Antifungal mechanism of essential oil from *Anethum graveolens* seeds against *Candida albicans*

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This work studied the antifungal mechanism of dill seed essential oil (DSEO) against *Candida albicans*. Flow cytometric analysis and inhibition of ergosterol synthesis were performed to clarify the mechanism of action of DSEO on *C. albicans*. Upon treatment of cells with DSEO, propidium iodide penetrated *C. albicans* through a lesion in its plasma membrane. DSEO also significantly reduced the amount of ergosterol. These findings indicate that the plasma membrane of *C. albicans* was damaged by DSEO. The effect of DSEO on the functions of the mitochondria in *C. albicans* was also studied. We assayed the mitochondrial membrane potential (ΔΨm) using rhodamine 123 and determined the production of mitochondrial dysfunction-induced reactive oxygen species (ROS) via flow cytometry. The effects of the antioxidant L-cysteine (Cys) on DSEO-induced ROS production and the antifungal effect of DSEO on *C. albicans* were investigated. Exposure to DSEO increased ΔΨm. Dysfunctions in the mitochondria caused ROS accumulation in *C. albicans*. This increase in the level of ROS production and DSEO-induced decrease in cell viability were prevented by the addition of Cys, indicating that ROS are an important mediator of the antifungal action of DSEO. These findings indicate that the cytoplasmic membrane and mitochondria are the main anti-*Candida* targets of DSEO.

INTRODUCTION

In recent years, the number of fungal infections has increased because of a growing number of high-risk patients, particularly immunocompromised hosts. *Candida albicans* is the causative agent most associated with serious fungal infections, accounting for more than 90% of cases (Douglas, 2003; Kauffman, 2006). In general, antibiotics are widely used in treating such fungal infections, which leads to resistant strains and various other drawbacks in terms of, for example, toxicity, drug–drug interactions and high cost (Runyoro et al., 2006). Antifungal drugs are still limited in number, despite the recent introduction of new ones (Rapp, 2004). Hence, the great demand for novel antifungal agents justifies the intense search for new drugs that are more effective and less toxic than the existing ones.

As natural products from aromatic and medicinal plants, essential oils are known to possess antiviral, antibacterial, antifungal and antioxidant activities (Kordali et al., 2005; Baratta et al., 1998; Bakkali et al., 2008). The anti-*Candida* activities of some essential oils have been established in previous studies. For example, anti-*Candida* activities have been demonstrated by essential oils extracted from *Origanum* (Giordani et al., 2004; Manohar et al., 2001), *Thymus* (Pinto et al., 2006; Pina-Vaz et al., 2004; Karaman et al., 2001), *Thymbra* (Salgueiro et al., 2004), *Melaleuca* (Hammer et al., 1998; Oliva et al., 2003), mint (Tampieri et al., 2005; Duarte et al., 2005), cinnamon (Tampieri et al., 2005), clove (Ahmad et al., 2005; Costa et al., 2000) and *Daucus* (Tavares et al., 2008).

Dill (*Anethum graveolens* L.) belonging to the family Umbelliferae is a traditional Chinese herb. For more than 2000 years, dill has been used as a flavouring agent in the food industry. Aside from its wide clinical applications, dill is also used to treat gastrointestinal ailments, such as flatulence, indigestion, stomach colic pain and intestinal...
gas (Editor Committee of National Chinese Medical Manage Bureau, 2005). Dill seed essential oil (DSEO) was used early in the last century to treat many pathological conditions, such as disease of the uterus and cervical ectropio, and as an antimicrobial (Editor Committee of National Chinese Medical Manage Bureau, 2005). Some researchers have reported that DSEO possesses anti-C. albicans activity (Yili et al., 2009; Jirovetz et al., 2003; López et al., 2005). Similarly, our laboratory reported that DSEO (from plant material derived from China) is composed of carvone (41.5 %), limonene (32.6 %) and apiol (16.8 %) (Tian et al., 2011), and has anti-Candida activities in vitro and in vivo (Zeng et al., 2011). Tian et al. (2011, 2012) revealed the antifungal activity of DSEO in vitro and in vivo and its mechanism of antifungal action on Aspergillus flavus. However, to the best of our knowledge, the mechanism of action of DSEO on C. albicans remains unknown. Accordingly, this study aimed to determine the mechanism of action of DSEO on C. albicans.

