

Original Research Article

In vitro Antimicrobial and Antibiofilm Activity of *Artocarpus Lakoocha* (Moraceae) Extract against Some Oral Pathogens

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Abstract

Purpose: To evaluate the antimicrobial and antibiofilm activity of *A. lakoocha* extract against oral pathogens by an *in vitro* method.

Methods: The dried powder of the aqueous extract of *A. lakoocha* was purchased from a Thai traditional drug store. Representative strains of oral pathogens (*Streptococcus mutans* ATCC 25175, *Streptococcus sobrinus* ATCC 33478, *Enterococcus faecalis* ATCC 19433, *Lactobacillus fermentum* ATCC 14931, *Lactobacillus salivarius* ATCC 11741, *Aggregatibacter actinomycetemcomitans* ATCC 33384, *Porphyromonas gingivalis* ATCC 33277, *Prevotella intermedia* ATCC 25611, *Prevotella nigrescens* ATCC 25261, *Fusobacterium nucleatum* ATCC 25586 and *Tanarella forsythia* ATCC 43037) were tested for minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) using a microdilution technique, as well as by a time kill assay. Antibiofilm activity was investigated by a 3-[4, 5-dimethyl-2-thiazoly]-2, 5-diphenyl-2H-tetrazolium-bromide (MTT) assay.

Results: All tested strains were susceptible to *A. lakoocha* extract with variable degrees of antimicrobial inhibition. The extract was effective against both Gram-negative bacteria (*Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*) and Gram-positive bacteria (*Streptococcus mutans*, *Streptococcus sobrinus*), with MIC ranging from 0.10 – 0.39 mg/ml and MBC from 0.10 – 3.12 mg/ml. Killing activity depended on time and concentrations of the extract. The extract acted as a potent antibiofilm agent with dual actions, preventing biofilm formation and also eradicating the existing biofilm.

Conclusion: *A. lakoocha* extract possesses compounds with good antimicrobial properties that may be used for oral infectious diseases caused by certain oral pathogens associated with dental caries and/or periodontal diseases. For the application, *A. lakoocha* extract may be incorporated in mouthwash or toothpaste.

Keywords: *Artocarpus lakoocha*, Antimicrobial, Biofilm, Dental caries, Periodontal diseases, Time-kill assay

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INTRODUCTION

Antimicrobial properties have been derived from a wide range of plant extracts. Natural products are sources of chemical compounds that can be

used as antimicrobial agents. *Artocarpus lakoocha* Roxb (Moraceae) is a traditional herbal medicine, which is commonly found in tropical areas such as India and Thailand. The oxyresveratrol, 2, 4, 3', 5'-tetrahydroxystilbene,

is the major constituent of aqueous extract of *Artocarpus lakoocha* which has been revealed as a natural anthelmintic [1] and anti-herpes simplex virus (HSV) both *in vitro* and *in vivo* studies [2,3]. It has also been reported to show *in vitro* anti-varicella zoster virus activity [4,5]. Another study has suggested that oxyresveratrol was neuroprotective and inhibited apoptotic cell death in transient ischemia in a rat model [6]. *A. lakoocha* extract has a potential application as a novel skin whitening agent in cosmetic preparations [7], due to its potent tyrosinase inhibitory [8] and antioxidant activities [9].

Oral diseases, including dental caries and periodontal diseases, are commonly caused by a wide range of microorganisms associated with oral biofilm or dental plaque [10,11]. Recently study reported that oxyresveratrol exhibited antibacterial activities against periodontal pathogens, *P. gingivalis* and *A. actinomycetemcomitans* [12]. Our previous study indicated that *A. lakoocha* extract may be a useful antimicrobial medication for endodontic treatment, due to its ability to inhibit growth of *Enterococcus faecalis* [13]. However, information of the antibacterial property of *A. lakoocha* extract against oral pathogens is still limited.

This study is aimed at investigating the antimicrobial and antibiofilm activities of *A. lakoocha* extract against oral pathogens as well as the potential health benefits of the extract.

