health by exerting cardioprotective, antibacterial, antifungal, antioxidative and anti-inflammatory properties [13–17].

Oxyresveratrol is a polyphenolic phytoalexin found in the roots, leaves, stem and fruits of plants belonging to Moraceae, Liliaceae and Gnetaceae families [18]. It possesses various biological activities, such as anti-obesity, anti-inflammatory and neuroprotective properties [19–21]. Moreover, it exerts antimicrobial activities against microorganisms, including methicillin-resistant Staphylococcus aureus and C. albicans, and also exerts anti-viral activity against herpes simplex virus [22–24]. Although the antifungal activity of oxyresveratrol has been reported, the underlying mechanisms have not yet been elucidated. Therefore, we aimed to elucidate the antifungal mechanisms of oxyresveratrol on C. albicans.
METHODS

Material preparation and cell culture condition

Oxyresveratrol (Fig. 1) and H$_2$O$_2$ were purchased from Sigma–Aldrich (St. Louis, MO, USA). C. albicans (ATCC 90028) and Candida parapsilosis (ATCC 22019) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Saccharomyces cerevisiae (KCTC 7296) and Trichosporon beigelii (KCTC 7707) were obtained from the Korean Collection for Type Cultures (KCTC) at the Korea Research Institute of Bioscience and Biotechnology (KRIIB) (Daejeon, Korea). For all experiments, the cells were grown in yeast extract peptone dextrose (YPD) broth (BD Difco, Flanklin Lakes, NJ, USA) under aerobic conditions at 28°C.

Antifungal susceptibility assay

The MIC of oxyresveratrol was determined on C. albicans, C. parapsilosis, S. cerevisiae and T. beigelii. The test was performed using twofold serial dilutions of test compounds according to the Clinical and Laboratory Standards Institute (CLSI) guidelines, with slight modifications [25]. The log-phased cells (1x10$^6$ cells ml$^{-1}$) suspended in YPD broth were inoculated in the wells of microtitre plates (0.1 ml well$^{-1}$) and followed by addition of various concentrations of oxyresveratrol and H$_2$O$_2$. After incubation for 48 h, the growth of fungus was determined by ELISA Reader (Molecular Devices Emax, Sunnyvale, CA, USA).

Haemolytic assay

A fresh human sample at the concentration of 8 % was suspended in PBS. The erythrocyte suspension was transferred to the 96-well plates and incubated with oxyresveratrol at 37°C for 1 h. After centrifugation for 10 min, an aliquot of supernatant was evaluated by determining the release of haemoglobin from the 8 % of the human erythrocytes at 414 nm with ELISA Reader (Molecu-

DNA strand break assay

A terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay was performed using an In Situ Cell Death Detection Kit, Fluorescein (Roche Applied Science, Penzberg, Germany) to evaluate DNA strand break. The addition of labelled dUTPs to the 3’-hydroxyl termini of the DNA is catalysed by terminal deoxynucleotidyl transferase. The log-phased C. albicans cells (1x10$^6$ cells ml$^{-1}$), resuspended in PBS, were treated with oxyresveratrol (2.5, 5.0 and 10.0 µg ml$^{-1}$) or 10 mM H$_2$O$_2$, incubated for 4 h at 28°C. The cells were then fixed with 4 % paraformaldehyde for 1 h. The fixed cells were collected, suspended in PBS, treated with 100 µl of permeabilization solution (0.1 % sodium citrate and 0.1 % Triton X-100), and incubated on ice for 2 min. Label solution and enzyme solution were added. After incubating for 1 h at 37°C, the samples were measured with a spectrofluorophotometer (Shimadzu RF-5301PC, Shimadzu, Kyoto, Japan).

Cell-cycle-arrest analysis

The DNA content of C. albicans was analysed for cell-cycle arrest. The log-phased cells (1x10$^6$ ml$^{-1}$), resuspended in YPD, were treated with 2.5, 5.0 and 10.0 µg ml$^{-1}$ oxyresveratrol and incubated for 4 h at 28°C. After washing with sodium citrate buffer and fixing for 12 h with 70 % ethanol at 4°C, the cells were washed twice with 50 mM sodium citrate buffer. RNase A was activated by heat shock (95°C for 10 min). The cells were treated with RNase A (200 µg ml$^{-1}$) for 2 h at 37°C, to ensure that only DNA is stained in the following step. The cells were washed and incubated with 1 µM SYTOX Green (Molecular Probes, Eugene, OR, USA). DNA contents were measured using FACSVerses flow cytometer (Becton–Dickinson, San Jose, CA, USA).