**METHODS**

**Plant materials.** Dill seeds were procured from Xinjiang Uighur Medical College in Hotan, Xinjiang, China. The plant material was initially identified via its morphological features and was finally confirmed by the corresponding author. Voucher specimen no. 581 was deposited at the herbarium of the Institute of Traditional Chinese Medicine and Natural Products, Wuhan University School of Pharmaceutical Sciences, China.

**Essential oil isolation.** Dried seeds (100 g) were smashed (size was less than 0.25 mm), and the essential oil was obtained via steam distillation in 1500 ml H2O for 4 h using a Clevenger apparatus (Chinese Pharmacopoeia Commission, 2010). DSEO was dried over anhydrous Na2SO4 and then preserved in a sealed vial at 4 °C until further use. The yield of essential oil from the dill seeds was 3.5 % (v/w).

**Micro-organisms.** One ATCC type strain (Candida albicans ATCC 64550) and two isolates of Candida were studied. C. albicans 09-1502 and C. albicans 09-5304 were isolated from the oral mucosa and vagina, respectively, of infected patients in the Department of Dermatology and Venerology of the Union Hospital in Wuhan, China. The two clinical isolates were identified according to their morphology on corn meal agar, and then by germ-tube formation, the presence of thick-walled spores and yeast spores and assimilation–fermentation profiles using the API 20 system (bioMérieux). The clinically isolated strains were stored in Sabouraud dextrose broth (SDB) with glycerol at −80 °C. Prior to antifungal susceptibility testing, each isolate was inoculated on Sabouraud dextrose agar (SDA) to ensure optimal growth characteristics and purity.

**Antifungal activity.** A macrodilution broth method was used to determine the MIC and minimum fungicidal concentration (MFC) according to our previously published method (Zeng et al., 2011).

Tests were performed briefly on sterile U-bottomed 96-well microplates. To each well of the first column of the plate was added 100 μl RPMI 1640 (without sodium bicarbonate and L-glutamine, pH 7.0). DSEO (100 μl) was added to the first well and twofold serially diluted to the 10th well. The 11th and last columns of the plate served as the negative (without inocula) and positive (without DSEO) control wells, respectively. Fluconazole (FCZ) and nystatin (Nys) were used as standard drugs. The concentration of DSEO ranged from 20 to 0.039 μl ml⁻¹, whereas that of FCZ and Nys ranged from 100 to 0.18 μg ml⁻¹. Next, 100 μl of the yeast colonies suspended in RPMI 1640 at a density of 1 × 10⁵ c.f.u. ml⁻¹ and adjusted to twice the final inoculum size was added to each well of the plate. The plates were incubated at 37 °C for 48 h for Candida spp. The MIC or the lowest concentration of the test substances that prevented visible growth of micro-organisms was determined.

The MFC or the lowest concentration yielding negative subcultures or comprising a single colony was determined by plating 100 μl from each negative and positive well on SDA.

**Effect of DSEO on membrane integrity.** Studies on membrane damage were conducted following a previously described procedure with slight modifications (Pinto et al., 2009). C. albicans ATCC 64550 suspensions were grown in SDB for 16 h at 30 °C in a shaker at 200 r.p.m. The suspensions were centrifuged, washed and resuspended in PBS with 2 % (v/v) d-glucose (PBS-G) at a density of 6 × 10⁸ c.f.u. ml⁻¹. DSEO was dissolved in Tween 20 to a final concentration of 0.01 % (v/v). Serial twofold dilutions of DSEO (1.250 to 0.156 μl ml⁻¹) and the positive control (Nys, 1.560 μg ml⁻¹) were then added to the cell suspensions for 16 h at 30 °C. Drug-free control tubes were included in each experiment (0.01 % Tween 20). After the incubation, the cells were washed and resuspended in 500 μl PBS-G for staining. A 5 μl propidium iodide (PI; Sigma) solution in PBS was added to the cell suspensions to obtain a final concentration of 1.0 μg PI ml⁻¹. PI is a fluorescent probe used to study the effect of drugs on membranes. It only penetrates cells with severe membrane lesions, resulting in increased red fluorescence (Pina-Vaz et al., 2001). All samples were then incubated at 30 °C for 30 min in the dark. Unstained cell suspensions served as autofluorescence controls. Scattergram analysis was performed to evaluate morphological changes (size and complexity). The percentage of PI-positive cells in the PMT4 channel (620 nm), which represents dead cells with severe lesions of the membrane, was quantified. The results were analysed using Expo32 v1.2 software.