EXPERIMENTAL

Preparation of *A. lakoocha* extract

The dried powder of the aqueous extract of *A. lakoocha* was purchased from a Thai traditional drug store at Chiang Mai province, Thailand, in January 2005. Oxyresveratrol, a major compound of *A. lakoocha* extract, was verified to be > 95 % purity using a high performance liquid chromatography by Dr Jindaporn Puripattanavong, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University. The extract was stored at -20 °C and a voucher specimen (no. SKP117011201) was deposited at the herbarium of the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai, Thailand. A 10 % (w/v) stock solution of *A. lakoocha* extract was prepared in 10 % v/v dimethyl sulfoxide (DMSO) for use in this study.

Bacterial strains and growth conditions

A total of 11 species were employed in the study including five (5) Gram positive cariogenic bacteria (*S. mutans* ATCC 25175, *S. sobrinus* ATCC 33478, *E. faecalis* ATCC 19433, *Lactobacillus fermentum* ATCC 14931, and *Lactobacillus salivarius* ATCC 11741) and six (6) Gram negative periodontopathogenic bacteria (*A. actinomycetemcomitans* ATCC 33384, *P. gingivalis* ATCC 33277, *Prevotella intermedia* ATCC 25611, *Prevotella nigrescens* ATCC 25261, *Fusobacterium nucleatum* ATCC 25586 and *Tanerella forsythia* ATCC 43037).

Organisms were maintained on either brain heart infusion agar (BHA) with 5 % (v/v) blood for facultative bacteria and supplemented with a 0.5 % (w/v) yeast extract, haemin and vitamin K for anaerobic bacteria. The strains were grown under aerobic or anaerobic (10 % H₂, 10 % CO₂ and 80 % N₂) conditions as appropriate.

Antibacterial assays

Agar diffusion test

The broth culture of each tested strain (approximately 10⁷ cfu/ml) was mixed thoroughly with the sterile BHA (20 ml) and then poured into a plate with 6-mm diameter metal cups. The metal cups were removed after the medium has set, and then in the wells were added 100 µl of 10 % *A. lakoocha* extract, while 10 % DMSO was used as the control. The plate was incubated in appropriate conditions according to microorganisms tested at 37 °C for 24 h. Antimicrobial activity was evaluated by measuring inhibition zone diameters; the experiments were conducted in triplicate.

Minimal inhibitory and minimal bactericidal concentration

The minimal inhibitory concentration (MIC) of *A. lakoocha* extract against each tested strain was determined using the Clinical and Laboratory Standards Institute-(CLSI) recommended broth microdilution assay as modified Briefly, two fold serial dilutions of *A. lakoocha* extract were prepared with brain heart infusion broth (BHI) at a total volume of 100 µl per well in the 96-well plates. The final concentrations of *A. lakoocha* extract ranged from 25-0.01 mg/ml. The microtiter plate wells were inoculated with 100 µl of 0.5 McFarland for each tested strain per well. After overnight incubation at 37 °C appropriate conditions, absorbance was measured at 600 nm using a microtiter plate reader (Expert Plus UV; ASYS Hitech GmbH Eugendorf, Austria) to

assess the cell growth. The negative control consisted of BHI broth and bacterial cell suspension without the agent, and the blank control contained only the medium. The MIC end-point was defined as the lowest concentration of the test agent that completely inhibited growth or produced at least 90 % reduction of absorbance in comparison with the negative control. All experiments were performed in triplicate and the average values were reported as MIC.

The minimal bactericidal concentration (MBC) was defined as the lowest concentration of wells that did not allow visible growth when 10 µl of the well contents was plated on agar and grown 24 - 48 h at 37 °C in appropriate conditions.