Genomic DNA extraction

To extract DNA, C. albicans cells in log phase (1x10$^6$ ml$^{-1}$) were harvested and lysed in 200 µl cell lysis buffer containing 100 mM NaCl, 1 % SDS, 2 % Triton X-100, 1 mM EDTA and 10 mM Tris-HCl (pH 8.0). The lysis was incubated with 10 mg ml$^{-1}$ proteinase K for 1 h at 37°C, and then with 10 mg ml$^{-1}$ RNase A for 1 h at 37°C. Two volumes of ice-cold 100 % EtOH and 1/25 vol of 5 M NaCl were added, and the samples were precipitated at −20°C to increase the yield. EtOH (70 %) was added after centrifugation for 7 min. The samples were then resuspended in TE buffer (1 mM EDTA, 10 mM Tris at pH 8.0) or water.

Oxidative DNA damage and DNA interaction assay

As oxidative DNA damage, the levels of 8-hydroxydeoxy-
guanosine (8-OHdG) were measured. Genomic DNA was extracted according to the method mentioned above. 8-OHdG levels were measured using the Oxiselect Oxidative DNA Damage ELISA Kit (Cell Biolabs, San Diego, CA, USA), according to the manufacturer’s instructions. The OD of 8-OHdG was measured using a spectrophotometer at 450 nm. To investigate the interaction of oxyresveratrol with DNA, the fluorescence spectrum of genomic DNA (1 µg) was measured. Genomic DNA (1 µg) upon addition of

Fig. 1. Structure of oxyresveratrol.
oxyresveratrol was also assessed to detect interaction of oxyresveratrol with DNA. The emission spectra were assessed using ELISA Reader.

Phosphatidylserine (PS) externalization assay
Determination of apoptosis or necrosis was detected using the Annexin V–FITC apoptosis detection kit (BD Pharmingen, San Diego, CA, USA). To measure PS externalization, protoplasts were prepared. d-sorbitol (1 M) and 0.15 g lysing enzyme (extracted from Trichoderma harzianum) were dissolved in 0.1 M potassium phosphate buffer (PPB) and mixed with cells. The centrifuged protoplasts were resuspended with the mixture of 1 M d-sorbitol and 0.1 M PPB. The protoplasts (1 × 10⁶ ml⁻¹) were treated with oxyresveratrol (2.5, 5.0 and 10.0 µg ml⁻¹), or H₂O₂ (10 mM and 20 mM) and incubated for 2 h at 28 °C. After incubation, the protoplasts were collected and resuspended in 1× Annexin V binding buffer, followed by addition of Annexin V–FITC and propidium iodide (PI). The mixtures were then incubated at room temperature for 15 min in the dark. The cells were analysed using a FACSVerse flow cytometer.

Morphological change analysis
Apopototic cells showed cell shrinkage and necrotic cells showed cell swelling. To confirm the morphological feature by oxyresveratrol, log-phased cells (1 × 10⁶ ml⁻¹) in PBS treated with 2.5, 5.0 and 10.0 µg ml⁻¹ oxyresveratrol were analysed by flow cytometer. Forward scatter shows cell size and side scatter indicates cell granularity. After incubation 4 h at 28 °C with oxyresveratrol, the cell samples were resuspended with PBS. The cells were evaluated in each sample by determining their position on forward scatter contour plots using a FACSVerse flow cytometer.

Metacaspase activation assay
The activation of metacaspase in C. albicans was detected by CasPASE FITC-VAD-FMK in situ marker (Promega, Madison, WI, USA). The log-phased cells (1 × 10⁶ ml⁻¹), resuspended in PBS, were incubated with oxyresveratrol (2.5, 5.0 and 10.0 µg ml⁻¹) or 10 mM H₂O₂ for 4 h at 28 °C. After incubation the cells were then stained with fluorescence-labelled pan-caspase inhibitor, FITC-VAD-FMK (2.5 µM), and incubated at 37 °C for 30 min. The samples were analysed using a FACSVerse flow cytometer.

