Anticandidal effect of *Syzygium aromaticum* on biofilm formation, cell surface hydrophobicity, and cell cycle

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Accepted 10 February, 2012

*Candida* infections are frequently associated with biofilm formation occurring on the surfaces of host tissues and medical devices. Biofilms have been shown to display significantly increased levels of resistance to the conventional antifungal agents. Higher cell surface hydrophobicity (CSH) is related with increased virulence of *C. albicans* due to stronger adherence to surfaces. Cloves, the aromatic dried flower buds of *Syzygium aromaticum*, and their oil have been widely used for medicinal purpose. The aim of this study was to assess the anticandidal potential of the *S. aromaticum* ethyl acetate extract by evaluating biofilm formation, CSH, and cell cycle of *C. albicans*. The growth and development of the biofilm was assessed using an XTT reduction assay, and the extract remarkably reduced (27.7 ± 14.0%) biofilm formation of the clinical strains. It significantly lowered CSH (61.1 ± 15.0%) as well. Moreover, the extract arrested the fungus cells at the G₀/G₁ and S phase. The *S. aromaticum* extract displayed fungistatic activity without any hemolytic activity. Based on the combined results of this study, *S. aromaticum* holds great promise for use in treating and eliminating biofilm-associated *Candida* infection.

**Key words:** Antifungal activity, biofilm, *Candida albicans*, cell cycle, cell surface hydrophobicity, *Syzygium aromaticum*.

**INTRODUCTION**

*Candida* species are opportunistic pathogens that cause superficial systemic diseases in critically ill or immunocompromised patients (Pfaller and Diekema, 2007). *C. albicans* is still documented as a leading fungal pathogen, although pathogenic non-*albicans* species, such as *C. krusei*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis* and *C. guilliermondii*, are continuously being discovered (Krcmery and Barnes, 2002).

The virulence of *Candida* species has been attributed to several factors, such as promoting the formation of hyphae and biofilms, secreting hydrolyses such as aspartic proteinases and phospholipases, adhering to host tissues, and responding to environmental changes and morphogenesis (Haynes, 2001). One of the most important characteristics of *C. albicans* is the ability to colonize on both biological and inert surfaces including intravascular catheters (Ramage et al., 2006), allowing for the formation of a biofilm structure that can subsequently infect human hosts. Recent studies suggest that the majority of diseases produced by this pathogen are associated with the biofilm growth (Blankenship and Mitchell, 2006). Fungal biofilms maintain their niche by evading host immune mechanisms, withstand the competitive pressure from other organisms, and resisting antifungal treatment (Chandra et al., 2001). Biofilm formation of *C. albicans* is a highly complex phenomenon, distinct from fungal adhesion and dependent on many factors (Chandra et al., 2001; Thein et al., 2007). A direct correlation between biofilm formation and CSH has been suggested, where higher CSH was shown to increase the virulence of *C. albicans* due to stronger adherence to substrates such as mucin, epithelial and endothelial cells, and increased resistance to phagocytic killing (Hazen, 1989; Glee et al., 1995; de Repentigny et al., 2000).

Treatment of *Candida* infections is difficult due to the
eukaryotic nature of fungal cells. For the last decades, treatment has relied on fungicidal polyene drugs such as amphotericin B, which binds to the fungal ergosterol. More recently, fungistatic drugs such as the azoles have become more widely used to treat fungal infection due to the comparative ease of their use (Oliver et al., 2008). However, extensive use of these available drugs has led to the emergence of resistant strains and a variety of associated problems due to serious side effects at the therapeutic dosage (Mahmoud and Louis, 1999). Thus, the discovery of novel antifungal agents with low toxicity and high therapeutic activity is urgently needed. Many different plant-derived compounds have gained interest for use as alternatives to traditional microbial control strategies, since these compounds are widely believed to be safe and have a long history of use in folk medicine for the prevention and treatment of diseases and infections (Guarrera, 2005).

