Abstract. – Background and Objective: *Candida* (*C.*) *albicans* infection in its biofilm mode of growth has taken centrepoint with the increasing recognition of its role in human infections due to the development of resistance to the commonly used antibiotic or phenotypic adaptation within the biofilm. Hence, in this study the inhibitory effect of methanol extract of *Cassia* (*C.*) *spectabilis* leaves was evaluated against biofilm forming *C. albicans*.

Materials and Methods: Anti-yeast activities were carried out using disc diffusion assay and broth dilution method against biofilm forming *C. albicans*. *C. spectabilis* leaves extract was assessed using XTT (2,3-bis-[2-Methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide) reduction assay for biofilm quantification with positive control fluconazole. Scanning electron microscopic (SEM) and confocal scanning laser microscopy (CLSM) analysis further revealed reduction in *C. albicans* biofilm by *C. spectabilis* leaves extract.

Results and Discussion: The methanol extract of *C. spectabilis* showed a favorable anti-yeast activity against *C. albicans* with MIC (Minimum Inhibitory Concentration) value of 6.25 mg/ml. Fluconazole and leaves extract showed 95.4% and 96.9% biofilm reduction respectively. The main changes observed under scanning electron microscopy after *C. spectabilis* leaves extract treatment were cellular damage and disruption in biofilms of *C. albicans*. The ultrastructural changes visualized by SEM were further confirmed using CLSM study.

Conclusions: The results from this research conclusively exhibit the in vitro anti-biofilm potential of *C. spectabilis* leaves extract against Candida biofilm.

Key Words:


Introduction

There are many antifungal agents commercially available in pharmacies. However, the action of these antifungal agents may be limited by their penetration and chemical reaction into biofilm matrix, the extracellular polymeric material\(^1\)\(^-\)\(^3\). Therefore, we need a new series of antifungal compounds that have a high efficiency and low cost. As a result, we have started to test natural products based materials such as edible medicinal plants. Malaysia is rich in medicinal plant diversity in all three levels of biodiversity such as species, genetic and habitat diversity\(^4\). One of such plant rich in medicinal value from Malaysia is *Cassia* (*C.*) *spectabilis*. The genus *Cassia*, comprising about 600 species widely distributed worldwide is well known for its diverse biological and pharmacological activities. *Cassia spectabilis* (*sin Senna spectabilis* (DC) Irwin et Barn (Leguminosae) is widely grown as an ornamental plant in tropical and subtropical areas. *Cassia spectabilis* has been commonly used in traditional medicine for many years. It has been used in traditional Brazilian medicine for the treatment of flu and cold, as laxative and purgative\(^5\). Moreover, various biological activities have been reported in literature such as antifungal\(^6\), antibacterial\(^7\), and antioxidant activities\(^8\).

In recent years, the interest in plant-based medicine has increased noticeably worldwide. At least 80% of the world’s population in developing countries uses plant materials as their source of primary health care\(^9\). Hence, the current research was attempted to explore new and effective natural products showing anti-biofilm activity against *Candida* (*C.*) *albicans* biofilm and the management of biofilm-associated *C. albicans*...
infections. The outlined work evaluates the potential of *C. spectabilis* leaves extract and their effect on biofilm formation by *C. albicans*.

**Materials and Methods**

**Plant Material**

Fresh *Cassia spectabilis* leaves were collected from University Sains Malaysia campus, Penang, Malaysia in April 2009 and authenticated by the botanist of the School of Biological Sciences, University Science of Malaysia, where a specimen was deposited in the Herbarium (voucher number of 11033). The leaves were separated and washed with water to remove dirt prior to the drying process (40°C for 3 days).

**Extraction of Plant Material**

The leaves extract was prepared by maceration of dried powdered plant material in methanol for 3 days\(^{10}\). Approximately 200 g of powdered leaves was macerated in methanol under agitation conditions for 72 h. The macerated extract was then filtered through No. 1 Whatman filter paper and further vaporized to dryness using the rotary evaporator (Buchi Rotary Evaporator R-110). The vaporized extract was then freeze dried.

**Antifungal Activity**

**Microorganism**

*Candida albicans* strain 1, 2 and 3 (local clinical isolates) were used as the test organism and was obtained from a laboratory stock culture. Newly streaked isolates on plates directly from frozen stocks were used. The yeast was cultured on Sabouraud dextrose agar (SDA) at 30°C for 24 h. The stock culture was maintained on SDA agar slants at 4°C.

**Screening for the Antifungal Effect**

The antifungal activity of the extract was determined following the method described by CLSI\(^{11}\).