**Determination of ergosterol content in the plasma membrane.** Intracellular sterols were extracted following the methods described by Arthington-Skaggs et al. (1999) with slight modifications. The ergosterol contents of C. albicans ATCC 64550, C. albicans 09-5304 and C. albicans 09-1502 incubated with different concentrations of DSEO or 3.12 μg FCZ ml⁻¹ (positive control) were measured. A single C. albicans colony from an overnight SDA culture plate was used to inoculate 60 ml SDB containing 0.078, 0.156 and 0.312 μl DSEO ml⁻¹ and suspended in 3.12 μg FCZ ml⁻¹. The cultures were incubated for 16 h at 30 °C in a shaker at 200 r.p.m. Stationary-phase cells were harvested by centrifugation at 5000 g for 5 min and washed once with sterile distilled water. The net wet weight of the cell pellet was determined. A 10 μl 25 % alcoholic potassium hydroxide solution was added to each pellet and vortex mixed for 10 min. The cell suspensions were transferred to clean glass tubes and incubated in a water bath at 85 °C for 2 h. Sterols were then extracted by adding a mixture of 1 ml sterile distilled water and 3 ml n-heptane, followed by vigorous vortex mixing for 10 min. The heptane layer was transferred to a clean tube and stored at −20 °C for 24 h. Prior to analysis, a 1 ml aliquot of the sterol extract was diluted twofold in 100 % ethanol and scanned spectrophotometrically between 230 and 300 nm with a spectrophotometer (UV-1700; Shimadzu). The presence of ergosterol and the late sterol intermediate 24(28)-dehydroergosterol (DHE) in the extracted sample resulted in a characteristic four-peak curve. The absence of detectable ergosterol in the extracts was indicated by a flat line. Ergosterol content was calculated as a percentage of the wet weight of the cell using the following equation:
% Ergosterol + % 24(28)DHE=[(A283/290) × F]/pellet weight, 
where

% 24(28)DHE=[A240/518 × F]/pellet weight, and

% Ergosterol=[% ergosterol + % 24(28)DHE−% 24(28)DHE],

with F being the factor for dilution in ethanol, and 290 and 518 the E values (in % cm⁻¹) determined for crystalline ergosterol and 24(28)DHE, respectively.

Measurement of mitochondrial membrane potential (△Ψm). The effect of DSEO on the △Ψm of C. albicans ATCC 64550 was measured by flow cytometry using rhodamine 123 (Rh123) according to a previously described procedure with some modifications (Tang et al., 2005). Cells exposed to various doses of DSEO (0.156 to 1.25 μl ml⁻¹) at 30 °C for 12 h were harvested by centrifugation at 5000 g for 5 min, washed twice and then resuspended at 6 × 10⁶ c.f.u. ml⁻¹ in PBS. Rh123 was added to a final concentration of 0.1 mg ml⁻¹ and incubated for 30 min in the dark at 30 °C. For all cytofluorimetric experiments, forward and side scatters were gated on the major population of cells, and 6 × 10⁶ cells per sample were measured. △Ψm was expressed as the fluorescence intensity of Rh123, which was read using a FACScan flow cytometer (EPICS ALTRA II; Beckman) with excitation at 488 nm and emission at 525 nm. The data were analysed using Expo32 v1.2 software.

Measurement of mitochondrial dehydrogenases. The effect of DSEO on the activities of mitochondrial dehydrogenases was determined using the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-(2H)-tetrazolium-5-carboxanilide (XTT) method as described previously with some modifications (Dulleeu et al., 2008). XTT (Sigma) was prepared as a saturated solution at 1 mg ml⁻¹ in acetone, was added to a final concentration of 1 mM to a stock solution of 1 mM to a final concentration of 10 mM, incubated for 30 min in the dark at 30 °C. After cytofluorimetric experiments, forward and side scatters were gated on the major population of cells, and 6 × 10⁶ cells per sample were measured. △Ψm was expressed as the fluorescence intensity of Rh123, which was read using a FACScan flow cytometer (EPICS ALTRA II; Beckman) with excitation at 488 nm and emission at 525 nm. The data were analysed using Expo32 v1.2 software.

