Anticandidal and antibiofilm activity of *Artocarpus lakoocha* extract

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Abstract

This study aimed to investigate the anticandidal and antibiofilm activity of *Artocarpus lakoocha* extract against various *Candida*. Anticandidal activity of *A. lakoocha* extract was determined using an agar well diffusion method. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were assessed using a method of the Clinical and Laboratory Standards Institute. A time kill assay was also performed. Antibiofilm activity was investigated using a 3-[4, 5-dimethyl-2-thiazolyl]-2, 5-diphenyl-2H-tetrazolium-bromide (MTT) assay. The extract was found to be effective against all tested *Candida* strains with MIC ranging from 0.05 to 3.12 mg/ml and MFC ranging from 0.10 to 25 mg/ml. The killing activity depended on the time and concentrations of the extract. *A. lakoocha* extract acts as a potent antibiofilm agent with dual actions, preventing and eradicating the biofilm. Results suggest that *A. lakoocha* extract is a potential source of natural anticandidal agent, which may be useful for prevention or treatment of candidiasis.

Keywords: *Artocarpus lakoocha*, *Candida*, anticandidal activity, antibiofilm activity

1. Introduction

There has been a significant increase in incidence of infections caused by *Candida* spp. (candidiasis), mainly due to the rise of the AIDS epidemic, an increasingly aged population, higher numbers of immunocompromised patients, the more widespread use of indwelling medical devices, and the use of broad spectrum antifungal drugs. *Candida albicans* is the main cause of candidiasis, however, non-*C. albicans* (NCAC) species such as *C. dubliniensis*, *C. tropicalis*, *C. glabrata* and *C. krusei* are now frequently reported as human pathogens (Moran et al., 2002).

The pathogenesis of candidiasis is facilitated by a number of factors including the ability to adhere to medical devices and/or host cells and to form biofilms. Biofilm formation is an important virulence factor for a number of *Candida* species, as it confers significant resistance to antifungal therapy by limiting the penetration of substances through the matrix and protecting cells from host immune responses (O’Toole et al., 2000; Kojic and Darouiche, 2004; Kuhn et al., 2002; Li et al., 2012). It has been demonstrated that biofilms formed by *C. albicans* and/or NCAC strains have been associated with higher morbidity and mortality rates compared with strains unable to form biofilms (Kumamoto, 2002).

*Artocarpus lakoocha* extract has been reported to contain antibacterial activity against a wide range of bacteria including *Mycobacterium tuberculosis* H37Ra, *Bacillus subtilis*, *B. pumilus*, *Proteus mirabilis*, *Shigella sonnei* and *Escherichia coli* (Pandey et al., 2009; Puntumchai et al., 2004). *A. lakoocha* is a valuable tropical tree species belonging to the Moraceae family; commonly found in tropical areas such as India and Thailand. The major constituent of *A. lakoocha* bark extract is oxyresveratrol.
(2, 4, 3′, 5′-tetrahydroxystilbene), which has also been reported to possess in vitro anti-virus activity (anti-herpes simplex virus (HSV), anti-varicella zoster virus) (Chuanaasa et al., 2008; Docherty et al., 2006; Likhitwityaywuid et al., 2005; Sasivimolphon et al., 2009; Sritulaluk et al., 1998). Another study suggested that oxyresveratrol was neuroprotective and inhibited apoptotic cell death in transient ischemia in a rat model (Andrabi et al., 2004). Due to its reported potent tyrosinase inhibitory and antioxidant activities (Sritulaluk et al., 1998; Kim et al., 2002), the material has potential application as a novel skin whitening agent in cosmetic preparations (Tengammuy et al., 2006). Most studies have reported its antiviral and antibacterial activities; however, data regarding the antifungal capability of A. lakoocha extract has been limited.

The aim of this study was to investigate the antifungal and antibiofilm activity of A. lakoocha extract against various Candida spp. via an in vitro study.