Time kill assay

Bactericidal activity of *A. lakoocha* extract was examined using a time kill assay. Growing cultures (10^6 cfu/ml) of each representative strain, Gram positive (*S. mutans* ATCC 25175) and Gram negative bacteria (*A. actinomycetemcomitans* ATCC 33384), were added to appropriate medium and exposed to 1×, 2× and 4× the MIC of *A. lakoocha* extract. Samples were taken for colony counts at 0, 30 min, 2, 4, 6, 8, 10, 12 and 24 h. The viable counts were determined after appropriate incubation and each experiment was performed in triplicate. Chlorhexidine diacetate monohydrate (CHX, 0.1 %) and extract free medium were used as the positive and negative controls, respectively.

Antibiofilm assay

Inhibition of biofilm formation

The effect of *A. lakoocha* extract on biofilm formation of each representative strain, *S. mutans* ATCC 25175 and *A. actinomycetemcomitans* ATCC 33384, was examined using the modified microdilution method of Tang *et al* [14]. Briefly, two-fold serial diluted concentrations from 25 to 0.01 mg/ml of *A. lakoocha* extract were made in a flat-bottom 96-well microtiter plate. The CHX (0.1 %), phosphate buffered saline and the medium alone were used as 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 agents, except in the well with medium alone (the blank control). Following incubation at 37 °C for 24 h, supernatants were discarded and washed three times with phosphate buffered saline. Biofilm formation was quantified using a 3-[4, 5-dimethyl-2-thiazolyl]-2,

5-diphenyl-2H-tetrazolium-bromide (MTT) assay. The numbers of surviving bacteria were determined by measuring their ability to reduce the yellow tetrazolium salt to a purple formazan product at 570 nm. The OD values indicated a number of surviving microorganisms in the biofilm. Percentage inhibition was calculated using an equation $[1 - (A_{570} \text{ of the test}/A_{570} \text{ of non-treated control})] \times 100$. The biofilm inhibition concentration (MBIC₅₀ and MBIC₉₀) was defined as the concentrations that showed 50 % and 90 % inhibition of biofilm formation.

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. Briefly, 200 µl (10^6 cfu/ml) of each representative strain, *S. mutans* ATCC 25175 and *A. actinomycetemcomitans* ATCC 33384, was inoculated into each well of the flat-bottom 96-well microtiter plate and incubated for 24 h in appropriate conditions at 37 °C. After biofilm formation, the medium was then blotted out and the well carefully washed three times with sterile phosphate buffered saline in order to remove non-adherent cells. *A. lakoocha* extract was then added to the biofilms in two-fold dilutions serially from 25 to 0.01 mg/ml and incubated for 24 h in appropriate conditions at 37 °C. At the end-point of the treatment of the biofilms with *A. lakoocha* extract, the adherent bacteria were washed three times with sterile phosphate buffered saline. The numbers of surviving bacteria were determined by a MTT assay. The MBEC value was defined as the concentrations that showed 50 % and 90 % inhibition of biofilm formation on the biofilm. And 0.1 % CHX, phosphate buffered saline and the medium alone were used as the positive, non-treated and blank controls, respectively. The percentage eradication was calculated using an equation $[1 - (A_{570} \text{ of the test}/A_{570} \text{ of non-treated control})] \times 100$.

Scanning electron microscopy

A scanning electron microscopy (SEM) was performed to examine the morphology changes of each representative strain in *S. mutans* ATCC 25175 and *A. actinomycetemcomitans* ATCC 33384 after treatment with 10 % DMSO or 0.1 mg/ml *A. lakoocha* extract as the control and treated sample, respectively. After 8 h incubation at 37 °C in an appropriate condition, the bacterial pellet was collected and washed twice with phosphate buffered saline with pH 7 by centrifugation at 1000 rpm at 4 °C for 5 min. The bacterial pellet was fixed overnight in 2.5 %

glutaraldehyde and 0.1 M cacodylate buffer at cool temperature, and then dehydrated in a graded series of ethanol solutions for 30 min. The samples were subsequently dried by a critical point drying method and coated with gold. The microbial morphology was observed with a field emission SEM.

Statistical analysis

Data were expressed as mean and standard deviation (S.D.) by computational analysis from triplicate independent experiments.

