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Antibacterial activities of Thankuni (*Centella asiatica*) leaf extract against *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*

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Abstract

Antimicrobial resistance is a major global health threat, driving the search for cost-effective alternatives. Plant-based drugs from traditional medicine are promising candidates due to their potential for reducing the development of resistance. An experimental study was conducted in the Department of Pharmacology & Therapeutics in collaboration with the Department of Microbiology of Mymensingh Medical College, Bangladesh, from July 2022 to June 2023, to evaluate the in vitro antibacterial activities of aqueous and ethanolic extracts of thankuni (*Centella asiatica*) leaves against *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Antibacterial activity against *Staphylococcus aureus, Escherichia coli*, and *Pseudomonas aeruginosa* was evaluated using the Kirby-Bauer disc diffusion method. The minimum inhibitory concentration (MIC) was determined by the broth dilution technique, with ceftriaxone as a control. All procedures were conducted in accordance with standard clinical laboratory protocols. For the aqueous extract (ATLE), the largest zone of inhibition (ZOI) at the highest tested concentration (100 mg/ml) was recorded against *S. aureus* (25 mm), followed by *E. coli* and *P. aeruginosa* (both 19 mm). A definite inhibitory effect (ZOI >8 mm) for ATLE began at 40 mg/ml for *S. aureus*, 60 mg/ml for *E. coli*, and 100 mg/ml for *P. aeruginosa*. The ethanolic extract (ETLE) exhibited superior antibacterial efficacy compared to ATLE ($p<0.001$). At 100 mg/ml, ETLE produced zones of inhibition of 26 mm against *S. aureus*, 25 mm against *E. coli*, and 24 mm against *P. aeruginosa*. Notably, the ethanolic extract began showing definite activity at much lower concentrations: 20 mg/ml for *S. aureus* and 40 mg/ml for both *E. coli* and *P. aeruginosa*. The two extracts at equivalent concentrations showed that the enhanced activity of the ethanolic extract was particularly significant against the Gram-negative organisms, *E. coli* and *P. aeruginosa* ($p<0.01$). The MIC values showed that ETLE was more potent than ATLE. The MIC of the aqueous extract was 30 mg/ml for *S. aureus*, 60 mg/ml for *E. coli*, and 75 mg/ml for *P. aeruginosa*. In contrast, the MIC of the ethanolic extract was significantly lower, at 20 mg/ml for *S. aureus* and 40 mg/ml for both *E. coli* and *P. aeruginosa*. This represents a 33.3% increase in potency against *S. aureus* and a 33.3% and 46.7% increase against *E. coli* and *P. aeruginosa* respectively, when using the ethanolic solvent for extraction. As expected, the standard antibiotic control, Ceftriaxone, demonstrated far greater potency, with MIC values in the microgram range: 1.5 µg/ml for *S. aureus*, 1.0 µg/ml for *E. coli*, and 1.5 µg/ml for *P. aeruginosa*. Both aqueous and ethanolic extracts of thankuni (*Centella asiatica*) leaves possess definite antibacterial activity against the tested organisms, with the ethanolic extract being more potent.

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Introduction

The relentless emergence and global spread of antimicrobial resistance (AMR) represent one of the most pressing public health crises of the 21st century.[1] The efficacy of conventional antibiotics is diminishing, leading to increased mortality, prolonged hospital stays, and higher healthcare costs. The pipeline of new antibacterial drugs is alarmingly sparse, a situation exacerbated by the high cost and lengthy timeline of de novo antibiotic discovery.[2] This urgent need has revitalized the exploration of alternative and complementary therapeutic strategies, with medicinal plants drawing significant scientific attention.[3] For millennia, plants have been the cornerstone of traditional medicine systems worldwide, offering a rich repository of bioactive compounds. It is estimated that over 80% of the world's population relies on plant-based remedies for primary healthcare.[4] This historical use provides a valuable starting point for modern drug discovery. Scientific investigations continue to validate the efficacy of many traditional plants, revealing their potential as sources of novel antimicrobial agents with diverse mechanisms of action.[5] A key hypothesis driving this research is that the complex phytochemical composition of plant extracts – often comprising synergistically acting alkaloids, flavonoids, tannins, and terpenoids – may present a multi-target challenge to pathogens, thereby reducing the propensity for resistance development compared to single-target synthetic antibiotics.[6] *Centella asiatica Linn*. Urb, commonly known in Bangladesh as thankuni or Indian pennywort, is one such plant with a venerable history in ethnomedicine. It is a perennial herbaceous plant belonging to the Apiaceae family, widely distributed in the tropical and subtropical regions of Asia, Africa, and the Americas.[7] In traditional practices, particularly in Ayurveda and Bengali folk medicine, it has been used for centuries to treat a wide array of ailments, including skin diseases, leprosy, lupus, wounds, and various inflammatory conditions.[8] Its renowned wound-healing properties are well-documented and are attributed to triterpenoid compounds such as asiaticoside, madecassoside, asiatic acid, and madecassic acid.[9] While its anti-inflammatory and antioxidant activities are extensively studied, its direct antibacterial properties warrant further systematic investigation, especially against contemporary clinical pathogens. Among the most challenging bacterial pathogens are *Staphylococcus aureus*, Escherichia coli, and *Pseudomonas aeruginosa*. *Staphylococcus aureus* is a leading cause of both community-acquired and hospital-onset infections, ranging from minor skin abscesses to life-threatening conditions like bacteremia and endocarditis, with methicillin-resistant strains (MRSA) posing a particular threat.[10] *Escherichia coli*, while a common commensal, is a predominant agent of urinary tract infections, gastroenteritis, and neonatal meningitis, with increasing incidence of multi-drug resistant (MDR) strains.[11] *Pseudomonas aeruginosa* is an opportunistic, MDR pathogen notorious for its intrinsic resistance to many antibiotics and its role in severe infections in immunocompromised individuals, cystic fibrosis patients, and those with burn wounds.[12] The rising resistance in these organisms to last-line antibiotics, including carbapenems, underscores the critical need for new therapeutic options.[13,14] Therefore, this study was designed to scientifically evaluate the in vitro antibacterial potential of both aqueous and ethanolic leaf extracts of Centella asiatica against these three clinically significant bacterial pathogens – *Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa*. By systematically determining the zone of inhibition and minimum inhibitory concentration (MIC), and

