

ผลของสารสกัดน้ำจากใบหูกวางต่อเชื้อ *Staphylococcus aureus* ภายใต้ความแตกต่างของความเข้มข้นและระยะเวลาการสัมผัส

Effects of Indian almond leaf aqueous extract on *Staphylococcus aureus* in relation to concentration and exposure time

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บทคัดย่อ

เชื้อ *Staphylococcus aureus* เป็นเชื้อก่อโรคที่ผิวหนังในทั้งสัตว์เลี้ยงและมนุษย์ที่มีแนวโน้มการดื้อยาปฏิชีวนะของเชื้อแบคทีเรียที่สูงขึ้นจึงทำให้สารต้านแบคทีเรียจากธรรมชาติได้รับความสนใจ ดังนั้นงานวิจัยนี้จึงมีวัตถุประสงค์เพื่อศึกษาประสิทธิภาพของและระยะเวลาของสารที่ได้จากใบหูกวาง (*Terminalia catappa* Linn.) ในการฆ่าเชื้อ *S. aureus* โดยมีวิธีการทดลอง ได้แก่ การเตรียมสารสกัดน้ำจากใบหูกวาง โดยวิธีการต้มและแช่ การหาความเข้มข้นของแทนนินจากสารสกัดแช่หมัก และระยะเวลาที่ออกฤทธิ์ฆ่าเชื้อเชื้อแบคทีเรีย จากการศึกษาพบว่าสารสกัดน้ำใบหูกวางที่เตรียมได้ด้วยวิธีการต้มและแช่มีความเข้มข้นของแทนนินทั้งหมดเท่ากับ 0.76 มก./มล. และพบว่ามีความเข้มข้นต่ำที่สุดสามารถฆ่าเชื้อแบคทีเรียด้วยวิธี broth macrodilution assay อยู่ที่ 0.35 ถึง 0.4 มก./มล. และจากการศึกษาระยะเวลาในการออกฤทธิ์ของสารสกัดน้ำใบหูกวางต่อการลดลงของจำนวนแบคทีเรีย พบว่าที่ความเข้มข้นของแทนนินในสารสกัดน้ำใบหูกวาง 0.35 มก./มล. นั้นทำให้จำนวนโคโลนีของ *S. aureus* ลดลงอย่างมีนัยสำคัญทางสถิติจาก 115 ที่ 2 ชั่วโมง เหลือ 0 ที่ 24 ชั่วโมง ($r_s = -1.000$, $p\text{-value} < 0.001$) และที่ความเข้มข้นของแทนนินในสารสกัดน้ำใบหูกวาง 0.4 มก./มล. ให้ผลในการกำจัดแบคทีเรียอย่างรวดเร็ว โดยจำนวนโคโลนีแบคทีเรียลดลงอย่างมีนัยสำคัญเหลือ 0 ที่ 8 ชั่วโมง ($r_s = -0.975$, $p\text{-value} = 0.02$) ผลการศึกษานี้ชี้ให้เห็นว่าสารสกัดน้ำใบหูกวางอาจเป็นทางเลือกจากธรรมชาติที่ช่วยลดการใช้ยาปฏิชีวนะในการรักษาการติดเชื้อ *S. aureus* ได้

คำสำคัญ: ใบหูกวาง, สารสกัดน้ำ, ฤทธิ์ต้านแบคทีเรีย, สแตปฟีโลคอคคัส ออเรียส

Abstract

Staphylococcus aureus is a pathogenic bacterium that causes skin infections in both pets and humans with the increasing trend of bacterial resistance to antibiotics, prompting interest in alternative antibacterial agents from natural sources. Therefore, this study aims to the efficacy of aqueous extract from Indian almond (*Terminalia catappa* Linn.) leaves, focusing on determining the minimum bactericidal concentration (MBC) and the time required for effective killing *S. aureus*. The experiment was conducted by preparing an aqueous extract of Indian almond leaves through boiling, followed by extended immersion for 7 days. The MBC and Time-dependent killing was performed by macrodilution method. The results show that the ferment extract of Indian almond leaf extract contained 0.76 mg/ml of tannin. The MBC of ferment extract against *S. aureus* was between 0.35 to 0.4 mg/ml. In addition, time-killing assay