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Measurement of reactive oxygen species (ROS) formation. The endogenous amounts of ROS in C. albicans ATCC 64550 were measured via flow cytometry using 2’,7’-dichlorofluorescin diacetate (DCFH-DA) as a ROS indicator as described previously with minor modifications (Helmerhorst et al., 2001). Exponentially growing C. albicans ATCC 64550 cells were suspended in sterile PBS to 6 × 10⁶ c.f.u. ml⁻¹. Serial twofold dilutions of DSEO (0.156 to 1.25 μl ml⁻¹) were added to the cell suspensions for 12 h at 30 °C. The cells were harvested by centrifugation at 5000 g for 5 min, washed twice and resuspended to 6 × 10⁶ c.f.u. ml⁻¹ in PBS. DCFH-DA was added from a stock solution of 1 μM to a final concentration of 10 μM using a ROS kit (C1300; Applygen Technology Co.). After 30 min of incubation at 30 °C, the cells were collected by centrifugation and resuspended in PBS to 6 × 10⁶ c.f.u. ml⁻¹. ROS was expressed as a fluorescence intensity of DCFH-DA, which was read using a FACScan flow cytometer (EPICS ALTRA II; Beckman) with excitation at 488 nm and emission at 525 nm. At the same time, these experiments were also conducted in the presence of the antioxidant l-cysteine (Cys) at 40 mM. The data were analysed using Expo32 v1.2 software.

Effect of the antioxidant Cys on DSEO-induced ROS production and antifungal effect of DSEO in C. albicans ATCC 64550. Exponentially growing C. albicans ATCC 64550 culture was treated with 0.312 μl DSEO ml⁻¹ in the presence or absence of different Cys concentrations (ranging from 20 to 80 mM) in SDB. After 12 h of incubation at 30 °C, 100 μl aliquots were placed on SDA plates. The number of c.f.u. was counted after 2 days of incubation at 30 °C. The percentage survival was calculated as the ratio of the number of c.f.u. after DSEO treatment compared with the initial inoculum.

Statistical analysis. All experiments were carried out in triplicate and results were reported as means ± sd (n=3). The data were analysed using one-way analysis of variance. Statistically significant effects were also analysed, and the means were compared using Duncan’s multiple range test. A P≤0.05 value was considered statistically significant.

RESULTS AND DISCUSSION

MICs and MFCs

The in vitro antifungal activity of DSEO was investigated against one ATCC type strain (C. albicans ATCC 64550) and two clinical strains of yeasts. The MICs and MFCs for the three C. albicans strains, obtained using the broth dilution method, are shown in Table 1. Evaluation of MIC and MFC values showed that DSEO was active against all the tested strains. For the three C. albicans strains, the MIC (0.625 μl ml⁻¹) and MFC (1.25 μl ml⁻¹) values were the same (Table 1). The MICs values ranged from 1.56 to 100 μg ml⁻¹ for FCZ against the three C. albicans strains. C. albicans 09-1502 was susceptible to FCZ, with a MIC value of 1.56 μg ml⁻¹. However, C. albicans 09-5304 was a non-susceptible strain, with a MIC value of 100 μg ml⁻¹. A previous study reported that FCZ MIC values of ≥2 and ≤2 μg ml⁻¹ indicate non-susceptibility and susceptibility, respectively (Pfaller et al., 2011). Dill has been used for cooking and in Uygur medicine since ancient times in China. DSEO is widely used as an aromatic agent in the

Table 1. Antifungal activities (MICs and MFCs) of DSEO, FCZ and Nys against the Candida spp. tested

<table>
<thead>
<tr>
<th>Strain</th>
<th>FCZ*</th>
<th>Nys*</th>
<th>DSEO†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>MFC</td>
<td>MIC</td>
</tr>
<tr>
<td>C. albicans ATCC 64550</td>
<td>3.12</td>
<td>6.25</td>
<td>1.56</td>
</tr>
<tr>
<td>C. albicans 09-5304</td>
<td>100</td>
<td>≥100</td>
<td>3.12</td>
</tr>
<tr>
<td>C. albicans 09-1502</td>
<td>1.56</td>
<td>3.12</td>
<td>1.56</td>
</tr>
</tbody>
</table>