comparing the activity to that of a standard antibiotic, this research aims to contribute validated data on the therapeutic potential of this widely available medicinal plant.

Methods

This experimental study was conducted from July 2022 to June 2023, in the Department of Pharmacology & Therapeutics in collaboration with the Department of Microbiology of Mymensingh Medical College, Bangladesh.

Plant material and extract preparation: Mature, fresh leaves of *Centella asiatica* were procured from a local market. The leaves were washed, shade-dried at room temperature for 12 days, and pulverized into a fine powder using an electrical blender. The aqueous extract (ATLE) was prepared by macerating 20 g of powder in 500 mL of hot distilled water for 7 days with continuous stirring. The ethanolic extract (ETLE) was prepared by soaking 20 g of powder in 500 mL of 95% ethanol for 24 hours. Both mixtures were filtered through Whatman No. 1 filter paper. The filtrates were evaporated to dryness in a pre-weighed petri dish at 50°C. The resulting dried extracts were reconstituted in sterile distilled water to a final stock concentration of 1 g/ml for antibacterial assays.[15]

Fig. 1: Air-dried thankuni leaves (left) and thankuni leaves powder (right).

Test microorganisms and standard antibiotics: Standard reference strains of *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), and *Pseudomonas aeruginosa* (ATCC 27853) were used. The standard antibiotic, ceftriaxone (1g/10ml, from Popular Pharma Ltd., Bangladesh) was used as a positive control.

Assessment of antibacterial activity: The antibacterial activity of both extracts was evaluated using the Kirby-Bauer disc diffusion method.16 Bacterial suspensions were standardized to 0.5 McFarland turbidity (approximately 1.5 × 10^8 CFU/ml) and swabbed onto Mueller-Hinton agar plates. Sterile 6 mm filter paper discs were impregnated with 10 µL of each extract at concentrations ranging from 10-100 mg/ml. A disc with 95% ethanol served as a negative control. The plates were incubated at 37°C for 24 hours, after which the zones of inhibition (ZOI) were measured in millimeters.

Determination of minimum inhibitory concentration (MIC): The MIC was determined for both extracts and Ceftriaxone using the broth macro-dilution technique.[17] Two-fold serial dilutions of the test agents were prepared in nutrient broth. Each tube was inoculated with a standardized bacterial suspension and incubated at 37°C for 24 hours. The MIC was defined as the lowest concentration that completely inhibited visible bacterial growth. Subcultures from clear tubes were performed on nutrient agar to confirm bactericidal activity (MBC). Categorical variables were summarized using frequencies and percentages. Associations were examined using unpaired Student's t-test test and two-way ANOVA as appropriate. Statistical analysis was conducted using IBM SPSS version 26.0 for Windows, with level of significance set at <0.05. Data was presented in tabulated form.

As no human or animal was used in the experimentation, this study protocol was exempted from ethical review by the permission of the Institutional Review Board of Mymensingh Medical College, Bangladesh.