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demonstrated a concentration and time-dependent bactericidal effect. At 0.35 mg/ml of aqueous extract, colony counts of *S. aureus* statistically significantly decreased from 115 at 2 hours to 0 at 24 hours ($r_s = -1.000$, p -value < 0.001). At 0.4 mg/ml, bacterial colony elimination occurred rapidly, with colony counts reduced significantly to 0 at 8 hours ($r_s = -0.975$, p -value = 0.02). These findings suggest that the aqueous Indian almond leaf extract may serve as a natural alternative to reduce antibiotic use against *S. aureus* infection.

Keywords: Indian almond leaf, *Staphylococcus aureus*, aqueous extract, tannin, antibacterial activity

Introduction

Pyoderma is a bacterial infectious skin disease characterized by various clinical presentations of skin such as erythema, alopecia, and itching with macules, papules, or pustules. The causative pathogen of pyoderma in animals is bacteria of the genus *Staphylococcus*, gram-positive bacteria, that live on the skin as normal flora, which infection typically secondary to skin damage (Heinrich et al., 2019). The infection may persist or recurrently infect due to contributing factors that promote the overgrowing and spreading of bacteria among animals including improper hygienic and veterinary care, stray dogs and cats, housing conditions, and pet and owner contact (Moon et al., 2022). These skin problems in animals commonly end with antibiotic treatment by veterinary practitioners. However, inappropriate treatment of pyoderma in animals with a lack of diagnostic data or improper selection and dose of antibiotics might lead to antimicrobial-resistant (AMR) bacteria (Malik et al., 2005), which commonly emerge among animals and humans. Moreover, bacterial resistance can be worsened in human and animal health due to multiple drug-resistant strains of bacteria. The methicillin-resistant *Staphylococcus aureus* (MRSA), for instance, resists not only methicillin but also antibiotics such as aminoglycosides, macrolides, tetracycline, chloramphenicol, and lacosamide (Nikaido, 2009). Hence, tackling this issue requires coordinated efforts in surveillance, antibiotic stewardship, and research into alternative treatments such as medicinal plants.

In this study, we have investigated the antibacterial effect of the leaf from the Indian almond tree (*Terminalia catappa* Linn.), which is commonly found in Thailand. This plant has been used as traditional medicine in aquatic animals due to its benefits for wound healing and antimicrobial properties (Malawa et al., 2022) against

S. epidermidis, *S. aureus*, *B. cereus*, *B. subtilis*, and *P. aeruginosa* (Chansue & Assawawongkasem, 2008). These therapeutic effects including antimicrobial activity are related to some chemical constituents of Indian almond leaf such as tannins, flavonoids, isovitexin, vitexin, isoorientin, rutin, and triterpenoids (Chansue & Assawawongkasem, 2008; Anand et al., 2015).

Although its antimicrobial potential is recognized, limited data exist on its bactericidal activity, specifically regarding the minimum bactericidal concentration (MBC) and the time required to achieve bacterial elimination. Therefore, this study aims to investigate the minimum concentration of aqueous Indian almond leaf extract against *S. aureus* and the suitable time for bacterial inhibition.

Materials and Methods

1. Preparation of aqueous extract from Indian almond leave

An aqueous extract of Indian almond leaves (*Terminalia catappa*) was prepared based on the methods of Chansue and Assawawongkasem (2008) and Muangthai et al. (2015) with modifications. Dried reddish-brown leaves, purchased from Thailand, were used for the preparation. A 32 g sample of the leaves was further dried at 60°C for 30 minutes and subsequently ground into a coarse powder. The aqueous extraction was performed by adding the ground Indian almond leaves to 250 ml of distilled water, followed by boiling at 100°C for 5 minutes. The resulting extract was then allowed to undergo an extended immersion period, stored in the dark at room temperature for 7 days, with daily pH monitoring and recording. No intentional microbial inoculum was added during this period. Finally, the mixture was passed through a stainless steel sieve to remove coarse particulate

matter. The resulting liquid was then sterile-filtered using a 0.22 µm rapid flow bottle top filter (Nalgene™, Thermo Scientific). The sterile aqueous extract was collected, aliquoted, and stored at -20°C until further use.