*MIC and MFC were determined using a macrodilution method and expressed as μg ml⁻¹ (w/v).
†MIC and MFC were determined using a macrodilution method and expressed as μl ml⁻¹ (w/v).
food and pharmaceutical industries, among others. It also covers a broad spectrum of antimicrobial activities, which may be attributed to its main chemical components of carvone and limonene (Bailer et al., 2001). Other authors have also reported that carvone and limonene have antimicrobial activities (Duarte et al., 2005; Filipowicz et al., 2003; Magiatis et al., 1999; Martins et al., 2000; Oumzil et al., 2002; Tzakou et al., 2001; Magwa et al., 2006; Duru et al., 2004).

**Effect of DSEO on membrane integrity**

Dying cells with injured membranes can incorporate PI, which stains nucleic acids (Bauer, 1993). Flow cytometry was used to investigate the effect of DSEO on the integrity of fungal cells, with PI as the fluorescent marker (Cox et al., 2000). Cells with severe membrane lesions that lead to inherent loss of viability internalize PI, resulting in an increase in red fluorescence (Pina-Vaz et al., 2001). Our results showed that PI penetrated over 99.6% of the yeast cells after incubation with 0.625 µl DSEO ml⁻¹ (Fig. 1), indicating that the structure of the cell membrane was disrupted by DSEO. The percentages of stained cells with 0.312 and 0.156 µl DSEO ml⁻¹ were 78.3 and 18.4%, respectively. Under the same experimental conditions, 1.56 µg Nys ml⁻¹ induced cell death in <6.8% of the cells (Fig. 1). Our results indicated that the mechanism of action of the drug involves a primary lesion of the cell membrane, leading to cell death. Furthermore, the effect of DSEO on the cell membrane was higher than that of Nys. A similar result was reported by other authors using different essential oils (Pinto et al., 2006, 2009; Cox et al., 2000).

**Determination of ergosterol amount**

Ergosterol, which maintains cell function and integrity, is the major sterol component of the fungal cell membrane (Rodriguez et al., 1985). We evaluated the effect of DSEO on ergosterol content in our three *C. albicans* strains to determine the target of DSEO in the cell membrane. The MIC values indicate that *C. albicans* 09-1502 was susceptible to FCZ, whereas *C. albicans* 09-5304 was not (Table 1). As shown in Table 2, the mean decrease in the total cellular ergosterol content for the three strains ranged from 33 to 75% after exposure to DSEO at different concentrations. Compared with the control, cells of *C. albicans* ATCC 64550, *C. albicans* 09-1502 and *C. albicans* 09-5304 grown in the presence of subinhibitory concentrations (0.078 to 0.312 µl ml⁻¹) of DSEO revealed lower quantities of ergosterol in their membrane (Table 2). Exposure to 3.12 µg FCZ ml⁻¹ did not affect the amount of ergosterol in *C. albicans* 09-5304, which is non-susceptible to FCZ. However, exposure to 3.12 µg FCZ ml⁻¹ decreased the ergosterol content in the FCZ-susceptible strains *C. albicans* 09-1502 and ATCC 64550 by 62.5%. Exposure to 0.312 µl DSEO ml⁻¹ decreased the ergosterol content in *C. albicans* 09-1502 and ATCC 64550 by 70% and 75%, respectively.