Intracellular and mitochondrial Ca²⁺ level assay
To evaluate the changes in intracellular Ca²⁺ levels, we used the intracellular calcium indicator, fura-2 acetoxymethyl ester (Fura-2AM) (Molecular Probes, Eugene, OR, USA). To evaluate the changes in mitochondrial Ca²⁺ levels, rhod-2 acetoxymethyl ester (Rhod-2AM) (Molecular Probes, Eugene, OR, USA) was used as a mitochondrial Ca²⁺ indicator. The log-phased cells (1 × 10⁶ ml⁻¹) in PBS were incubated with oxyresveratrol (2.5, 5.0 and 10.0 µg ml⁻¹) or 10 mM H₂O₂ for 4 h at 28 °C. The cells were washed and treated with 1 % bovine serum albumin and 0.01 % Pluronic F-127 (Molecular Probes, Eugene, OR, USA). The suspensions were then incubated with 5 µM Fura-2AM or 10 µM Rhod-2AM at 37 °C for 30 min. The samples were washed with calcium-free Krebs buffer. The fluorescence intensity of the samples was measured using a spectrophotometer at wavelengths of 340 nm (excitation) and 510 nm (emission) for Fura-2AM or 552 nm (excitation) and 581 nm (emission) for Rhod-2AM.

Mitochondria membrane potential (ΔΨ𝑚) assay
Mitochondrial membrane depolarization was measured by 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolercyanine iodide (JC-1) (Molecular Probes, Eugene, OR, USA) staining assay. The log-phased cells (1 × 10⁶ ml⁻¹) in PBS were treated with oxyresveratrol (2.5, 5.0 and 10.0 µg ml⁻¹) or 10 mM H₂O₂ and incubated for 4 h at 28 °C. The cells were suspended in 1 ml warm PBS and treated with 0.5 mg ml⁻¹ JC-1. The fluorescence shift from green (FL1) to red (FL2) was indicated using a FACSVerse flow cytometer.

Cytochrome c release assay
The cells in log phase (1 × 10⁶ ml⁻¹), which is re suspended in PBS, were incubated with oxyresveratrol (2.5, 5.0 and 10.0 µg ml⁻¹) or 10 mM H₂O₂ for 4 h at 28 °C. Then, the cells were suspended in homogenization buffer (50 mM Tris, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, pH 7.5). Cells with beads were homogenized by vortexing and cooling in ice four times, followed by the addition of 2 % of glucose. After centrifugation, the supernatants were transferred to a new tube. For the separation of mitochondrial and cytoplasm, the samples were centrifuged for 45 min. Then, 5 % TCA was added to the supernatants and pellets. After acetone washing, buffer A (50 mM Tris, 2 mM EDTA, pH 7.5) was added to the samples. For quantification of cytochrome c, equal amounts of cytoplasmic and mitochondrial proteins were suspended in buffer B (1 M Tris, 0.1 M EDTA, pH 5.0). After treatment with 1 g ml⁻¹ ascorbic acid, cytochrome c contents were measured using a spectrophotometer at 550 nm (DU530, Beckman).

Statistical analysis
Measurements were performed in three independent experiments and values are reported as means±SD. After confirming the normality of distribution using the Shapiro–Wilk test, group statistical comparisons were assessed by ANOVA. This was followed by a post hoc analysis with Tukey’s test for three-group comparisons using SPSS software (SPSS, version 23, SPSS/IBM, Chicago, IL, USA). Differences between samples were considered significant for P-values <0.05.

RESULTS
Antifungal and haemolytic effects of oxyresveratrol
The antifungal activity of oxyresveratrol on different fungal strains (C. albicans, C. parapsilosis, S. cerevisiae and T. beigelii) was investigated. H₂O₂ was used as a positive control. As shown in Table 1, the fungal strains were susceptible to oxyresveratrol, with an MIC value of 5.0 µg ml⁻¹. Oxyresveratrol showed a low percentage haemolysis.
Oxyresveratrol can be a potential antifungal agent having no haemolytic effects on human erythrocytes less than 25.0 µg ml⁻¹ (Table 2). Because the MIC of oxyresveratrol was 5.0 µg ml⁻¹ without haemolysis, we decided to test different concentrations of oxyresveratrol (2.5, 5.0 and 10.0 µg ml⁻¹) to elucidate the antifungal mechanisms.

DNA cleavage by oxyresveratrol

DNA strand break assay was conducted to determine DNA damage and was measured in cells treated with oxyresveratrol (2.5, 5.0 and 10.0 µg ml⁻¹). *C. albicans* treated with oxyresveratrol showed increased TUNEL fluorescence intensity in a dose-dependent manner (Fig. 2). Thus, we confirmed that oxyresveratrol induced DNA damage by DNA strand break.