RESULTS

A. lakoocha extract was evaluated for antimicrobial potential against oral pathogens by an agar diffusion assay, the results of which are shown in Table 1. All tested strains were susceptible to *A. lakoocha* extract with variable degrees of inhibition zones. The extract was found effective against both Gram positive bacteria and Gram negative bacteria. The solvent control, 10 % DMSO, did not affect microorganism growth. The MIC and MBC of *A. lakoocha* extract as evaluated by a microdilution assay are shown in Table 1. The most susceptible strains were *S. mutans*, *P. gingivalis*, *F. nucleatum* and *T. forsythia* with MIC of approximately 0.1 mg/ml and MBC 0.2 mg/ml.

Time-kill curves were performed for 2 representative oral pathogens (*S. mutans* and *A. actinomycetemcomitans*); the killing activity depended on time and concentrations of *A. lakoocha* extract. Generally, 1× MIC could reduce the number of the CFU by approximately 50 %, although complete sterility was not achieved. At 4× MIC and 2× MIC, *S. mutans* was

killed after 8 and 12 h, while *A. actinomycetemcomitans* was killed after 6 and 8 h, respectively (Figure 1). The killing of the positive control (CHX) was observed within 30 min.

The concentrations of *A. lakoocha* extract required to inhibit of the ≥ 50 % biofilm formation (MBIC₅₀) of *S. mutans* and *A. actinomycetemcomitans* were 0.39 ± 0.11 and 0.10 ± 0.09 mg/ml, and for ≥ 90 % inhibition of biofilm growth (MBIC₉₀) were 3.12 ± 0.02 and 0.39 ± 0.10 mg/ml, respectively (Figure 2A). The eradication of the biofilm formation of *S. mutans* and *A. actinomycetemcomitans* by *A. lakoocha* extract at various concentrations and with 0.1 % CHX is demonstrated in Figure 2B. The concentrations of *A. lakoocha* extract to eradicate of the ≥ 50 % biofilm formation (MBEC₅₀) of *S. mutans* and *A. actinomycetemcomitans* were 1.56 ± 0.13 and 0.78 ± 0.12 mg/ml, and for ≥ 90 % of the eradication of biofilm growth (MBEC₉₀) were 6.25 ± 0.34 and 3.12 ± 0.18 mg/ml, respectively.

In Figure 3, the change in morphology of *S. mutans* and *A. actinomycetemcomitans* was observed using SEM after treating 0.1 mg/ml of *A. lakoocha* extract in comparison with the control (10 % DMSO). Bacterial cells of *S. mutans* and *A. actinomycetemcomitans* in control groups showed a regular, smooth surface as shown in Figure 3A and C, respectively. It revealed that the bacterial cells after treatment with *A. lakoocha* extract lost their original shape showing a distorted, irregular cell wall structure, which was clearly observed in *A. actinomycetemcomitans* (Figure 3D).

Table 1: Inhibition zone, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *A. lakoocha* extract against oral pathogens (mean ± SD, n = 3)

Bacteria strain	inhibition zone (mm)	MIC (mg/ml)	MBC (mg/ml)
Gram positive bacteria			
<i>S. mutans</i> ATCC 25175	30.5±0.00	0.10±0.00	0.20±0.00
<i>S. sobrinus</i> ATCC 33478	16.0±0.35	0.39±0.00	1.56±0.00
<i>E. faecalis</i> ATCC 19433	18.2±0.00	0.39±0.00	3.12±0.00
<i>L. fermentum</i> ATCC 14931	17.7±0.35	0.39±0.00	0.78±0.00
<i>L. salivarius</i> ATCC 11741	15.0±1.41	0.10±0.05	0.78±0.00
Gram negative bacteria			
<i>P. gingivalis</i> ATCC 33277	21.0±1.41	0.10±0.05	0.10±0.00
<i>P. intermedia</i> ATCC 25611	30.5±0.70	0.20±0.05	0.20±0.00
<i>P. nigrescens</i> ATCC 25261	25.5±0.70	0.39±0.00	0.39±0.00
<i>F. nucleatum</i> ATCC 25586	30.7±0.35	0.10±0.00	0.20±0.00
<i>T. forsythia</i> ATCC 43037	29.0±0.00	0.10±0.00	0.20±0.00
<i>A. actinomycetemcomitans</i> ATCC 33384	29.5±2.12	0.10±0.00	0.20±0.00