The flow cytometry data and impairment in the biosynthesis of ergosterol both agree with the mechanism of action proposed: lesion of the cytoplasmic membrane. Many essential oils exhibit wide-spectrum antimicrobial activity. Some authors have proposed that different modes of action are involved in the antimicrobial activity of essential oils, such as disruption of cell-membrane structures, leading to an alteration of permeability and a consequent leakage of cell contents. The ability of *Candida* spp. to form drug-resistant biofilms is an important factor, and contributes to human disease. The virulence of *Candida* spp. will be greatly reduced by drugs associated with disruption of biofilms synthesis. The antifungal effects of polyene and azole are due to their actions on the fungal cell membrane (Sardi et al., 2013). Recent investigations on the antifungal action of some essential oils have shown disruption of the fungal membrane (Bakkali et al., 2008; Cox et al., 2000, 2001). These reports suggested that this antimicrobial mechanism results from membrane damage. The results of the present study support this viewpoint. Therefore, the anti-*Candida* activity of DSEO may be

![Fig. 1. Effect of DSEO and Nys on the percentage of PI-stained *C. albicans* ATCC 64550 cells analysed using flow cytometry for 12 h compared with an untreated control. Bars with different letters (a–e) differ significantly (P<0.05).](Image)

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**Table 1.** Total cellular ergosterol content of *C. albicans* strains treated with DSEO for 12 h compared with control.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Control 1.56 Nys 1.25 0.625 0.312 0.156</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>78.3 73.4 68.5 61.2 54.3 47.5</td>
</tr>
<tr>
<td>30</td>
<td>68.5 61.2 54.3 47.5 40.6 33.8</td>
</tr>
</tbody>
</table>

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**Table 2.** Mean percentage decrease in ergosterol content of *C. albicans* strains treated with DSEO for 12 h compared with control.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Control 1.56 Nys 1.25 0.625 0.312 0.156</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
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</tr>
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<td>68.5 61.2 54.3 47.5 40.6 33.8</td>
</tr>
</tbody>
</table>

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**Fig. 1.** Effect of DSEO and Nys on the percentage of PI-stained *C. albicans* ATCC 64550 cells analysed using flow cytometry for 12 h compared with an untreated control. Bars with different letters (a–e) differ significantly (P<0.05).
attributed to its ability to disrupt the permeability barrier of microbial membrane structures.

**Measurement of \( \Delta \psi \)**

The effect of DSEO on \( \Delta \psi \) of the cells was first measured by flow cytometry using Rh123, a potential-dependent distributional probe. The results are shown in Fig. 2. The \( \Delta \psi \) values (calculated from the mean fluorescence of all measured cells) of the cells untreated or treated with DSEO at concentrations of 0.156, 0.312, 0.625 and 1.25 \( \mu \)m l\(^{-1}\) were 61.56 ± 3.51, 74.63 ± 2.64, 179.3 ± 4.13 and 265.76 ± 9.64, respectively. The results showed that DSEO increased the intensity of fluorescent cells in a dose-dependent manner. In addition, DSEO increased \( \Delta \psi \) in *C. albicans* ATCC 64550 in a dose-dependent manner.

The mitochondria play vital roles in the cell, such as participation in apoptosis, pH homeostasis, redox (Zorov et al., 1997) and ATP production. In the mitochondria, ATP is derived from mitochondrial oxidative phosphorylation, in which \( \Delta \psi \) plays an essential role (Jouaville et al., 1999). \( \Delta \psi \) is a sensitive indicator of the energetic state of the mitochondria and the cell (Brand et al., 1994). This indicator can also be used to assess the activity of mitochondrial proton pumps, electrogenic transport systems and mitochondrial permeability transition (Crompton, 1999). The mitochondria of healthy cells maintain \( \Delta \psi \) across their inner membranes, which is the result of an electrochemical gradient maintained through the electron transport chain (Simbula et al., 1997). Inhibitors of mitochondrial electron transport, such as antimycin A and potassium cyanide, decrease \( \Delta \psi \) by inhibiting the proton-pumping function of the respiratory chain, leading to decreased ATP production and cell death. A previous study showed that the antifungal action of farnesol is involved in \( \Delta \psi \) hyperpolarization in *C. albicans* (Machida & Tanaka, 1999).