G₂/M phase arrest by oxyresveratrol

To investigate the effects of DNA strand break, cell-cycle arrest was analysed. Assessment of cell-cycle distribution in *C. albicans* was performed with different doses of oxyresveratrol. Compared with untreated cells, oxyresveratrol-treated cells showed a decrease in cell population in the G₁ phase from 66.36±0.50 to 64.48±1.16, 49.53±0.77, 39.36±0.31 %, respectively. In contrast, the population in the G₂/M phase increased significantly from 8.82±0.08 to 12.14±0.15, 23.98 ±0.15, 33.27±0.27 % (Fig. 3). We confirmed that oxyresveratrol induced cell-cycle arrest at the G₂/M phase.

Oxidative stress and binding affinity of oxyresveratrol on DNA

DNA lesions can be generated by oxidative stress. To test this directly, we checked whether oxyresveratrol induces oxidative DNA damage by measuring OD value. Exposure of *C. albicans* cells to oxyresveratrol resulted in no increase of oxidative DNA damage in the form of 8-OHdG, while H₂O₂ treatment increases 8-OHdG production compared to untreated cells (Fig. 4a). The data suggest that oxyresveratrol did not increase 8-OHdG level, thus oxidative DNA damage is not an inducer of DNA strand break. To provide evidence for the interaction of oxyresveratrol with DNA, the affinity process was monitored by measuring the changes in fluorescence intensity. Oxyresveratrol interacting with DNA affects the fluorescence value of DNA, leading to a change in fluorescence intensity. The fluorescence intensity of genomic DNA treated with oxyresveratrol decreased compared to the fluorescence intensity of DNA alone (Fig. 4b).

**Detection of apoptosis**

To confirm whether DNA damage by oxyresveratrol induces apoptosis or necrosis, we examined PS exposure and membrane disruption by staining fluorescein isothiocyanate (FITC)-conjugated annexin V and PI. Annexin V+/PI+ or annexin V+/PI– indicate apoptosis, and annexin V–/PI+ indicates necrosis. Protoplasts treated with oxyresveratrol (2.5, 5.0 and 10.0 µg ml⁻¹) exhibited a FITC-annexin V fluorescence percentage of 12.15±1.70, 18.50±1.28 and 22.86 ±1.51 %, respectively. As shown in Fig. 5(a), the histogram showed that oxyresveratrol induced PS externalization in a concentration-dependent manner. Oxyresveratrol triggered apoptosis rather than necrosis via PS exposure.

Cell morphological change was conducted to verify oxyresveratrol trigger apoptosis. In the quadrants (Fig. 5b), oxyresveratrol-treated cells showed a decrease in FSC values (57.64, 50.44 and 44.36 %, respectively) as the concentration increases. For further investigation of oxyresveratrol-induced apoptosis, metacaspase activation was measured. Metacaspase activation due to oxyresveratrol was assessed using FITC-VAD-FMK. This assay showed that metacaspases were activated (30.31±0.64, 37.86±0.64 and 39.98 ±1.06 %) in cells treated with different concentrations of oxyresveratrol (2.5, 5.0 and 10.0 µg ml⁻¹), respectively (Fig. 5c). These results proved that oxyresveratrol induced apoptosis by cell shrinkage and metacaspase activation.

**Intracellular Ca²⁺ changes and mitochondrial dysfunction by oxyresveratrol**

Intracellular Ca²⁺ changes are observed in apoptotic cells. In this study, Ca²⁺ accumulation from cytosol and the mitochondria were measured using a spectrofluorophotometer. Fura-2AM was used to evaluate intracellular Ca²⁺ change. Mitochondrial Ca²⁺ change was evaluated using Rhod-2AM. Oxyresveratrol triggered the increase in cytosolic and mitochondrial Ca²⁺ contents in a dose-dependent manner (Fig. 6).

Mitochondrial dysfunction is associated with changes in ∆Ψₘ. To investigate the relationship between apoptosis and mitochondria, mitochondrial membrane depolarization was measured by JC-1. At high ∆Ψₘ, JC-1 spontaneously formed J-aggregates with red fluorescence, whereas at low ∆Ψₘ, JC-1 remained in the monomeric form with green fluorescence. Therefore, JC-1 changes the colour from red to green as the membrane potential decreases. As the concentration of oxyresveratrol increased, the mean values of FL2 FL1⁻¹ decreased (Fig. 7a). These results indicated that the mitochondrial membrane was depolarized in a dose-dependent manner. The release of cytochrome c from the mitochondrial intermembrane space was verified using the annexin V/PI double-staining method (Fig. 7b).