Syzygium aromaticum, belonging to Myrtaceae, is a moderate-sized, conical, evergreen tree that attains a height up to about 12 m. The flower bud of S. aromaticum, commonly known as clove, is one of the oldest aromatic spices of the world and a popular remedy for dental disorders, respiratory disorders, headache and sore throat in traditional medicines over the centuries due to its pharmacological properties, including anti-inflammatory, antioxidant, anticarcinogenic, antibacterial and antifungal effects (Lee and Shibamoto, 2001; Mishra and Singh, 2008; Pinto et al., 2009).

The present study was aimed to assess the antifungal effects of the S. aromaticum extract on C. albicans pathogens and its mode of action for antifungal activity against fungal pathogens.

MATERIALS AND METHODS

Plant material and extraction

Flower buds of S. aromaticum were purchased from jchanbang.com, Korea. A voucher specimen was deposited at the Department of Oriental Medicine, Semyung University. Dried S. aromaticum flower buds (200 g) were soaked in 2 L of distilled water for 1 h and boiled for 2 h. They were then centrifuged at 2000 g for 20 min, and the supernatant was concentrated using a vacuum evaporator and lyophilized to yield an aqueous extract. The dried extract (14.4 g) was resuspended in 200 ml distilled water and then serially fractionated with n-hexane, chloroform, ethyl acetate and n-butanol. Each fraction was concentrated, lyophilized and subjected to an antifungal susceptibility test, and the ethyl acetate fraction (0.26 g) was determined to be the most active. The ethyl acetate fraction, which was called the S. aromaticum extract, was dissolved in methanol to 5 mg/ml, and kept at -20°C until used.

Strains and growth

Candida albicans (KCCM 50235, ATCC 18804), C. glabrata (KCCM 50044, ATCC 2001), C. tropicalis (KCCM 50075, ATCC 750) and C. kruzie (KCCM 11426, ATCC 32196) were obtained from Korean Culture Center of Microorganisms (KCCM). A total of 16 clinical C. albicans isolates were used in this study, and kindly provided by Prof. K. H. Lee (Yonsei University, Wonju, Korea). Biofilm formation was assessed with the XTT [2, 3-Bis (2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide] reduction assay using menadione in 98 clinical isolates from candidemic patients (Park et al., 2009). High biofilm formers (H1 to H8) and low biofilm formers (L1 to L8) were defined as isolates that exhibited XTT activity between 1.87 and 2.00, and between 0.42 and 0.75. Isolates were retrieved from storage at -70°C and subcultured twice on YM agar to ensure optimal growth.

Antifungal susceptibility test

Antifungal susceptibility was determined according the CLSI M27-A3 microdilution protocol with minor modifications, which is based on the colorimetric broth microdilution method using resazurin (Liu et al., 2007). All assays were repeated three times. The minimum inhibitory concentration (MIC) was chosen using two criteria: the highest MIC of the three tests if they were different or the MIC that was reproduced in at least two assays. Sterility and growth controls in the presence of organic solvents employed in sample preparation were also included. No inhibitory effects were observed in the presence of the solvent control at the highest concentration used (1% v/v). The positive control drug amphotericin B was purchased from Sigma.

Time-kill assay

The C. albicans cell suspension was diluted in RPMI 1640 buffered to pH 7.0 with 0.165 M MOPS to give a final inoculum concentration of 2 × 10⁶ CFU/ml, which was used in a broth microdilution. Experimental cultures were grown in the absence and presence of the plant extract (25 μg/ml) at 37°C with shaking. At predetermined time points, a 100 μl aliquot was removed from the cell suspension and diluted with distilled water. A 100 μl aliquot from each dilution was streaked on YM agar plates, and colony counts were determined after incubation at 37°C for 24 h. The colony counts represented the average measurements conducted in triplicate of one of the three independent assays.

Microscopic evaluation of the effect of S. aromaticum on C. albicans

C. albicans cells at the exponential growth phase (1 × 10⁵ CFU/ml) were incubated with 12.5, 25 and 50 μg/ml of the plant extract along with a negative control at 37°C. The cells were examined using an inverted phase contrast microscope.