In the present research, Rh123 was used to examine the effect of DSEO on mitochondrial potentials. Rh123 is a cationic and lipophilic dye that permeates the negatively

### Table 2. Inhibition of ergosterol biosynthesis in *Candida* spp. by DSEO and FCZ

Results are expressed as a percentage wet weight of the cell ± SD (followed in parentheses by the percentage reduction in the mean cellular ergosterol content compared with the control).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Control</th>
<th>FCZ (3.12 ( \mu )g ml(^{-1}))</th>
<th>Mean ergosterol content of cells grown with DSEO at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.078 ( \mu ) ml(^{-1})</td>
</tr>
<tr>
<td><em>C. albicans</em> 09-5304</td>
<td>1.40 ± 0.10</td>
<td>1.35 ± 0.12 (3.51)</td>
<td>0.90 ± 0.13 (37.51)*</td>
</tr>
<tr>
<td><em>C. albicans</em> ATCC 64550</td>
<td>1.20 ± 0.02</td>
<td>0.45 ± 0.05 (62.50)*</td>
<td>0.75 ± 0.11 (37.50)*</td>
</tr>
<tr>
<td><em>C. albicans</em> 09-1502</td>
<td>1.20 ± 0.01</td>
<td>0.45 ± 0.01 (62.50)*</td>
<td>0.80 ± 0.01 (33.33)*</td>
</tr>
</tbody>
</table>

*P < 0.05 compared with the control.

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**Fig. 2.** Effect of DSEO on *C. albicans* ATCC 64550 \( \Delta \psi \) assayed using confocal microscopy (top panels; magnification ×1000) and flow cytometry (bottom panels; excitation at 488 nm and emission at 525 nm) stained with Rh123. (a) Control; (b) DSEO at 0.156 \( \mu \)l ml\(^{-1}\); (c) DSEO at 0.312 \( \mu \)l ml\(^{-1}\); (d) DSEO at 0.625 \( \mu \)l ml\(^{-1}\); (e) DSEO at 1.25 \( \mu \)l ml\(^{-1}\).
charged mitochondria and reflects mtΔψ. Exposure to different concentrations of DSEO for 12 h showed significant hyperpolarization of C. albicans ATCC 64550 mtΔψ in a dose-dependent manner, suggesting mitochondrial dysfunction.

Measurement of mitochondrial dehydrogenases

The effects of DSEO on the activities of mitochondrial dehydrogenases were assayed through a colorimetric method using XTT as the substrate, which can be metabolically reduced to an orange water-soluble formazan product by mitochondrial dehydrogenases in viable cells (Kuhn et al., 2003). DSEO exhibited a dose-dependent inhibitory effect on the activity of the mitochondrial dehydrogenases of C. albicans ATCC 64550 (Fig. 3). Compared with the control group, the relative activities of the mitochondrial dehydrogenases treated with DSEO at concentrations of 0.078, 0.156, 0.312, 0.625, 1.25, 2.5, 5 and 10 μl ml⁻¹ were 98.89, 91.46, 86.69, 75.04, 55.01, 45.87, 38.28 and 28.37 %, respectively.

Mitochondrial dehydrogenases are important catalysing enzymes in ATP biosynthesis. The mitochondrial dehydrogenase activities measured in this assay included lactate dehydrogenase, malate dehydrogenase and succinate dehydrogenase (Lippold, 1982). Lactate dehydrogenase is an enzyme that catalyses the conversion of lactate to pyruvate, the last step in anaerobic glycolysis, and is an important step in cellular energy production. Malate dehydrogenase catalyses the interconversion of malate to oxaloacetate in the tricarboxylic acid cycle, and succinate dehydrogenase catalyses the oxidation of succinate to fumarate in the tricarboxylic acid cycle and transfers the electrons from succinate to ubiquinol (Balietti et al., 2005). Our results showed that the activities of the mitochondrial dehydrogenases were significantly inhibited by DSEO through disturbance of the citric acid cycle and inhibition of ATP synthesis in the mitochondria of C. albicans ATCC 64550.

Measurement of ROS formation and antifungal effect of DSEO in C. albicans ATCC 64550