2. Quantitative estimation of total tannin

The total tannin content in the Indian almond leaf aqueous extract was determined using the Folin-Ciocalteu colorimetric method, following the protocols of Muangthai *et al.* (2015) and Kahlaoui *et al.* (2019) with slight modifications. A set of tannic acid standards (Gallotannin, Himedia™) was prepared in distilled water at concentrations of 0, 2, 4, 6, 8, and 10 mg/ml. The aqueous extract sample was prepared for analysis by performing undiluted and a two-fold water-diluted extract samples. For the assay, 100 µl of each standard concentration and the diluted aqueous extract were placed into separate test tubes. To each tube, 500 µl of 10% Folin-Ciocalteu reagent (Loba Chemie, India) was added, followed by 400 µl of 7.5% sodium carbonate. The mixtures were immediately vortexed and incubated at room temperature in the dark for 30 minutes to allow for color development. The absorbance of each standard and sample was then measured at 700 nm using a spectrophotometer, with the 0 mg/ml standard serving as the blank.

To calculate the concentration, a linear regression curve was established from the tannic acid standards (Figure 1). The absorbance of both the undiluted and diluted samples was used to determine their concentrations from this equation. The result for the diluted sample was multiplied by the dilution factor of 2. The final tannin content was taken from the sample whose absorbance fell within the linear range of the assay and expressed as mg/ml of tannic acid equivalent.

3. Minimum bactericidal concentration (MBC) of Indian almond leaf aqueous extract against *S. aureus*

The *S. aureus* (TSTR 1840) sample provided by the Immunology Lab, Faculty of Veterinary Medicine, Mahanakorn University of Technology, was used to evaluate the bactericidal effect of the Indian almond leaf ferment extract using the broth dilution technique. To

ensure specificity, bacterial samples were selectively cultured on mannitol salt agar to confirm Staphylococcal presence, followed by isolation of a single colony and culturing in nutrient broth at 37°C for 48 hours. The bacterial density was quantified as colony-forming units per milliliter (CFU/ml) prior to testing.

For the experiment, 100 µl of fresh bacterial culture at 1.14×10^9 CFU/ml was added to each 1.5 ml microcentrifuge tube containing Mueller-Hinton broth, alongside 10 µg of amoxicillin (positive control, Andrews, 2004) and various concentrations of estimated tannin equivalents from the Indian almond leaf ferment extract (0.1, 0.15, 0.2, 0.25, 0.3, 0.35, and 0.4 mg/ml). After incubation at 37°C for 24 hours, treated bacterial samples were subjected to serial dilutions (10^1 , 10^2 , 10^4 , and 10^6) in Mueller-Hinton agar and cultured for an additional 24 hours. The recovered bacterial colonies were counted and quantified as CFU/ml, allowing for assessment of the minimum bactericidal concentration.

4. Time-dependent bactericidal effect of Indian almond aqueous extract against *S. aureus*

The time-dependent bactericidal activity of Indian almond aqueous extract against *S. aureus* was evaluated using two selected minimum bactericidal concentrations determined in the previous experiment. A total of 100 µl of freshly prepared *S. aureus* suspension (1.14×10^9 CFU/ml) was added into individual sterile microcentrifuge tubes containing either 10 µg of amoxicillin (positive control) or the two tested MBC concentrations of the aqueous extract.

All treated samples were incubated at 37°C and sampled at specific time intervals: 0, 2, 4, 6, 8, and 24 hours. At each time point, 20 µl of the bacterial suspension from each treatment group was collected and serially diluted with sterile water to 10^{-1} , 10^{-2} , 10^{-4} , and 10^{-6} . Aliquots from each dilution were plated onto mannitol salt agar and incubated for 48 hours at 37°C.