**Disk Diffusion Technique**

The test microbe was removed aseptically with an inoculating loop and transferred to a test tube containing 5 ml sterile distilled water. Sufficient inoculums were added until the turbidity was equal to 0.5 McFarland (10\(^{8}\) colony-forming units; CFU mL\(^{-1}\)) standard (bioMerieux, Marcy Peteole, France). 1 ml of the cells suspension from the test tube was added to 15-20 ml of SDA before setting aside the seeded plate (9 cm in diameter) to solidify for 15 min. Nine Whatman’s filter paper No. 1 disks of 6 mm diameter were used to screen for the fungicidal activity. Each sterile disk was impregnated with 20 µl of extract (corresponding to 100 mg/ml of crude extract); miconazole nitrate (30 µg/ml, as positive control); 10% dimethyl sulfoxide (DMSO) (v/v) (as negative control). The disks were placed on the surface of the seeded plates, incubated at 37°C overnight, and examined for zones of growth inhibition.

**Determination of the Minimal Inhibitory Concentration (MIC)**

An 18-h culture was diluted with a sterile physiologic saline solution [PS; 0.85% (w/v) sodium chloride] with reference to the 0.5 McFarland standard to achieve inoculums of approximately 10\(^{8}\) colony forming unit (CFU) mL\(^{-1}\). A serial dilution was carried out to give final concentrations between 1.563 and 200.00 mg crude extract per milliliter. The tubes were inoculated with 500 µL yeast suspension per milliliter Sabouraud dextrose broth (SDB), homogenized, and incubated at 37°C. After incubation, 20 µl was withdrawn from each tube, inoculated on SDA agar plates, and incubated at 37°C for 24 h. The MIC value was determined as the lowest concentration of the crude extract in the broth medium that inhibited a visible growth of the test microorganism.

**Anti-biofilm Activity**

**Biofilm Formation and Quantification**

Biofilms were formed and quantified on microtitre plate (MTP) surfaces, as described earlier\(^{12}\). Briefly, 200 µL of 5 × 10\(^{8}\) cfu/mL of *C. albicans* was suspended in yeast peptone dextrose broth supplemented with 50 mM glucose (YPD, Himedia, India) and added to MTP for 90 min of adhesion phase. The wells were washed with sterilized phosphate buffered saline (PBS) to remove loosely adhered cells. The washed wells were added with 6.25 mg/ml concentration of *C. spectabilis* extract, and sterilized Yeast Peptone Dextrose (YPD) broth was added to 200 µl final volumes and incubated at 37°C for 24 h.
Quantification of biofilm formed was done using XTT reduction assay using MTP reader (Asys UVM 340, Biochrom Ltd., Cambridge, United Kingdom) at 490 nm. Testing was performed in triplicate. Fluconazole was used as a positive control in the study.

**Confocal Scanning Laser Microscopy Study (CCLM)**

18 h *Candida* biofilms was developed by culturing the *C. albicans* on SDA agar for 18 h. Controls without the plant extract or antimicrobials were also included as control groups. The 48 h biofilms were gently transferred into a 12-well microtiter plate and rinsed with PBS for 15 s. The discs were then immersed in 1 ml of the plant extract or antimicrobial agents and incubated at 37°C in an aerobic incubator for 24 h. Subsequently, the extract or antimicrobial was removed and the viability of the biofilm cells was assessed by Molecular Probes Live/Dead BacLight Bacterial viability kit which comprise Syto-9 and propidium iodide (PI) (Molecular Probes, Eugene, OR, USA). After incubation with the dyes, the polymethylmethacrylate discs with biofilms were placed on glass slides and live/dead ratio of biofilm cells observed using the CSLM system.

**Statistical Analysis**

All experiments were performed in triplicate and results were expressed as mean ± standard deviations. Statistical analyses of the differences between mean values obtained for experimental groups were performed using Student’s t-test. *p*-values of 0.05 or less were considered significant.

**Results**

**Antifungal Activity**

The results of antifungal activity of the *C. spectabilis* leaves extract against *C. albicans* are given in Table I. The extract exhibited a favorable activity against the yeast tested. The zone of inhibition produced by the extract was 20.00 to 25.00 mm.

**Table I.** Anti-yeast activity (zone of inhibition and MIC) of *Cassia spectabilis* leaves extract compared with commercial antibiotic miconazole nitrate.