Flow cytometry analysis on fungal cell cycle

C. albicans cells (1 × 10⁵ cells) at the exponential growth phase were treated without or with 25 μg/ml S. aromaticum. After incubation at 37°C with shaking, the cells were harvested, washed with phosphate-buffered saline (PBS) and fixed with 70% ethanol overnight at 4°C to permeabilize the cell membrane. The cells were treated with 200 μg/ml RNase A (Sigma), and the mixture was left to react for 2 h at 37°C. For DNA staining, propidium iodide (PI) was added to a final concentration of 50 μg/ml, and the mixture was incubated for 1 h at 4°C in the dark. Flow cytometry analysis was performed using a FACS Calibur flow cytometer (Becton Dickinson, USA). Data obtained from one run out of two independent experiments, which were conducted in duplicate, are presented.
Effect on cytoplasmic membrane

The C. albicans cell suspension was diluted in RPMI 1640 medium to give a final inoculum concentration of $1 \times 10^5$ CFU/ml. Experimental cultures were grown without or with 25 µg/ml of the plant extract at 37°C for 4 h. PI was added to a final concentration of 1 µg/ml and incubated for 30 min at room temperature. Confocal laser microscopy (Olympus) was used to evaluate the effect of S. aromaticum.

Germ-tube formation assays

Serial dilutions of the plant extract ranging from 50 to 12.5 µg/ml were made in a 96-well microtiter plate containing 0.1 ml of sterile fetal bovine serum. 10 µl of a C. albicans suspension ($1 \times 10^6$ CFU/ml) was added to each well. After incubation at 37°C for 3 h, the microtiter plate was evaluated for formation of germ tubes, using an inverted phase contrast microscope.

Determination of antibiofilm activity

For each strain tested, the C. albicans cell suspension was diluted in Yeast Nitrogen Base supplemented with 5% glucose (YNB) to a concentration of $1 \times 10^6$ cells/ml. Biofilm formation was achieved by aliquoting 0.1 ml of the fungal culture into a 96-well microtiter plate, and the plate was incubated at 37°C for 3 h to allow C. albicans to form biofilms. The plate was washed twice with YNB to remove planktonic yeasts, and the biofilms were treated with 0.2 ml of the plant extract to investigate the inhibitory activity during the initial steps of biofilm formation. The plate was incubated for an additional 24 h at 37°C with moist air. The viability of the biofilm remaining on the surfaces of the wells was quantified using the XTT reduction assay (Ramage et al., 2001). All the tests were performed in triplicate. The baseline values were normalized to 100 and the results were expressed in percentages of inhibition. The data from one of two independent experiments are presented.

Cell surface hydrophobicity (CSH)

For each strain tested, the C. albicans cell suspension was diluted in yeast nitrogen base (YNB) to a concentration of $1 \times 10^6$ cells/ml and incubated in the absence and presence of the plant extract (25 µg/ml) at 37°C for 3 h. The culture was washed twice and resuspended in 1 ml PBS. For each cell suspension, the OD$_{660}$ was determined after diluting with PBS. The CSH of the cells in the suspension was determined using the biphasic separation method (Klotz et al., 1985) with slight modifications. For each strain tested, 1.2 ml of the cell suspension, which was overlaid with 0.3 ml of n-octane (Sigma), was vortexed vigorously for 3 min and then the phases were allowed to separate. The upper octane layer was carefully removed and the OD$_{660}$ of the lower aqueous phase was measured after diluting with PBS. Optical density values of the Candida strains without octane overlay were used as controls. The relative CSH was calculated as follows: $\frac{[\text{OD}_{660} \text{ of the control} - \text{OD}_{660} \text{ after n-octane overlay}]/\text{OD}_{660} \text{ of the control}}{100}$. The data are one of the results of two independent experiments.