After incubation, bacterial colonies were counted, and viable bacterial counts were expressed as colony-forming units per milliliter (CFU/ml). The percentage of bacterial reduction at each time point was calculated

based on the initial CFU/ml, following the method described by Balouiri *et al.* (2015), to assess the time-dependent bactericidal effect of the extract.

5. Data collection and analysis

In this study, the concentration of total tannin in Indian almond leaf aqueous extract was presented as mg/ml. The number of *S. aureus* observed during the experiments was recorded as bacterial colonies and CFU/ml. Then, the relationship between the exposure time and the number of bacterial colonies was statistically determined by Spearman correlation using IBM SPSS statistics version 23. The statistical significance was set at a confidence level of 95% ($p < 0.05$).

Results and discussion

1. Characteristics of aqueous extract from Indian almond leave and total tannin content

The Indian almond leaf aqueous extract was prepared by hot water extraction using 32 g of homogenized reddish-brown dried leaves in 250 ml of distilled water. The mixture was allowed to stand in dark conditions at room temperature for 7 days, yielding a brownish, water-soluble extract with an acidic final pH of 3.65. A gradual decrease in pH was observed over the 7-day extraction period, with pH values recorded at 3.98, 3.77, 3.69, 3.73, 3.64, 3.60, 3.61, and 3.65 from day 0 to day 7, respectively. This acidification may be attributed to the release of phenolic compounds and hydrolyzable tannins

during extended aqueous extraction. Similar observations were reported by Chansue and Assawawongkasem (2008), where the application of Indian almond leaf extract at increasing concentrations (0–10 mg/ml) decreased the pH of aquaria water from 7.60 ± 0.14 to 6.09 ± 0.16 . The acidity of the extract is likely due to the presence of hydrolyzable tannins, which are known to contain esters of phenolic acids such as gallic acid and ellagic acid (Bule *et al.*, 2020). During hot water extraction, hydrolysis of tannins that are bound to plant cell wall components such as cellulose can be enhanced, resulting in a greater release of free phenolic acids (Das *et al.*, 2020; Bolling, 2017).

The total tannin content of the Indian almond leaf aqueous extract was estimated at 0.76 mg/ml (Figure 1B), based on a standard gallotannin curve (2–10 mg/ml) and absorbance at 700 nm (Figure 1A). Although a high leaf-to-water ratio was used (32 g in 250 ml; 12.8% w/v), the yield was lower than expected. This may be due to differences in leaf color, maturity, and extraction efficiency (Muangthai *et al.*, 2015; Mueller-Harvey, 2001). Indian almond leaves contain hydrolyzable tannins such as gallotannins, which can release phenolic acids under heat and polar solvent conditions (Bule *et al.*, 2020; Desam & Al-Rajab, 2022; Arapitsas, 2012). These factors, along with natural variability, may explain the moderate tannin concentration observed. Tannin content was measured once per sample; future studies should include replicates to enhance accuracy.