ATCC = American Type Culture Collection

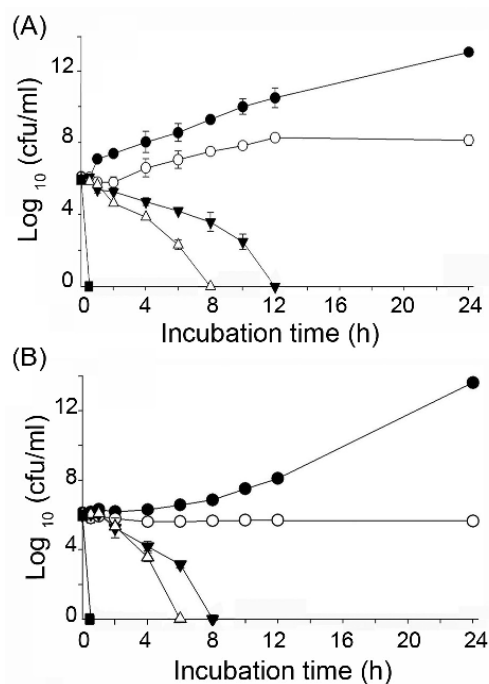


Figure 1: Time-kill curves of *A. lakoocha* extract against *S. mutans* (A) and *A. actinomycetemcomitans* (B). Bacteria stains were incubated with 0x MIC (●), 1x MIC (○), 2x MIC (▲) and 4x MIC (△); 0.1 % (w/v) CHX (■) over time; CFU = colony forming units

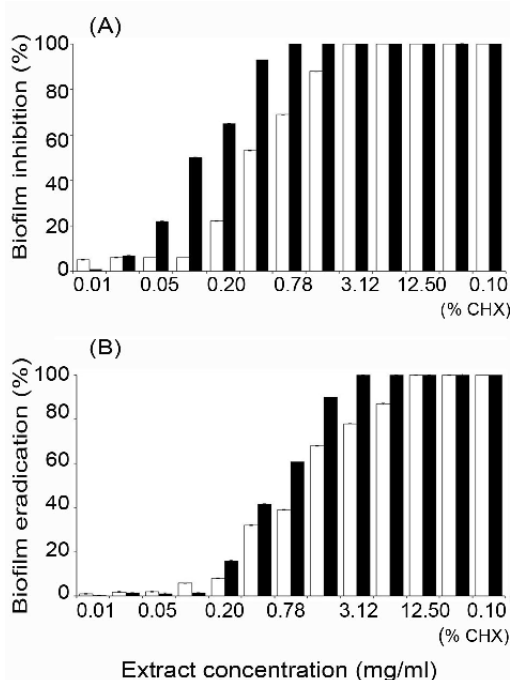


Figure 2: Inhibition of biofilm formation (A) and eradication of biofilm formation (B) of *S. mutans* (□) and *A. actinomycetemcomitans* (■) by *A. lakoocha* extract at various concentrations and with 0.1 % (w/v) CHX (positive control); error bars denote standard deviation (n = 6)

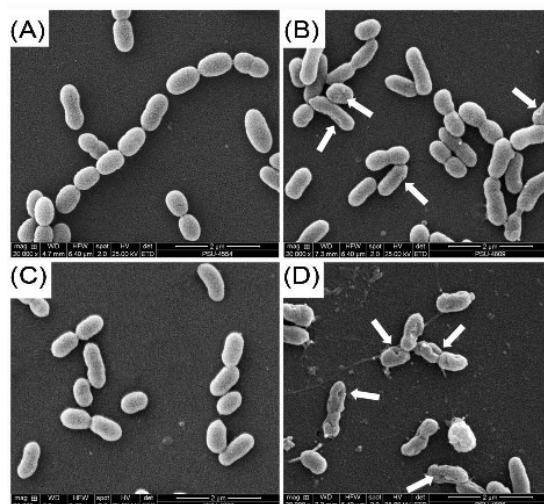


Figure 3: Scanning electron microscope images of *S. mutans* (top) and *A. actinomycetemcomitans* (below) cells, in the control (BHI broth) (A and C), in the presence of *A. lakoocha* extract (B and D)