Statistical analysis

To compare variation and correlation, means and SDs of biofilm formation and CSH were calculated for each sample. The statistical significance in the difference among samples was compared using the Student t-test. Regression analysis, Pearson’s correlation index (r), and multiway analysis of variance were used to determine the relationship between CSH and biofilm formation of untreated and S. aromaticum-treated test groups. The correlation coefficient ranges from -1 (perfect negative correlation) through 0 (no correlation) to 1 (perfect positive correlation). A p value of < 0.05 was considered statistically significant.

Hemolytic activity

To assess the cytotoxicity effects of S. aromaticum against human blood cells, hemolytic activity was evaluated by the percentage of hemolysis (Sharma and Sharma, 2001). The hemolysis percentage was calculated using the following equation: % hemolysis = $\frac{[A_{450} \text{ of test compound treated sample} - A_{450} \text{ of buffer treated sample}]/A_{450} \text{ of 1% Triton X-100 treated sample}]}{100}$. Hemolysis was also checked using an inverted contrast microscope.

RESULTS

The antifungal activity of S. aromaticum fractions was evaluated against C. albicans, C. glabrata, C. krusei and C. tropicalis using microdilution assays (Table 1). The ethyl acetate fraction of S. aromaticum extract showed the best activity against C. albicans ATCC 18804 with the MIC of 25 µg/ml. The plant extract was also tested against 16 C. albicans clinical isolates, and the MIC values ranged between 12.5 and 50 µg/ml. The MICs of 13 out of 16 isolates tested were 25 µg/ml. C. albicans ATCC 18804 was used for the experiment and 8 C. albicans strains that were high biofilm formers (H1 to H8) were used in the biofilm and CSH experiments.

The antifungal activity of the plant extract against C. albicans was determined using a colony count assay. The results of time-kill curve analysis are presented in Figure 1. The plant extract at a concentration of 25 µg/ml weakly inhibited growth compared to the control. The relative viability was less than 10% at 24 h.

The effect of 1× MIC of the plant extract (25 µg/ml) on the growth and morphology of C. albicans cells ($2 \times 10^4$ CFU/ml) was examined using an inverted phase contrast microscope (Figure 2). Analyses of the microscopic images showed that control cells displayed a normal budding profile, but irregular budding patterns and elongated pseudohyphae were observed in yeasts treated with the 0.5× MIC of the plant extract for 24 h (Figure 2B). The density of C. albicans cells decreased remarkably in yeasts treated with the 1× or 2× MIC of the extract, and the fungus cells were yeast forms (Figure 2C and 2D). Similarly, pseudohyphae were observed in yeasts incubated with 1× MIC of the extract for a few hours (data not shown). When 1× MIC of the plant extract was incubated with $1 \times 10^5$ CFU/ml of C. albicans cells for 6 h, chlamydospores were widely detected (Figure 2E).

To understand how S. aromaticum affects cellular physiology, the effects of S. aromaticum on the cell cycle progression of C. albicans were examined. The cells
Table 1. Minimum inhibition concentrations (µg/ml) of the S. aromaticum extract.

<table>
<thead>
<tr>
<th>Candida species</th>
<th>MIC (µg/ml)</th>
<th>Aqueous extract</th>
<th>Fractions</th>
<th>Amphotericin B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ethyl acetate</td>
<td>Butanol</td>
</tr>
<tr>
<td>C. albicans ATCC 18804</td>
<td>782</td>
<td>25.0</td>
<td>50.0</td>
<td>0.25</td>
</tr>
<tr>
<td>C. glabrata ATCC 2001</td>
<td>25.0</td>
<td>6.3</td>
<td>12.5</td>
<td>0.50</td>
</tr>
<tr>
<td>C. krusei ATCC 750</td>
<td>98.0</td>
<td>12.5</td>
<td>12.5</td>
<td>0.25</td>
</tr>
<tr>
<td>C. tropicalis ATCC 32196</td>
<td>3,125</td>
<td>25.0</td>
<td>50.0</td>
<td>0.25</td>
</tr>
<tr>
<td>C. albicans (clinical, n =16)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H1~H8</td>
<td>ND*</td>
<td>25.0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>L1, L2, L4, L7, L8</td>
<td>ND</td>
<td>25.0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>L3, L6</td>
<td>ND</td>
<td>50.0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>L5</td>
<td>ND</td>
<td>12.5</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*ND: Not determined.