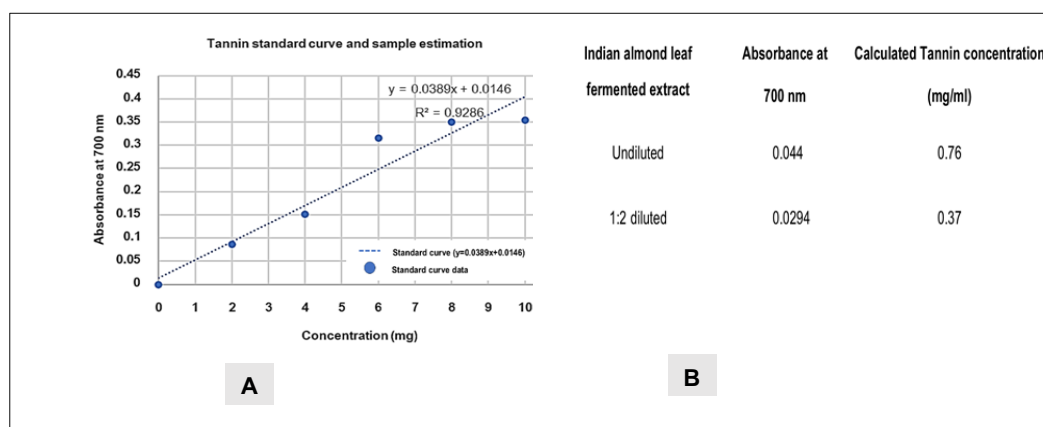


Figure 1 Panel A shows the tannin standard curve with the regression equation and R^2 value for concentration estimation. Panel B presents OD measurements for undiluted and two-folded diluted Indian almond leaf aqueous extract and estimated tannin concentration.

2. Minimum bactericidal concentration (MBC) of Indian almond leaf aqueous extract against *S. aureus*

The bactericidal activity of Indian almond leaf aqueous extract against *Staphylococcus aureus* was evaluated using the broth dilution technique followed by bacterial recovery on Mueller-Hinton agar. Post-treatment bacterial suspensions were serially diluted (10^{-1} , 10^{-2} , 10^{-4} , and 10^{-6}) and plated to determine viable colony counts. At a 10^{-4} dilution, over 300 colonies were observed in the group treated with 0.1 mg/ml of the crude extract, indicating limited bactericidal effect. At a 10^{-6} dilution, over 300 colonies were observed, which exceeds the reliable counting range and is considered too numerous to count

(TNTC) due to potential overlap and miscounting (Madigan *et al.*, 2019). In contrast, lower concentrations of the extract (0.15 to 0.4 mg/ml) showed a dose-dependent reduction in bacterial count: 260 colonies (2.60×10^6 CFU/ml), 245 colonies (2.45×10^6 CFU/ml), 38 colonies (3.80×10^5 CFU/ml), 185 colonies (1.85×10^6 CFU/ml), 51 colonies (5.10×10^5 CFU/ml), and 31 colonies (3.10×10^5 CFU/ml), respectively. As a positive control, amoxicillin-treated samples yielded 15 colonies at 10^{-6} dilution (1.50×10^8 CFU/ml). Notably, no bacterial colonies were recovered from the 10^{-6} dilution in samples treated with Indian almond leaf aqueous, suggesting that bactericidal activity occurred at this concentration (Table 1).

Table 1 Minimum bactericidal concentration (MBC) of 0.1–0.4 mg/ml tannin from Indian almond leaf aqueous extract against *S. aureus*

Indian almond leaf aqueous extract (mg/ml)	Dilution 10^{-4}		Dilution 10^{-6}	
	Colony	CFU/ml	Colony	CFU/ml
0.10	>300	-	0	-
0.15	260	2.60×10^6	0	-
0.20	245	2.45×10^6	0	-
0.25	38	3.80×10^5	0	-
0.30	185	1.85×10^6	0	-
0.35	51	5.10×10^5	0	-
0.40	31	3.10×10^5	3	3×10^6
Amoxicillin 10 µg (Positive control)	>300	-	15	1.50×10^8

Note: > 300 bacterial colonies considered too numerous to count (TNTC) (Madigan *et al.*, 2019).

Based on the results, the minimum bactericidal concentration (MBC) of Indian almond leaf aqueous extract against *S. aureus* was identified at 0.35–0.4 mg/ml, at which no viable colonies were recovered. This aligns with previous reports on tannins' antibacterial activity. Akiyama *et al.* (2001) found that tannic acid at 280 mg/L inhibited 14 *S. aureus* strains, and full bactericidal activity was observed above 4,500 mg/L. Similarly, Dong *et al.* (2017) demonstrated that tannic acid at 40–160 µg/ml (mean 80 µg/ml) inhibited both MSSA and MRSA. Regarding *T. catappa*, Thansuwan *et al.* (2019) reported that aqueous extract at 32.25 mg/ml produced a 4.33 mm inhibition