**Table 1. The antifungal effect of oxyresveratrol and H₂O₂**

<table>
<thead>
<tr>
<th>Fungal strains</th>
<th>MIC (µg ml⁻¹)</th>
<th>H₂O₂ (mM)</th>
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</thead>
<tbody>
<tr>
<td><em>C. albicans</em> ATCC 90028</td>
<td>5.0</td>
<td>10.0</td>
</tr>
<tr>
<td><em>C. parapsilosis</em> ATCC 22019</td>
<td>5.0</td>
<td>10.0</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> KCTC 7296</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td><em>T. beigeli</em> KCTC 7707</td>
<td>5.0</td>
<td>5.0</td>
</tr>
</tbody>
</table>

**Table 2. The haemolysis of oxyresveratrol and amphotericin B**

<table>
<thead>
<tr>
<th>Compound (µg ml⁻¹)</th>
<th>Haemolysis (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>100.0</td>
</tr>
<tr>
<td>Oxyresveratrol</td>
<td>6.72</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>100</td>
</tr>
</tbody>
</table>
mitochondria to the cytosol was also investigated. The release of pro-apoptotic factors from the mitochondria to the cytoplasm is a crucial step in apoptosis. Cytochrome c is a pro-apoptotic factor and its release is associated with mitochondrial dysfunction. Mitochondrial cytochrome c levels decreased with an increase in oxyresveratrol concentration, while cytosolic cytochrome c levels increased when compared with the levels in the control cells (Fig. 7b).

**DISCUSSION**

Phytoalexins are defensive substances produced by plants in response to infection [18]. Among those compounds, oxyresveratrol showed several biological activities, including anticancer and anti-inflammatory activity [20, 27]. It has more of the hydroxyl group compared to resveratrol, a well-known phytoalexin as an anticarcinogenic, antifungal and antibacterial agent [28–30]. The functional group of oxyresveratrol provides lower cytotoxicity and better antioxidant activity than resveratrol [18]. In this study, oxyresveratrol showed efficient antifungal activities with low haemolytic effect on human erythrocytes. The results suggested that oxyresveratrol might be a candidate for controlling fungal diseases, with selective toxicity toward fungal cells. Fungal strains, which are conduced antifungal activities in this study, cause human infections. Among them, *C. albicans* is the most frequent cause of fungal infections causing life-threatening systemic disease in predisposed patients [6, 31–34]. Therefore, we attempted to investigate the antifungal mode of action of oxyresveratrol in *C. albicans*.

Several phytoalexins including resveratrol, oxyresveratrol and catechin are involved in DNA damage [35]. We confirmed oxyresveratrol provoke DNA strand break using TUNEL assay. DNA damage interferes with DNA replication and transcription, which ultimately leads to mutations and chromosomal aberrations [36]. DNA strand break makes repair highly difficult and leads to inappropriate rejoining, which results in genomic instability [37]. Cell-cycle arrest at a critical stage before or during DNA replication (G1/S and intra-S checkpoints) and before cell division (G2/M checkpoint) prevents duplication and segregation of damaged nucleic acids [36]. Thus, we compared cell-cycle progression of untreated cells and cells upon exposure to oxyresveratrol. In response to lesions induced by oxyresveratrol, G2/M phase checkpoints activate cell-cycle arrest prior to mitosis. Among the cell-cycle checkpoints, G2/M checkpoint plays a major role in controlling DNA damage [38]. G2/M phase arrest is induced when the cell detects DNA damage and transduces signals that control mitotic entry [39].