Figure 1. Effect of S. aromaticum extract on the viability of C. albicans. The fungus cells (2 × 10⁴ CFU/ml) were incubated in the absence (control) and presence of S. aromaticum (25 µg/ml) at 37°C, and a colony count assay was used to determine the candidacidal activity of the plant extract against the fungus.

were cultured in the absence or presence of S. aromaticum, and the DNA content was determined via flow cytometry after staining with PI. PI is a DNA staining dye that intercalates between the bases of DNA or double-stranded RNA molecules. Analysis of the cell cycle indicated that treatment with the plant extract arrested the cell cycle of the fungus at the G₀/G₁ and S phase (Figure 3).

C. albicans cells treated without and with the plant extract were not stained with the fluorescent dye PI (Figure 4), indicating that the extract did not induce cell membrane lesions. Effect on germ tube formation was examined, but no differences in germ tube formation were observed between treated and untreated yeast cells (data not shown).

The major consequence of biofilm formation is the increased resistance of sessile cells to antibiotic treatment (Ramage et al., 2006). Adherence is a key step in biofilm formation. In order to investigate the effect of the plant extract on the early phase of biofilm formation, the metabolic activity of biofilms aged 3 h, which is considered the initial phase of biofilm formation, was
Figure 2. Microscopic view of *C. albicans* culture treated without (A) and with 12.5 µg/ml (B), 25 µg/ml (C), and 50 µg/ml (D) of the plant extract for 24 h, respectively. Panel E shows the result (6 h) of the experiment with 25 µg/ml of the plant extract using a starting culture of $2 \times 10^6$ CFU/ml on a different day. The arrowhead indicates the formation of chlamydosores.

measured after exposure to *S. aromaticum*. As shown in Table 2, the reduction in metabolic activity of *C. albicans* isolates reached 50.2% (27.8 ± 14.0%) after treatment with 25 µg/ml of the plant extract. Biofilms of 8 *C. albicans* strains showed less metabolic activity ranging from 23.6 to 60.9% (40.8 ± 12.0%) after treating with 50 µg/ml of the plant extract.

The effect of the plant extract on the CSH of *C. albicans* was examined. The mixture of hydrophobic and hydrophilic cells was separated using the biphasic separation method. The CSH of the 8 clinical isolates of *C. albicans* ranged from 12.5 to 27.2% (21.4 ± 4.7%). *S. aromaticum* treatment for 3 h decreased the CSH of *C. albicans* to 8.6 ± 4.4%. The reduction in CSH after treatment with the plant extract was 61.1 ± 15.0% (Table 3).

Correlation of CSH values of *C. albicans* isolates with their biofilm formation gave a Pearson coefficient of $r = 0.418$ (n = 16). The calculated t value (1.721) was less than the tabulated value of t (1.761; p = 0.05). Therefore, biofilm formation of *C. albicans* isolates was not correlated with the CSH values of the respective isolates.

The hemolysis at various concentrations of *S. aromaticum* was measured against human erythrocytes, and no hemolytic activity was observed up to 50 µg/ml of the plant extract. However, crenated erythrocytes were
Figure 3. Effect of *S. aromaticum* extract on the cell cycle progress of *C. albicans*. Subcultured *C. albicans* cells (1 × 10^8 cells) were treated without A and B, and with 25 µg/ml of the plant extract and incubated for 30 and 120 min, respectively. After washing with PBS, the cells were fixed in 70% ethanol overnight, and then stained with 50 µg/ml of PI.

seen only in 50 µg/ml *S. aromaticum*-treated erythrocytes out of tested concentrations of 50, 25 and 12.5 µg/ml (data now shown). These results demonstrate that the *S. aromaticum* extract was safe at 1 × MIC (25 µg/ml).