2. Materials and Methods

2.1 Preparation of A. lakoocha stem bark extract

The extract of A. lakoocha was obtained by boiling small pieces of stem bark in water. After removing the remaining wood fragments and other insoluble residues, the aqueous extract was dried to give a yellow-brown powder for use in this study. The content of oxyresveratrol in the dried extract was determined to be >80% (w/w) by using high performance liquid chromatography. A 10% (w/v) stock solution of A. lakoocha extract was prepared in 10% (v/v) dimethyl sulfoxide (DMSO, Merck) for use in this study.

2.2 Candida strains and growth conditions

The tested Candida strains included Candida albicans ATCC 90028, Candida albicans ATCC 10231, Candida dubliniensis MYA-577, Candida dubliniensis MYA-646, Candida glabrata ATCC 66032, Candida glabrata ATCC 90030, Candida kruzei ATCC 34135, Candida krusei ATCC 6258, Candida tropicalis ATCC 66029, Candida tropicalis ATCC 750, and Candida tropicalis ATCC 13803. All strains were cultured on Sabouraud Dextrose Agar (SDA; Difco Laboratories, Detroit, Michigan) aerobically at 37°C for 24 h.

2.3 Anticandidal assay

2.3.1 Agar well diffusion assay

The broth culture of each tested strain (approximately 10^5 CFU/ml) was mixed thoroughly with the sterile SDA (20 ml), and then poured into a plate with 6-mm diameter metal cups. The metal cups were removed after the medium set, and then the wells were added with 100 µl of 10% A. lakoocha extract, while 10% (w/v) DMSO was used as the negative control and 0.1% (w/v) CHX was used as the positive control. Plates were incubated at 37°C for 24 h. The antifungal activity was evaluated by measuring inhibition zone diameters in millimeters. Duplicates were maintained and the experiment was repeated thrice.

2.3.2 Broth microdilution assay

The minimal inhibitory concentration (MIC) of A. lakoocha extract against each tested strain was determined by broth microdilution method (NCCLS, 2002). Briefly, two-fold serial dilutions of A. lakoocha extract were prepared with SDB at a total volume of 100 µl per well in 96-well microtiter plates. The final concentrations of A. lakoocha extract ranged from 0.02 to 25.00 mg/ml. The microtiter plate wells were inoculated with 100 µl of each tested strain at the final concentration of 1×10^5 CFU/ml, and incubated at 37°C for 24 h. The negative control consisted of SDB broth and Candida suspension without the agent, and the blank control contained only the medium. The MIC was defined as the lowest concentration of the test agent that completely inhibited growth in comparison with the negative control. All experiments were performed in triplicate.

The minimal fungicidal concentration (MFC) was defined as the lowest concentration in a well that did not allow visible growth when 10 µl of the well content was plated on agar and grown for 24 h at 37°C.

2.4 Killing kinetics assay

Candidal activity of A. lakoocha extract was studied using a time-kill kinetic method. Growing cultures (10^5 CFU/ml) of each representative strain, C. albicans ATCC 90028, C. tropicalis ATCC 66029 and C. dubliniensis MYA-577 were added to SDB and were exposed to 1×, 2× and 4× the MIC of A. lakoocha extract. Samples were taken for colony counts at 0, 0.5, 1, 2, 4, 6, 8, 10, 12 and 24 h. The viable counts were determined using the serial dilution method after incubation at 37°C for 24 h. Each experiment was performed three times to confirm the results. Chlorhexidine (CHX, 0.1% (w/v)) and extract free were used as the positive and negative controls, respectively.