**Fig. 2.** Effects of oxyresveratrol on DNA. Spectrofluorophotometric analysis of DNA strand break by TUNEL staining. Numbers 1, 2 and 3 correspond to different concentrations of oxyresveratrol (2.5, 5.0 and 10.0 µg ml⁻¹, respectively). H2O2 was treated with 10 mM as a positive control. The data are displayed as mean±SD from three independent experiments. *P<0.1, **P<0.05, ***P<0.01 versus control (one-way ANOVA followed by post hoc Tukey’s test).
To elucidate the cause of DNA damage by oxyresveratrol, 8-OHdG level and fluorescence intensity of DNA were measured. Oxidative stress is particularly hazardous and reactive oxygen species (ROS), caused by oxidative stress, pose a significant threat to cellular integrity in terms of damage to DNA [40, 41]. Interaction of ROS with guanine leads to the formation of 8-OHdG [42]. 8-OHdG can lead to double strand breaks after replication, which can result in recombination, thus producing permanent genome rearrangements [40]. To investigate whether oxyresveratrol induces DNA oxidation, assessment of 8-OHdG levels confirmed that DNA oxidation is not a cause of DNA strand break. Furthermore, we assessed the interaction with DNA in *C. albicans* following exposure to oxyresveratrol. DNA was altered by oxyresveratrol and this change means direct interaction of oxyresveratrol. Antimicrobial activity is

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**Fig. 3.** Detection of cell-cycle arrest. Flow cytometric analysis of cell-cycle arrest by SYTOX Green. Numbers 1, 2 and 3 correspond to different concentrations of oxyresveratrol (2.5, 5.0 and 10.0 µg ml⁻¹, respectively). H₂O₂ was added at a concentration of 10 mM as a positive control. The data are displayed as mean±SD from three independent experiments. *P<0.1, **P<0.05, ***P<0.01 versus control (one-way ANOVA followed by post hoc Tukey’s test).

**Fig. 4.** Ability of oxyresveratrol on DNA strand break. Numbers 1, 2 and 3 correspond to different concentrations of oxyresveratrol (2.5, 5.0 and 10.0 µg ml⁻¹, respectively). H₂O₂ was treated with 10 mM as a positive control. (a) Spectrophotometric analysis of oxidative DNA damage (8-OHdG quantitation). (b) Spectrofluorophotometric analysis of DNA interaction with oxyresveratrol. gDNA indicates genomic DNA. Data are displayed as mean±SD from three independent experiments. *P<0.1, **P<0.05, ***P<0.01 versus control (one-way ANOVA followed by post hoc Tukey’s test).
caused by direct interaction with DNA. It has suggested that DNA-binding ability contributes to DNA damage and cell death [43, 44]. Oxyresveratrol has resorcinol at both ends of the structure which has a hydroxyl group at the 1,3-carbon of benzene ring. It has been reported that resorcinol is known to cleavage DNA strands [35, 45]. The decrease of DNA fluorescence indicated that oxyresveratrol interacts with DNA by possessing DNA-binding affinity. In this

Fig. 5. Detection of apoptotic features. Numbers 1, 2 and 3 correspond to different concentrations of oxyresveratrol (2.5, 5.0 and 10.0 µg mL⁻¹, respectively). H₂O₂ was used as a positive control. (a) Flow cytometric analysis to distinguish between apoptosis and necrosis by Annexin V-FITC/PI staining. Percentage of cells that are classified as apoptotic [annexin(+) PI(+/-); grey bars] and necrotic [annexin(−) PI(+); black bars]. (b) Flow cytometric analysis of cell size change. (c) Flow cytometric analysis of metacaspase activation by caspase FITC-VAD-FMK. Data are displayed as mean±SD from three independent experiments. *P<0.1, **P<0.05, ***P<0.01 versus control (one-way ANOVA followed by post hoc Tukey’s test).

Fig. 6. Detection of cytosolic and mitochondrial calcium overload. Spectrofluorophotometric analysis of cytosol calcium accumulation by Fura-2AM and mitochondrial calcium accumulation by Rhod-2AM. Numbers 1, 2 and 3 correspond to different concentrations of oxyresveratrol (2.5, 5.0 and 10.0 µg ml⁻¹, respectively). H₂O₂ was treated with 10 mM as a positive control. Data are displayed as mean±SD from three independent experiments. *P<0.1, **P<0.05, ***P<0.01 versus control (one-way ANOVA followed by post hoc Tukey’s test).
respect, the ability of oxyresveratrol to interact with DNA may disrupt DNA directly.