<table>
<thead>
<tr>
<th>Yeast</th>
<th>Zone of inhibition (mm)</th>
<th>Methanol extract</th>
<th>MIC (mg/ml) of the extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crude extract</td>
<td>Miconazole nitrate</td>
<td></td>
</tr>
<tr>
<td><em>Candida albicans</em> strain 1</td>
<td>20.00</td>
<td>22.00</td>
<td>6.25</td>
</tr>
<tr>
<td><em>Candida albicans</em> strain 2</td>
<td>21.00</td>
<td>25.00</td>
<td>6.25</td>
</tr>
<tr>
<td><em>Candida albicans</em> strain 3</td>
<td>23.00</td>
<td>24.00</td>
<td>6.25</td>
</tr>
</tbody>
</table>

*a*Agar dilution method, mean value n = 3. *b*The values (average of triplicate) are diameter of zone of inhibition at 100 mg/ml crude extract and 30 µg/ml Miconazole Nitrate.
23.00 mm. The solvent only negative control disk produced no zone of inhibition. The broth dilution method recorded the MIC value of 6.25 mg/ml against all tested strains. Hence, *C. albi-cans* strain 1 was used for further study.

**Biofilm Formation and Quantification**

Screened *C. spectabilis* extract was further checked against *C. albicans* biofilm up to 6.25 mg/ml (MIC) concentration. XTT (2, 3-bis [2-Methoxy-4-nitro-5-sulphophenyl]-2H-tetrazoli-um-5-carboxanilide) reduction assay showed that 1024 µg/ml concentration of fluconazole (used as standard drug) was able to reduce 95.4% biofilm (Table II), whereas 6.25 mg/ml concentration of *C. spectabilis* gave 96.9% biofilm reduction (Table II). The result shows that there is no significant difference between the results obtained with fluconazole and the extracts tested.

**Scanning Electron Microscopy**

To evaluate the relevance of XTT reduction assay, SEM was employed. Scanning electron microscopic analysis of control biofilms, and those treated with the *C. spectabilis* leaves extract and

| Table II. The inhibition of *Candida albicans* biofilms treated with *Cassia spectabilis* leaves extract (6.25 mg/ml) and fluconazole (1.024 mg/ml). The reduction of biofilm metabolic activity was measured using the XTT reduction assay. |
|------------------|------------------|
| **Extract (6.250 mg/ml)** | **Fluconazole (1.024 mg/ml)** |
| 96.88 ± 0.35* | 95.39 ± 0.23 |

*Mean ± SD, *P* >0.05.

positive control fluconazole are shown in Figure 1, 2 and 3. Untreated cells in the control *C. albicans* biofilms were generally smooth-walled bodies, spherical to elongated in shape entangled in a thick biofilm mass (Figure 1a, 2a and 3a). In *C. spectabilis* extract (MIC) treated biofilms, the cells were ruptured, damaged or bloated with most cell walls demonstrating rough, irregular topographic features (Figure 1b-d). In addition, similar results were also exhibited by fluconazole treated biofilms (Figure 3b-d). The surface of the fluconazole treated cells appeared uniformly

![Figure 1. SEM visualization of *Candida albicans* biofilm treated with *Cassia spectabilis* extract. A, Untreated *Candida albicans* biofilm (control). B-D, Candida albicans biofilm treated with MIC concentrations of *Cassia spectabilis* extract at 12, 24 and 36 h respectively.](image-url)
Anti-biofilm activity of *Cassia spectabilis*

rough because of a well-defined wrinkling of the cell wall. Fluconazole and *C. spectabilis* extract showed the comparable activity on cellular damage and disruption in biofilms of *C. albicans*. However, the only difference observed was several yeasts with true “holes” resulting from the action of fluconazole. However, such effects were not observed with the leaf extract. Figure 3b shows the enlarge morphology of the *Candida* biofilm treated with *C. spectabilis* extract, revealed that the destruction to biofilm architecture and constituents was caused by the extract as compared to the untreated biofilm.

**Confocal Scanning Laser Microscopy Study (CCLM)**

The ultrastructural changes in extract treated Candida biofilm visualized by SEM were fur-

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**Figure 2.** Enlarged image of the biofilm ultrastructure. **A,** Untreated *Candida albicans* biofilm (control). **B,** *Candida albicans* biofilm treated with MIC concentrations of *Cassia spectabilis* extract.

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**Figure 3.** SEM visualization of *Candida albicans* biofilm treated with Fluconazole. **A,** Untreated *Candida albicans* biofilms (control). **B-D,** *Candida albicans* biofilm treated with 1.024 mg/ml concentrations of Fluconazole at 12, 24 and 36 h respectively.
ther confirmed using CLSM study. As shown in Figure 4a, 4b and 4c the untreated control biofilms cells were predominantly viable (green) with only a few red stained non-viable cells interspersed. The dead cells were evident after 12 h (Figure 4d and 4g) incubation and was greater after 24 h (Figure 4e and 4h) incubation for cells treated with both the extract and fluconazole. The proportion of dead cells (red) of *C. albicans* increased as the time of incubation was increased. After 36 h (Figure 4f and 4i) of incubation with extract or fluconazole, CSLM observation of viable/nonviable cells exhibited the highest number of dead cells (red) compared with untreated cells.