2.5 Antibiofilm assay

2.5.1 Inhibition of biofilm formation

The effect of A. lakoocha extract on biofilm formation of each representative strain, C. albicans ATCC 90028, C. tropicalis ATCC 66029 and C. dubliniensis MYA-577 was examined using the modified microdilution method (Taweewchaisupapong et al., 2010; Wu et al., 2013). Briefly, two-fold serial diluted concentrations (0.02-25.0 mg/ml) of A. lakoocha extract were made in a flat-bottom 96-well microtiter plate. The CHX (0.1%, w/v), PBS and the medium alone were used as the positive, non-treated and blank controls, respectively.
An equal volume of the tested strains (1×10^6 CFU/ml) was added and mixed with the agent, except in the well with medium alone (the blank control). Following incubation at 37°C for 24 h, supernatants were discarded and washed 3 times with PBS. Biofilm formation was quantified using a 3-[4, 5-dimethyl-2-thiazolyl]-2, 5-diphenyl-2H-tetrazolium-bromide (MTT) assay. After washing, 100 µl PBS with 0.5% (w/v) MTT (Sigma-Aldrich, USA) solution was added and allowed to incubate for 3 h at 37°C. The MTT solution was replaced with 100 µl DMSO and allowed to incubate for 15 min at room temperature. The number of surviving cells was determined by measuring their ability to reduce the yellow tetrazolium salt to a purple formazan product at 570 nm. Higher OD values indicate an increased number of surviving Candida in the biofilm. Percentage inhibition was calculated using this equation: [1-(A570 of the test/A570 of the non-treated control)] ×100%. The biofilm inhibitory concentrations (MBIC \(50\) and MBIC \(90\)) were defined as the concentrations that showed 50% and 90% inhibition of biofilm formation. All experiments were performed in triplicate.

### 2.5.2 Eradication of biofilm formation

The antimicrobial activity of A. lakoocha extract in the biofilm was also examined using the minimum biofilm eradication concentration (MBEC) assay (Brambilla et al., 2009; Wu et al., 2013). Briefly, 200 µl (10^6 CFU/ml) of each representative strain, C. albicans ATCC 90028, C. tropicalis ATCC 66029 and C. dubliniensis MYA-577 was inoculated into each well of the flat-bottom 96-well microtiter plate and incubated for 24 h at 37°C. After biofilm formation, the medium was then blotted out and the well carefully washed three times with sterile PBS to remove non-adherent cells. A. lakoocha extract was then added to the biofilms in two-fold serial dilutions (0.02-25 mg/ml) and incubated for 24 h at 37°C. At the end-point of the treatment of the biofilms with A. lakoocha extract, the adherent Candida was washed three times with sterile PBS. The numbers of surviving Candida were determined by a MTT assay. The MBEC value was defined as the concentrations that showed 50% and 90% eradication of Candida in the biofilm. The 0.1% (w/v) CHX, PBS and the medium alone were used as the positive, non-treated and blank controls, respectively. The experiments were performed in triplicate.

### 2.6 Statistical analysis

The data were expressed as mean and standard deviation (SD) by computational analysis from the three experiments with duplicate independent experiments. Data from biofilm assay were analyzed statistically using Mann-Whitney U test. Differences were considered statistically significant at P<0.05.

### 3. Results

The anticandidal activity of A. lakoocha extract was evaluated using an agar well diffusion assay, which demonstrated that all tested strains were susceptible to A. lakoocha extract with variable degrees of inhibition zones (Table 1). An example of an inhibition zone of A. lakoocha extract is shown in Figure 1; the vehicle control (10% DMSO) did not affect Candida growth. The MIC and MFC of A. lakoocha extract as evaluated by a microdilution assay are shown in Table 1. A. lakoocha extract exhibited anticandidal activity against most tested Candida strains with MICs ranging from 0.05 to 3.12 mg/ml and MFCs ranging from 0.10 to 25.00 mg/ml (Table 1).