**DISCUSSION**

The opportunistic fungal pathogen *C. albicans* can grow in a variety of morphological forms depending on environmental conditions. *C. albicans* typically forms buds, but under different conditions elongated pseudohyphae, filamentous chains of cells termed hyphae, or large rounded cells termed chlamydospores can be induced to form (Calderone and Fonzi, 2001; Kumamoto, 2002; Martin et al., 2005). The dimorphic transition of *C. albicans* from yeast to the mycelia form is responsible for pathogenicity, where mycelia shapes are predominantly found during host tissue invasion and are thought to be important for biofilm formation (Kumamoto, 2002). The present study showed that the plant extract did not exert antifungal activity towards the serum-induced mycelial structure of *C. albicans*, but rather reduced biofilm formation at an early stage of biofilms. The plant extract was not or less effective against a high density of *C. albicans* cells.

PI is a fluorescent probe that has been used to study the effect of drugs on membranes. It penetrates cells with severe membrane lesions only, resulting in increased red fluorescence (Pina-Vaz et al., 2001). PI did not penetrate the cell membranes of *S. aromaticum*-treated *C. albicans* cells irrespective of incubation time up to 30 h, indicating that the plant extract did not damage the cell membrane or kill *C. albicans* cells directly. The fungistatic effect of *S. aromaticum* was confirmed by cell cycle analysis with the fungus. The analysis demonstrated that a high proportion of *S. aromaticum*-treated *C. albicans* cells stay at the G₂/M phase due to inhibition of cell cycle progression to the G₂/M phase. The data indicate that *S. aromaticum* inhibits some cellular progresses that are...
Figure 4. Images of *C. albicans* using confocal laser microscope. Cells with membrane damage were stained with PI (red signals): (A) untreated control cells; (B) cells treated with 25 µg/ml of the *S. aromaticum*; (C) the fungus cells of a single colony which were grown on a YM agar plate and stored for 7 days at 4°C.

Table 2. Metabolic activity and percent inhibition of *C. albicans* biofilms after *S. aromaticum* treatment.

<table>
<thead>
<tr>
<th><em>C. albicans</em> strains</th>
<th>Concentration of <em>S. aromaticum</em> (µg/ml)</th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td><strong>Metabolic activity</strong>a &amp; <strong>Inhibition</strong>b (%)</td>
<td><strong>Metabolic activity</strong>a &amp; <strong>Inhibition</strong>b (%)</td>
<td><strong>Metabolic activity</strong>a &amp; <strong>Inhibition</strong>b (%)</td>
</tr>
<tr>
<td>H1</td>
<td>0.312 ± 0.026 &amp; 33.0</td>
<td>0.122 ± 0.006** &amp; 60.9</td>
<td></td>
</tr>
<tr>
<td>H2</td>
<td>0.265 ± 0.041 &amp; 27.2</td>
<td>0.166 ± 0.007 &amp; 37.4</td>
<td></td>
</tr>
<tr>
<td>H3</td>
<td>0.217 ± 0.040 &amp; 50.2</td>
<td>0.102 ± 0.011* &amp; 53.0</td>
<td></td>
</tr>
<tr>
<td>H4</td>
<td>0.194 ± 0.029 &amp; 32.0</td>
<td>0.115 ± 0.004* &amp; 40.7</td>
<td></td>
</tr>
<tr>
<td>H5</td>
<td>0.152 ± 0.007 &amp; 13.8</td>
<td>0.096 ± 0.001** &amp; 36.9</td>
<td></td>
</tr>
<tr>
<td>H6</td>
<td>0.124 ± 0.004 &amp; 21.8</td>
<td>0.087 ± 0.001** &amp; 29.8</td>
<td></td>
</tr>
<tr>
<td>H7</td>
<td>0.123 ± 0.001 &amp; 5.7</td>
<td>0.094 ± 0.007* &amp; 23.6</td>
<td></td>
</tr>
<tr>
<td>H8</td>
<td>0.096 ± 0.011 &amp; 38.6</td>
<td>0.053 ± 0.001* &amp; 44.8</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>27.7 ± 14.0</td>
<td>40.8 ± 12.0</td>
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</table>

