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Original Article

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 anti-fungal 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 soneii* 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,

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(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) (Chuanasa *et al.*, 2008; Docherty *et al.*, 2006; Likhitwitayawuid *et al.*, 2005; Sasivimolphan *et al.*, 2009; Sritulaluk *et al.*, 1998). Another study suggested that oxyresveratrol was neuro-protective 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 (Tengamnuay *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 anti-candidal 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 krusei* 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 10^7 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 micro-titer 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 1×10^3 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 (10^6 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 (Tawee-chaisupapong *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 in order 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.

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

[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 ($MBIC_{50}$) 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 ($MBIC_{90}$) 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 ($MBEC_{50}$) 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 ($MBEC_{90}$) 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

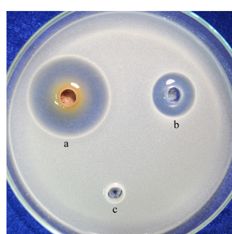

Figure 1. Agar well diffusion assay of 0.1% (w/v) *A. lakoocha* extract against *C. tropicalis* ATCC 66029 (a) showing by inhibition clear zone, 0.1 g% (w/v) CHX served as positive control (b) and 10% (v/v) DMSO (c) served as negative control.

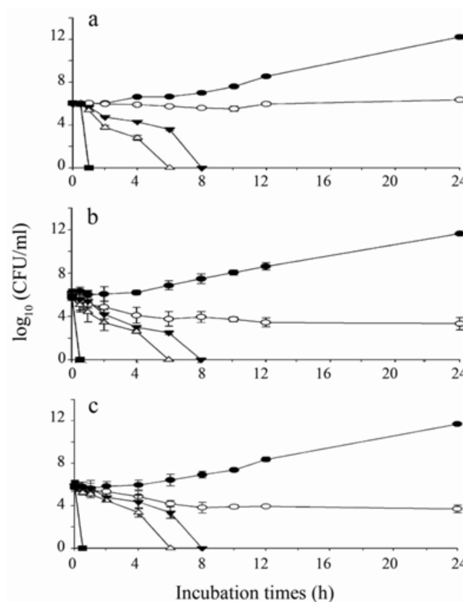

Figure 2. Time kill curves of *A. lakoocha* extract against of *C. albicans* ATCC 90028 (a), *C. tropicalis* ATCC 66029 (b) and *C. dubliniensis* MYA-577 (c). Bacteria stains were incubated with 0× MIC (●), 1× MIC (○), 2× MIC (▼) and 4× MIC (Δ); 0.1% (w/v) CHX (■) over time. CFU, Colony Forming Units.

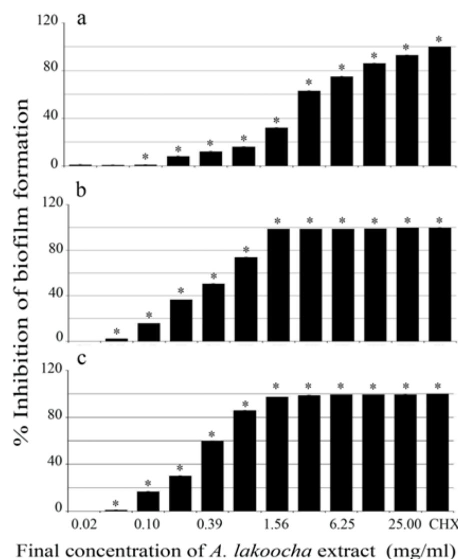

Figure 3. Inhibition of biofilm formation of *C. albicans* ATCC 90028 (a), *C. tropicalis* ATCC 66029 (b) and *C. dubliniensis* MYA-577 (c) by *A. lakoocha* extract at various concentrations and with 0.1% (w/v) CHX (the positive control) is demonstrated. Error bars indicate standard deviations; n=6. *, differences (compared to the blank control) were considered statistically significant at P<0.05.

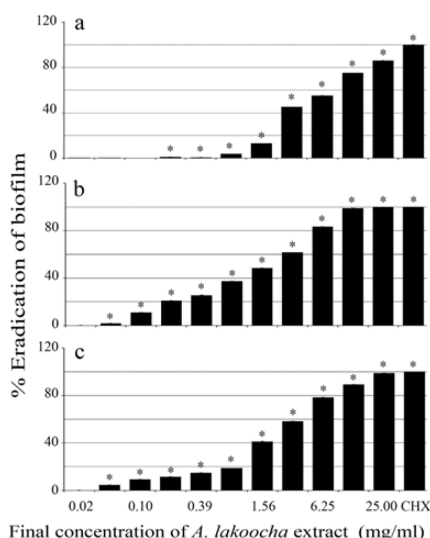

Figure 4. Eradication of biofilm formation of *C. albicans* ATCC 90028 (a), *C. tropicalis* ATCC 66029 (b) and *C. dubliniensis* MYA-577 (c) by *A. lakoocha* extract at various concentrations and with 0.1% (w/v) CHX (the control) is demonstrated. Error bars indicate standard deviations; n=6. *, differences (compared to the blank control) were considered statistically significant at $P<0.05$.

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 dorsum of the tongue, and desquamative lesions of oral mucosa (Hiom *et al.*, 1992; Giuliana *et al.*, 1997).

Many studies have demonstrated that traditional herbs possess anticandidal 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 anticandidal 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.*, 2005). 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*.

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