DNA strand breaks are deleterious because the broken ends of DNA are liable to be dissociated [37]. DNA damage can lead to cell death by activating DNA damage signal [46]. This signal elicits cell death by apoptosis and necrosis and these have distinctive morphological and biochemical features [47–49]. To exert which cell death occurs by oxyresveratrol, PS exposure/membrane integrity were investigated. The plasma membrane comprises lipids, proteins and glycosans, and each leaflet has a different composition including PS, normally distributed in the cytosolic leaflet [50]. During apoptosis, phospholipid scramblases transfer the PS component of lipid bilayer from the inner plasma membrane to the outer membrane with an intact cell membrane [51]. However, in cells undergoing necrosis the plasma membrane is the major site of damage and thus cells lose membrane integrity, leading to lysis [52]. Oxyresveratrol-treated protoplasts showed PS exposure without membrane disruption which related to apoptosis. Further consolidate apoptotic cell death, cell shrinkage and metacaspase activation were confirmed. During apoptosis, cellular shrinkage is

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**Fig. 7.** Analysis of mitochondrial dysfunction. Numbers 1, 2 and 3 correspond to different concentrations of oxyresveratrol (2.5, 5.0 and 10.0 µg ml$^{-1}$, respectively). H$_2$O$_2$ was treated with 10 mM as a positive control. (a) Flow cytometric analysis of mitochondrial membrane depolarization by JC-1. (b) Spectrophotometric analysis of cytochrome c release. Data are displayed as mean±SD from three independent experiments. *P<0.1, **P<0.05, ***P<0.01 versus control (one-way ANOVA followed by post hoc Tukey’s test).
observed with condensation of the cytoplasm and nucleus [53]. Oxyresveratrol-treated cells showed cell shrinkage called apoptotic volume decrease. Metacaspases exist as inactivate zymogens, with an N-terminal prodomain and a caspase domain comprising P20 and P10 subunits [54]. Metacaspase is required for cell death following loss of ubiquitination control, altered mRNA stability, and defective initiation of DNA replication, and plays a role in activating apoptosis in yeast [55]. Following an apoptotic stimulus, metacaspase was activated by proteolytic removal of a 14 kDa peptide [56]. In this study, we observed metacaspase activation in response to oxyresveratrol exposure. Taken together, unrepaired DNA damage induced by oxyresveratrol led to apoptotic features.

Ca$^{2+}$ is a universal intracellular messenger that regulates several aspects of cellular function, and is a decisive element of apoptosis [57]. During apoptotic response, cytosolic Ca$^{2+}$ levels increase via opening of the membrane Ca$^{2+}$ channel and release from intracellular stores, such as endoplasmic reticulum. Treatment with oxyresveratrol induces Ca$^{2+}$ uptake into the cytosol. Elevated cytosolic Ca$^{2+}$ levels induce Ca$^{2+}$ uptake into the mitochondria, which in turn stimulates the formation of permeability transition pores and break down of ΔΨm [58]. Inappropiate low values of ΔΨm have insufficient ability of producing ATP and lead to loss of cell function [59]. Oxyresveratrol triggered a Ca$^{2+}$ increase in the mitochondria, followed by induction of mitochondrial membrane depolarization. Perturbation of the mitochondrial membrane is related to the release of mitochondrial apoptotic factors such as cytochrome c [60]. Cytochrome c is electrostatically bound to the inner membrane surface of the mitochondria. Mitochondrial membrane depolarization induced by oxyresveratrol caused exposure of cytochrome c to the intermembrane space and its subsequent release to the cytosol. Cytosolic cytochrome c acts as one of the downstream death executioners of yeast mitochondria and initiates apoptotic cascades by activating metacaspases [56, 61]. Oxyresveratrol triggered leakage of Ca$^{2+}$ into the cytosol and leads to Ca$^{2+}$ uptake by the mitochondria. Mitochondrial Ca$^{2+}$ overload resulted in mitochondrial membrane depolarization and cytochrome c release. Therefore, we assumed that oxyresveratrol induces mitochondrial dysfunction and subsequently leads to apoptosis.

This study investigated how oxyresveratrol leads to apoptosis upon treatment to C. albicans. Oxyresveratrol does not induce DNA damage by oxidative stress. However, direct interaction of oxyresveratrol with DNA triggers DNA strand breaks and results in cell cycle arrest. During DNA damage, oxyresveratrol results in mitochondrial dysfunction. Cells show mitochondrial Ca$^{2+}$ accumulation, mitochondrial membrane depolarization and cytochrome c release to cytosol. Several apoptotic responses sequentially occur with PS externalization, apoptotic volume decrease and metacaspase activation. In summary, oxyresveratrol exerts antifungal activity through mitochondria-mediated apoptosis in a dose-dependent manner. This study may offer novel insight for controlling pathogenic fungi.

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**Conflicts of interest**
The authors declare that they have no conflicts of interest.

**References**