*aMetabolic activity was assessed using the XTT assay measuring the absorbance at 492 nm. bInhibition percentages were calculated as 100 × (1 - A<sub>492</sub> with *S. aromaticum*/A<sub>492</sub> without *S. aromaticum*). The differences in the optical density (mean ± standard deviation) of individual biofilms incubated with *S. aromaticum* extract and control values were calculated using Paired Student’s t-test, and were considered statistically significant when p-value was ≤ 0.05. All experiments were performed three times with four replicates. *p ≤ 0.05, **p ≤ 0.01.*
involved in normal bud growth or DNA replication. Chlamydospores are generally defined as thick-walled asexual spores that are derived from a hyphal cell (Martin et al., 2005). The role of chlamydospores is unclear, but they act as a resting form in other species. During chlamydospore morphogenesis, cells switch to filamentous growth and develop elongated suspensor cells that give rise to chlamydospores (Martin et al., 2005). In our experiment, some enlarged chlamydospores were easily seen, usually after a few hours of exposure of the plant extract to the fungus cells. Chlamydospores were detected when the density of \(C.\) \textit{albicans} was relatively high in the \(S.\) \textit{aromaticum}-treated \(C.\) \textit{albicans} culture. In contrast, when the cell density of the fungus culture was low, a large portion of the fungus cells were yeast forms in the \(S.\) \textit{aromaticum}-treated culture compared with the large and oval healthy controls (Figures 2C and 4B). It seems that the cellular stress induced by \(S.\) \textit{aromaticum} made the fungus cells change their shapes into pseudohyphae, chlamydospores and smaller yeasts through the arrest of cell cycle progression.

Microbial infections in human beings have been estimated to be correlated with biofilm formation in 65% of cases (Ramage et al., 2006). During biofilm formation, \(C.\) \textit{albicans} cells express several genes that are involved in adhesion including the amyotrophic lateral sclerosis (ALS) protein family (Hoyer, 2001), carbohydrate synthesis, drug resistance, such as efflux pumps, and quorum sensing (Chandra et al., 2001; Karkowsk-Kuleta et al., 2009). A tighter adherence of \(C.\) \textit{albicans} to epithelial and endothelial cells is achieved through a higher CSH, and Csh1p is one of the proteins that enhance the CSH of the fungus, which facilitates specific receptor-ligand interactions (Singleton et al., 2001). CSH is also associated with the cell wall architecture of the fibril structure, which is composed of mannoproteins (Masuoka and Hazen, 2004). \(C.\) \textit{albicans} regulates CSH by altering the conformation of the mannoprotein fibrils, and yeast CSH is determined by the presence of hydrophilic surface fibrils (Hazen and Hazen, 1992). Adhesion of \(C.\) \textit{albicans} to host cells depends on the interactions between mannoproteins with lectin-like properties and fucosyl or glucosaminyl glycosides on the surface of epithelial cells (Ruiz-Herrera et al., 2006). In this study, the CSH of 8 clinical isolates of \(C.\) \textit{albicans} with high virulence was examined as well as the effect of \(S.\) \textit{aromaticum} on CSH. The CSH of the fungus isolates ranged between 12.5 and 27.2%, which coincides with the CSH of the \(C.\) \textit{albicans} genotype A, B and C (Borecka-Melkusova and Bujdaková, 2008). Exposure of the plant extract to the fungus isolates significantly decreased the CSH (61.2 ± 15.0%). The same concentration of the plant extract also inhibited biofilm formation fairly (27.7 ± 14.0%). Reports on the correlation between CSH and adhesion to polystyrene, acrylic and tissue surfaces are conflicting. Our report shows that biofilm formation of \(C.\) \textit{albicans} isolates did not correlate with the CSH values of the respective isolates. These results indicate that many other factors are involved in reducing biofilm formation of \(C.\) \textit{albicans}.