zone, while 70% and 97% ethanol extracts at 32.25 and 15.63 mg/ml yielded 3.33 mm and 6.67 mm zones, respectively. These results suggest concentration-dependent bactericidal effects, likely related to tannin content (Anand *et al.*, 2015). Wang *et al.* (2023) showed that tannic acid disrupts *S. aureus* membranes by downregulating genes like *murB*, *murG*, and *tagA*. Dong *et al.* (2017) further reported preferential binding of tannic acid to the bacterial peptidoglycan layer, with 41.2% binding in *S. aureus* versus 21.3% in *E. coli*, suggesting that cell wall composition influences susceptibility.

3. Time-dependent bactericidal effect of Indian almond leaf aqueous extract

The bactericidal effect of Indian almond leaf aqueous extract against *S. aureus* was assessed over time. At 10^4 CFU/ml, treatment with 0.35 and 0.4 mg/ml extracts showed a progressive reduction in colony counts from 0 to 24 hours. After 2 hours, colony numbers were 115 and 113 (61.66% and 62.33% inhibition), which decreased to 39 and 17 at 4 hours (87% and 94.33%

inhibition). Complete inhibition (100%) occurred at 8 hours for 0.4 mg/ml and at 24 hours for 0.35 mg/ml. Colony counts declined with time for both concentrations, showing statistically significant negative correlations ($r_s = -1.000$, $p < 0.001$ and $r_s = -0.975$, $p = 0.02$, respectively). In contrast, 145 colonies were observed at 24 hours in the amoxicillin-treated group (10 µg/ml). These results confirm a time- and concentration-dependent bactericidal effect, as summarized in Table 2.

Table 2 Time-dependent bactericidal activity of Indian almond leaf aqueous extract

Indian almond leaf aqueous extract (mg/ml)	Hour	Colony count	% Bactericidal activity	Correlation coefficient (r_s)	p-value
0.35	0	>300	0	-1.000	< 0.001
	2	115	61.66		
	4	39	87		
	6	16	94.66		
	8	3	99		
	24	0	100		
0.4	0	>300	0	-0.975	0.02
	2	113	62.33		
	4	17	94.33		
	6	5	98.33		
	8	0	100		
	24	0	100		
Amoxicillin 10 µg	0	>300			
(Positive control)	2-8	>300	NA	NA	NA
	24	145			

Note: r_s stands for Spearman correlation coefficient, and NA stands for not analyzed. The correlation is significant at the 0.05 level ($p < 0.05$).

These findings indicate that Indian almond leaf aqueous extract exerts a statistically significant time-dependent bactericidal effect against *S. aureus*. The progressive reduction in viable bacterial colonies from 2 to 24 hours suggests that tannins, flavonoids, and phenolic acids gradually interact with bacterial cell structures, particularly the cell wall (Dong *et al.*, 2017). Supporting this, Olchowik-Grabarek *et al.* (2023) demonstrated that gallotannin (PGG) at its MIC (76 µg/ml) sustained suppression of *S. aureus* growth over 12 hours. Wang *et al.* (2023) further demonstrated that

tannic acid disrupts cell envelope integrity, ribosome assembly, and amino acid metabolism, inducing bacterial stress and metabolic exhaustion. This aligns with prior findings showing tannic acid progressive weakening of bacterial viability, ultimately leading to clearance within 24 hours depending on concentration. Together, the present results and prior studies support that tannins in Indian almond leaf extract contribute to time and concentration-dependent bactericidal activity, with bacterial clearance occurring between 2 to 8 hours, and complete elimination by 24 hours.

Conclusion

Indian almond leaf aqueous extract demonstrates promising bactericidal activity against *S. aureus*, with an MBC observed at 0.35–0.4 mg/ml. The effect was both concentration- and time-dependent, achieving complete bacterial elimination within 8–24 hours. While these findings highlight the potential of tannin-rich plant extracts for antibacterial use, further studies on extract standardization, safety, and formulation are essential to advance their application in veterinary or medical settings.

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