**Discussion**

*C. spectabilis* is traditionally used for the treatment of various types of ailments and latest research on this plant claimed to have broad spectrum antimicrobial properties. Today we are observing a great deal of public interest in the use of herbal remedies because they are relatively safe, easily available and affordable to masses. The World Health Organization has recommended the evaluation of the effectiveness of plants in conditions where there is lack of safe synthetic drugs. However, azole antifungal agents and derivatives continue to be the first choice of drugs to treat *Candida* infections. Subsequently, repeated use of azole antifungal agents on biofilms can select drug-resistant microbes. Eventually, this episode leads to new problems in treating drug-resistant microbes. In these perspective, new agents that can inhibit the growth of biofilm-associated *C. albicans* is greatly needed. Hence, the present study was carried out to evaluate the antifungal and antibiofilm activity of *C. spectabilis* extract against *C. albicans* and its biofilm. Moreover, exploring additional natural resources for new antifungal agents with an-
Anti-biofilm activity could possibly reveal new antifungal agents with different modes of actions or affect different sites in the Candida cell.

In this study the tetrazolium salt, XTT reduction assay was used to monitor the effect of C. spectabilis leaves extract on C. albicans biofilm formation by colorimetric determination. The salt reduced by mitochondrial dehydrogenase to brown colour water-soluble tetrazolium formazan product was determined spectrophotometrically (absorbance at 492 nm)\(^2\). C. spectabilis leaves extract gave 96.88% biofilm reduction by using this assay at 6.25 mg/ml concentration. Further visualization of the biofilm ultrastructure by SEM revealed that damage to the biofilm constituents was caused by C. spectabilis leaves extract as compared to the untreated biofilm suggesting that C. spectabilis leaves extract may interfere with the normal metabolic functions in Candida biofilm. Furthermore, CLSM study shows that the number of viable cells (green) decreased when the time of exposure to the extract increased. This suggests that bioactive compounds of C. spectabilis leaves extract have strong potential to affect C. albicans cell growth and biofilm formation by interfering in normal metabolic functions in biofilm development. Our results also showed that C. spectabilis leaves extract is not only able to kill C. albicans cells efficiently but also inhibit biofilm formation. Our results clearly demonstrate that C. spectabilis leaves extract not only act as an effective antifungal agent against C. albicans and its biofilm but a potentially equal antifungal agent compared to fluconazole as positive control.

Similar results also reported by Agarwal et al\(^1\) by using plants oils from 30 medicinal plants. They evaluated the inhibitory effect of 30 plant oils against biofilm forming C. albicans strain (CA I). They reported 18 among the 30 plant oils tested showed anti-Candida activity by disc diffusion assay. Effective plant oils were assessed using XTT reduction assay for biofilm quantification. Four oils (4 oils) from eucalyptus, peppermint, ginger grass and clove showed 80.87%, 74.16%, 40.46% and 28.57% biofilm reduction respectively. In addition, scanning electron microscopic analysis further revealed reduction in C. albicans biofilm in response to effective oils in their study.

From this study, it appears that the C. spectabilis leaves extract exhibits a favourable anti-yeast activity against C. albicans with the MIC value to be only 6.25 mg/ml. The methanol extract used in this study was standardized in our previous study\(^19\). Hence, Candida infections could be treated with the extract, as the MIC for this yeast was found to be only 6.25 mg/ml. In addition, C. spectabilis leaves extract may be used as an anti-yeast agent in known dosages, especially in rural communities where conventional drugs are unaffordable or unavailable and the health facilities inaccessible particularly in developing countries with conclusive studies on the reproducibility between results obtained in vitro with in vivo. Our data also indicate C. spectabilis leaves extract is not only able to inhibit C. albicans cells efficiently but also inhibit biofilm formation. Such combinations may have translational value in the development of new drugs for the treatment of C. albicans biofilm which are much more resistant to antimicrobial agents.

**Conclusion**

In conclusion, the finding from this study conclusively demonstrates the in vitro anti-biofilm potential of C. spectabilis leaves extract against Candida biofilm. The C. spectabilis leaves have never been evaluated in detail for anti-biofilm activity before, and thus, it has been shown for the first time in this study to have detailed anti-biofilm activity. Therefore, further purification of active compounds, its individual anti-biofilm activity studies and in vivo experiments of the extracts from C. spectabilis may be suggested on the basis of the present study.

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**References**