**Table 1. Antimicrobial activity of A. lakoocha extract against Candida spp.**

<table>
<thead>
<tr>
<th>Stains of Candida spp.</th>
<th>Inhibition zone (mm) Mean ± SD</th>
<th>Concentration of A. lakoocha extract (mg/ml)</th>
<th>MIC(^a)</th>
<th>MFC(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans ATCC 90028</td>
<td>20±0.13</td>
<td></td>
<td>0.78</td>
<td>1.56</td>
</tr>
<tr>
<td>C. albicans ATCC 10231</td>
<td>15±0.14</td>
<td></td>
<td>1.56</td>
<td>3.12</td>
</tr>
<tr>
<td>C. dubliniensis MYA-577</td>
<td>15±0.21</td>
<td></td>
<td>0.78</td>
<td>1.56</td>
</tr>
<tr>
<td>C. dubliniensis MYA-646</td>
<td>20±0.15</td>
<td></td>
<td>0.78</td>
<td>1.56</td>
</tr>
<tr>
<td>C. glabrata ATCC 66032</td>
<td>20±0.13</td>
<td></td>
<td>1.56</td>
<td>25.00</td>
</tr>
<tr>
<td>C. glabrata ATCC 90030</td>
<td>18±0.06</td>
<td></td>
<td>1.56</td>
<td>12.50</td>
</tr>
<tr>
<td>C. krusei ATCC 34135</td>
<td>13±0.06</td>
<td></td>
<td>3.12</td>
<td>25.00</td>
</tr>
<tr>
<td>C. krusei ATCC 6258</td>
<td>14±0.10</td>
<td></td>
<td>3.12</td>
<td>25.00</td>
</tr>
<tr>
<td>C. tropicalis ATCC 66029</td>
<td>28±0.05</td>
<td></td>
<td>0.05</td>
<td>0.10</td>
</tr>
<tr>
<td>C. tropicalis ATCC 750</td>
<td>19±0.15</td>
<td></td>
<td>0.78</td>
<td>12.50</td>
</tr>
<tr>
<td>C. tropicalis ATCC 13803</td>
<td>20±0.06</td>
<td></td>
<td>0.78</td>
<td>25.00</td>
</tr>
</tbody>
</table>

\(^a\) MIC – Minimum Inhibitory Concentration

\(^b\) MFC – Minimum Fungicidal Concentration
Time kill curves were performed for 3 representative Candida spp. (C. albicans ATCC 90028, C. tropicalis ATCC 66029 and C. dubliniensis MYA-577); the killing activity depended on time and concentrations of A. lakoocha extract. Generally, 1× MIC could reduce the number of CFU by approximately 50% after 10 h of incubation; however, complete sterility was not achieved. At 4× MIC and 2× MIC, all strains were killed after 6 and 8 h, respectively (Figure 2). Killing by the positive control (CHX) was observed within 30 min.

The concentrations of A. lakoocha extract required to inhibit ≥50% biofilm formation (MBIC<sub>50</sub>) of C. albicans ATCC 90028, C. tropicalis ATCC 66029 and C. dubliniensis MYA-577 were 3.13±0.23, 0.39±0.12 and 0.39±0.18 mg/ml, respectively, and for ≥90% inhibition of biofilm growth (MBIC<sub>90</sub>) the concentrations were 25.00±0.18, 1.56±0.05, and 1.56±0.29 mg/ml, respectively. At the concentration of A. lakoocha extract > 0.10 mg/ml, there was a statistically significant inhibition of biofilm growth of all tested strains compared to non-treated control (Figure 3). The amount of A. lakoocha extract required to eradicate ≥50% biofilm growth (MBEC<sub>50</sub>) of C. albicans ATCC 90028, C. tropicalis ATCC 66029 and C. dubliniensis MYA-577 were 6.25±0.20, 3.12±0.13 and 3.12±0.23 mg/ml, respectively, and for ≥90% of the eradication of biofilm growth (MBEC<sub>90</sub>) the amounts were > 25.00, 12.50±0.20 and 25.00±0.26 mg/ml, respectively. At the concentration of A. lakoocha extract > 0.20 mg/ml, there was a statistically significant eradication of biofilm growth of all tested strains compared to non-treated control (Figure 4).