DISCUSSION

The search for novel antimicrobial agents from plants has been of great interest in the last few decades. Throughout human history, infectious diseases are known to have been treated with compounds derived from plants and herbal remedies.

Results of the present study clearly demonstrated that *A. lakoocha* extract revealed good antibacterial agent activity against both cariogenic bacteria (e.g. *S. mutans*, *S. sobrinus*) and periodontopathogens including *P. gingivalis*, *F. nucleatum*, *T. forsythia* and *A. actinomycetemcomitans*. Gram negative bacteria were generally found to be more susceptible than Gram positive bacteria. Our results are in agreement with Phoolcharoen *et al* [12] who demonstrated the antimicrobial activity of *A. lakoocha* extract against periodontopathogens Gram negative bacteria. However, cariogenic bacteria were not susceptible to *A. lakoocha* extract in that study [12]. This may be due to the different strains used in that study; however, no details of the strains were given. Besides, the *in vitro* antimicrobial activity of *A. lakoocha* extract against oral pathogen planktonic cells, the antibiofilm activity of *A. lakoocha* extract was also assessed in this study. Experiments comparing biofilms of oral strains 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 bacteria in the biofilm are strongly protected and less

susceptible to antimicrobial agents than in planktonic form. It was shown that *A. lakoocha* extract was able to eradicate oral biofilm 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 eradication of existing biofilm.

Oral infections are commonly caused by imbalance of microorganisms in dental plaque or biofilm. Pathologies such as dental caries or periodontitis arise when an imbalance of microorganisms occurs in the biofilm resulting in a decrease of the indigenous bacteria and in favour of pathogenic strains. An effective approach for controlling dental plaque is to prevent formation of biofilm or to remove biofilm by using antimicrobial or chemical agents [15]. The use of CHX solution, a commercial antimicrobial agent, for clinical application is limited due to its bitter taste and staining of the tooth surface [16]. Thus, it is reasonable to develop alternative antiplaque agents from natural sources that exhibit few or no side-effects. It was found that *A. lakoocha* extract has an efficacy for the prevention of plaque-related diseases such as dental caries and periodontitis. Although the MIC and MBC of *A. lakoocha* extract obtained were higher than for CHX, the extract exhibited a better antimicrobial activity than other herb extract such as garlic [17], *Polygonum cuspidatum* root [18], *Coco cravo* [19] and *Rhei Rhizoma* [20] with MICs against *S. mutans* of 71.4, 1 - 2, 4 and > 5 mg/ml, respectively.

The exact mechanism of action exerted by oxyresveratrol, the major constituent of *A. lakoocha* extract, has not been previously reported. SEM images in this study indicated that oxyresveratrol might have effect on the cell wall integrity of bacterial cells. Exposure of *S. mutans* and *A. actinomycetemcomitans* to *A. lakoocha* extract revealed an irregular cell wall structure. All the results above indicated that *A. actinomycetemcomitans* is more sensitive to *A. lakoocha* extract than *S. mutans*. This may be due to Gram positive bacteria contained in the thicker peptidoglycan layer than Gram negative bacteria.

CONCLUSION

This study suggests that *A. lakoocha* extract possesses compounds with potential antimicrobial properties that may be useful for treatment of oral infectious diseases caused by certain oral pathogens. However, since this study was conducted on artificial biofilm *in vitro*, the

actual effects of *A. lakoocha* extract on dental plaque would need to be evaluated clinically.

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