The fluorescent probe DCFH-DA was used to monitor ROS formation in C. albicans ATCC 64550 (Bonini et al., 2006). DSEO caused a dose-dependent increase in the fluorescence intensity of this C. albicans strain (Fig. 4). The dose-dependent enhancement of ROS generation was evident after 12 h of incubation with DSEO. Compared with the control, the increase corresponded to 3.77-, 7.06-, 9.24- and 10.29-fold at concentrations of 0.156, 0.312, 0.625 and 1.25 μl DSEO ml⁻¹, respectively. These data indicated that DSEO induced ROS accumulation in C. albicans ATCC 64550. We assessed the effect of the antioxidant Cys on DSEO-induced ROS generation and its antifungal action against C. albicans ATCC 64550 to determine whether the observed ROS induction by DSEO was directly involved in its antifungal activity and was not
merely a secondary effect of the action of DSEO. As shown in Fig. 5, the generation of ROS in *Candida* cells treated with DSEO increased significantly after 12 h of incubation. In the presence of Cys (40 mM), the amount of ROS in the cells treated with a high concentration of DSEO (1.25 µl ml⁻¹) was only increased slightly. This finding indicated that Cys can prevent DSEO-induced ROS generation. At the same DSEO concentration, the amount of ROS in the *Candida* cells was clearly less when Cys was present than when Cys was absent. In addition, the c.f.u. assay also showed that Cys prevented DSEO-induced cell death (Fig. 6). The *Candida* cells were treated with DSEO (0.312 µl ml⁻¹) at different concentrations of Cys (0 mM to 80 mM) for 12 h at 30 °C. The c.f.u. were counted after 24 h of incubation at 30 °C. Cys caused a dose-dependent increase in the cell survival. These data indicate that ROS formation was the key event in the DSEO-induced death of *C. albicans* ATCC 64550.

The mitochondria are a major subcellular source of ROS in eukaryotic cells (Lesnfsky *et al.*, 2001; Lenaz, 2001). The individual radical species involved in fungi are superoxide, hydroxyl radicals and peroxynitrite. Many factors cause endogenous ROS production in cells, such as irradiation and cytotoxic drugs (Moradas-Ferreira *et al.*, 1996). ROS lead to enzyme inactivation, cell dysfunction and then cell death; they also play physiological roles in the regulation of apoptosis (Perrone *et al.*, 2008). Recent research has demonstrated that the antifungal action of many antifungal agents is involved in the induction of ROS formation in *C. albicans* ATCC 64550. Many antifungal agents, such as azole antifungals, amphotericin B and Nys (Kobayashi *et al.*, 2002), interact with ergosterol in the fungal membrane, cause membrane permeabilization and then induce ROS in susceptible fungi. Moreover, the isoprenoid alcohol farnesol (Machida *et al.*, 1998), natural perylenequinonoid pigments (Xing *et al.*, 2003) and several antifungal peptide proteins can induce ROS in yeast species. To the best of our knowledge, the present study is the first to show that the antifungal activity of DSEO is causally linked with an intracellular process in *C. albicans* ATCC 64550, which is the induction of endogenous ROS. Normally, higher Δψm is accompanied by a more efficient electron transportation process, which in turn leads to the leakage of more electrons to generate ROS. Previous studies have indicated that ROS generation is exponentially dependent on Δψm (Starkov & Fiskum, 2003), which is consistent with the results of our study. That is, both Δψm and endogenous ROS were obviously augmented in *C. albicans* ATCC 64550 after DSEO treatment.

In the present study, DSEO caused mitochondrial hyperpolarization in *C. albicans* ATCC 64550, indicating mitochondrial dysfunction. Consequently, the effects of DSEO on ROS production in *C. albicans* ATCC 64550 were assayed. The results showed that DSEO caused intracellular ROS production in *C. albicans* ATCC 64550. Furthermore, the addition of the antioxidant Cys prevented both the generation of ROS and the antifungal effect of DSEO. This finding indicates that ROS is an important mediator of the antifungal action of DSEO. The mitochondrial dysfunctions promoted ROS accumulation in *C. albicans* ATCC 64550, causing cell death through oxidative damage to biomacromolecules or mediation of apoptosis. The results indicate that DSEO exerts its antifungal activity through mitochondrial dysfunction-induced ROS accumulation in

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**Fig. 5.** Effect of the antioxidant Cys on DSEO-induced ROS generation in *C. albicans* ATCC 64550 cells analysed using flow cytometry (excitation at 488 nm and emission at 520 nm). AU, arbitrary units.

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**Fig. 6.** Effect of the antioxidant Cys on the antifungal effect of DSEO in *C. albicans* ATCC64550. Treatment of *Candida* cells with (+) or without (−) DSEO is indicated.
C. albicans ATCC 64550. All of the above-mentioned data indicate that the cytoplasmic membrane and mitochondria are the main anti-Candida targets of DSEO.

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