The previously reported antifungal activity of \(S.\) \textit{aromaticum} was mostly based on eugenol, which is the major constituent of clove essential oil (85.3% of the total composition) (Pinto et al., 2009). However, the antifungal activities of the plant extract used in this study did not originate from eugenol for the following reasons: First, eugenol was not detected in the plant extract by thin layer chromatography (TLC) analysis with eugenol as a reference compound (data not shown). Second, eugenol inhibited germ tube formation (Pinto et al., 2009), but the plant extract did not. Third, eugenol caused an extensive lesion of the cell membrane in \(C.\) \textit{albicans} leading to fungicidal effect (Pinto et al., 2009), but \(S.\) \textit{aromaticum}

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**Table 3.** Effect of the \(S.\) \textit{aromaticum} extract (25 µg/ml) on the cell surface hydrophobicity (mean ± standard deviation) of \(C.\) \textit{albicans} isolates.

<table>
<thead>
<tr>
<th>(C.) \textit{albicans} isolates</th>
<th>Control</th>
<th>(S.) \textit{aromaticum}</th>
<th>Amount of reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>22.9 ± 2.8</td>
<td>8.7 ± 5.2**</td>
<td>62.0</td>
</tr>
<tr>
<td>H2</td>
<td>27.2 ± 2.7</td>
<td>10.6 ± 4.1**</td>
<td>61.0</td>
</tr>
<tr>
<td>H3</td>
<td>26.4 ± 3.9</td>
<td>10.2 ± 2.4**</td>
<td>61.3</td>
</tr>
<tr>
<td>H4</td>
<td>12.5 ± 1.9</td>
<td>4.3 ± 1.9**</td>
<td>65.6</td>
</tr>
<tr>
<td>H5</td>
<td>16.3 ± 1.4</td>
<td>3.9 ± 1.9**</td>
<td>76.0</td>
</tr>
<tr>
<td>H6</td>
<td>18.7 ± 3.0</td>
<td>3.2 ± 3.1**</td>
<td>82.8</td>
</tr>
<tr>
<td>H7</td>
<td>24.4 ± 3.3</td>
<td>14.9 ± 6.3</td>
<td>38.9</td>
</tr>
<tr>
<td>H8</td>
<td>22.5 ± 5.6</td>
<td>13.1 ± 4.3*</td>
<td>41.7</td>
</tr>
<tr>
<td>Mean</td>
<td>21.3 ± 4.7</td>
<td>8.6 ± 4.4</td>
<td>61.1 ± 15.0</td>
</tr>
</tbody>
</table>

The differences in the CSH (mean ± standard deviation) of individual \(C.\) \textit{albicans} incubated with \(S.\) \textit{aromaticum} extract and control values were calculated using Paired Student’s t-test, and were considered statistically significant when the \(P\)-value was ≤ 0.05. *\(p\) ≤ 0.05, **\(p\) ≤ 0.01.
extract did not induce the lesion of the cell membrane in the fungus. Consequently, the results of the antifungal activity come from novel compounds in the plant extract.

Many antifungal agents have limited clinical applications, because they also cause cytolysis of human erythrocytes. The results of the hemolysis test showed that 25 µg/ml of the plant extract did not produce cytolysis of human erythrocytes.

Conclusion

The results of this paper indicate that the antifungal activity of the *S. aromaticum* extract against *C. albicans* is due to multiple effects including inhibiting biofilm formation, decreasing CSH and arresting cell cycle at the G$_2$/G$_1$ and S phase. The findings indicate that the plant extract have a good potential for use in the treatment of biofilm-associated *Candida* infection.

ACKNOWLEDGEMENT

This work was supported by a Korea University Grant to H. S. Lee.

REFERENCES