4. Discussion

Candida species are the most common fungal pathogens in humans and are the causative agents at various locations in the body, giving rise to severe morbidity in millions of individuals worldwide (Calderone and Fonzi, 2001; Ruhnke, 2002). In the oral cavity, C. albicans is the organism that most frequently causes a range of mucosal infections including oral candidiasis e.g. oropharyngeal candidiasis, angular cheilitis, oral thrush and denture stomatitis (Richardson and Warnock, 1997; Ruhnke, 2002), NCAC have also been found dramatically in oral candidal infection. The most common treatment is the use of antifungal agents such as azoles (uconazole, itraconazole, miconazole and ketoconazole) and polyenes (amphotericin B and nystatin). However, those who use such agents are often faced with several problems including the
limited number of effective antifungal agents, their high toxicity and cost, the recurrence of the infection, and increasing emergence of antifungal resistance (Klepser, 2001; Khan et al., 2003). A topical use of CHX mouthwash is the most common antimicrobial substance to control oral candidiasis. However, CHX has been reported as having many unwanted effects over long-term use including unpleasant taste and disturbance in taste sensation, brown discoloration of the denture, and desquamative lesions of oral mucosa (Hiom et al., 1992; Giuliana et al., 1997). Many studies have demonstrated that traditional herbs possess antifungal activity against Candida spp. (Boroujeni et al., 2012; Rukayadi et al., 2006; Yigit et al., 2009). In the present study, it was found that A. lakoocha extract has a good antifungal activity against Candida strains. A. lakoocha has previously been reported to have antiviral and antibacterial properties, and this study is the first to reveal the antifungal activity of A. lakoocha.

In addition, A. lakoocha extract has shown antibiofilm activity. Biofilms of C. albicans and NCACs are associated with high incidence of hospital morbidity and mortality (Nett and Andes, 2006; O'Toole et al., 2000; Silva et al., 2010) due to the increased use of medical devices such as lens, implants and dentures. Such biomaterials facilitate Candida strains to colonize and form biofilms leading to the development of resistance to antifungal agents, e.g. amphotericin B, micronazole, ketoconazole and fluconazole (Chandra et al., 2001; Kuriyama et al., 2003). Experiments comparing biofilms of Candida with broth cultures have demonstrated that higher concentrations of A. lakoocha extract are required to significantly inhibit existing biofilm cells. This is an expected result since Candida in the biofilm is strongly protected and less susceptible to antifungal agents than Candida in planktonic form (Baillie and Douglas, 2000; Chandra et al., 2012). It was shown that A. lakoocha extract was able to remove Candida in a dose- and time-dependent manner. Our results indicate that A. lakoocha extract acts as a potent antibiofilm agent that has dual actions preventing biofilm formation and removing existing biofilms.

The exact mechanism of action exerted by A. lakoocha extract on Candida is still unclear. Among previous studies, one study of interaction of resveratrol with Botrytis cinerea, a gray mold that infects grapevines, showed that the proposed mode of action involved an interference with the functionality of membrane proteins, especially those of the mitochondria. The interaction leads to an immediate decrease in oxygen uptake by the fungal cells. At the ultra-structural level, mitochondrial and nuclear membranes are affected first, followed by a complete disorganization of organelles and disruption of the cell membrane. Another study of the effect of A. lakoocha extract in Fasciola gigantica, a liver fluke that causes fasciolosis, implied that A. lakoocha contains a very high content of oxyresveratrol that could cause tegument changes by affecting the oxidative phosphorylation in mitochondria. It has been explained that oxyresveratrol (the major constituent of this crude extract has similar chemical structure to the halogenated phenol group of drugs, such as nitroxynil) could act via a similar mechanism. It was reported that nitroxynil acts as an uncoupler of oxidative phosphorylation (Fairweather et al., 1984; McKinstry et al., 2003). As a result, the decreased production of ATP would affect the Na⁺−K⁺ pump, leading to the influx of Na⁺ and water, and consequently the swelling of the syncytium as observed in the study of F. hepatica treated with nitroxynil (McKinstry et al., 2003).

In conclusion, the current study supports the traditional advantages of the studied plant, and suggests that the stem bark A. lakoocha extract is a potential source as a natural antifungal agent. It possesses compounds with good antifungal properties that may be used for oral infectious diseases caused by certain Candida spp. After this screening experiment, further work should be performed to describe the antifungal activities in more detail as well as their activity in vivo.

Acknowledgments

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