Results

The present study systematically evaluated the in vitro antibacterial potential of aqueous (ATLE) and ethanolic (ETLE) extracts of *Centella asiatica* against three clinically significant bacterial pathogens – *Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa*. The results, obtained through disc diffusion and broth dilution assays, demonstrated significant and differential antibacterial activity for both extracts. The initial screening using the Kirby-Bauer disc diffusion method revealed that both ATLE and ETLE possessed notable antibacterial activity, which was directly proportional to the concentration of the extract. The negative controls (sterile disc and ethanol-treated disc) showed no zone of inhibition, confirming that the observed activities were due to the plant extracts. For the aqueous extract (ATLE), the largest zone of inhibition (ZOI) at the highest tested concentration (100 mg/ml) was recorded against *S. aureus* (25 mm), followed by *E. coli* and *P. aeruginosa* (both 19 mm). A definite inhibitory effect (ZOI >8 mm) for ATLE began at 40 mg/ml for *S. aureus*, 60 mg/ml for *E. coli*, and 100 mg/ml for *P. aeruginosa*, indicating that *S. aureus* was the most susceptible among the three to the aqueous extract (Table-I). The ethanolic extract (ETLE) exhibited superior antibacterial efficacy compared to ATLE ($p<0.001$). At 100 mg/ml, ETLE produced zones of inhibition of 26 mm against *S. aureus*, 25 mm against *E. coli,* and 24 mm against *P. aeruginosa.* Notably, the ethanolic extract began showing definite activity at much lower concentrations: 20 mg/ml for *S. aureus* and 40 mg/ml for both *E. coli* and *P. aeruginosa* (Table-II). Statistical analysis comparing the ZOIs of the two extracts at equivalent concentrations showed that the enhanced activity of the ethanolic extract was particularly significant against the Gram-negative organisms, *E. coli* and *P. aeruginosa* ($p<0.01$) (Table-III).

Table-I: Zone of inhibition (in mm) of aqueous thankuni leaf extract (ATLE) at different concentrations

Concentration (mg/ml)	*S. aureus*	*E. coli*	*P. aeruginosa*
100	25	19	19
80	20	15	12
60	18	14	8
40	16	8	6
20	6	6	6
10	6	6	6
Control (Disc)	6	6	6

Table-II: Zone of inhibition (in mm) of ethanolic thankuni leaf extract (ETLE) at different concentrations

Concentration (mg/ml)	*S. aureus*	*E. coli*	*P. aeruginosa*
100	26	25	24
80	24	19	21
60	18	15	19
40	16	13	13
20	15	8	8
10	6	6	6
Control (Ethanol)	6	6	6

Two-way ANOVA was done; the effect of extract type and concentration on ZOI was statistically significant ($p<0.001$).

Table-III: Comparison of ZOI (mm) between ATLE and ETLE at 100 mg/ml

Test Organism	ATLE	ETLE
S. aureus	25	26
E. coli	19	25
P. aeruginosa	19	24

Unpaired Student's t-test was done; the difference in ZOI between extracts was significant for E. coli ($p=0.003$) and P. aeruginosa ($p=0.002$).

The MIC values aligned with the trends observed in the diffusion tests, solidifying the finding that ETLE was more potent than ATLE. The MIC of the aqueous extract was 30 mg/ml for *S. aureus*, 60 mg/ml for *E. coli,* and 75 mg/ml for *P. aeruginosa*. In contrast, the MIC of the ethanolic extract was significantly lower, at 20 mg/ml for *S. aureus* and 40 mg/ml for both *E. coli* and *P. aeruginosa*. This represents a 33.3% increase in potency against *S. aureus* and a 33.3% and 46.7% increase against *E. coli* and *P. aeruginosa* respectively, when using the ethanolic solvent for extraction. As expected, the standard antibiotic control, Ceftriaxone, demonstrated far greater potency, with MIC values in the microgram range: 1.5 µg/ml for *S. aureus*, 1.0 µg/ml for *E. coli*, and 1.5 µg/ml for *P. aeruginosa* (Table-IV&V).

Table-IV: Minimum inhibitory concentration (MIC) of ATLE, ELTE, and ceftriaxone

Organism	MIC of ATLE (mg/ml)	MIC of ETLE (mg/ml)	MIC of Ceftriaxone (µg/ml)
S. aureus	30	20	1.5
E. coli	60	40	1.0
P. aeruginosa	75	40	1.5

This underscores the difference in potency between crude plant extracts and a purified, modern antibiotic, while also validating the sensitivity of the test organisms and the experimental protocol. To confirm whether the observed MIC represented a bacteriostatic or bactericidal effect, subculturing was performed from the clear, non-turbid tubes in the MIC test onto fresh nutrient agar plates. The results were unequivocal: no bacterial growth was observed on any of the subculture plates from the tubes containing the extract at its MIC value and above. This confirms that the MIC determined for both ATLE and ETLE against all three test organisms was, in fact, a minimum bactericidal concentration (MBC), indicating that the extracts killed the bacteria rather than merely inhibiting their growth.

Table-V: Subculture results from MIC tubes confirming bactericidal activity

Organism	ATLE MIC (mg/ml)	Subculture	ETLE MIC (mg/ml)	Subculture	Ceftriaxone MIC (µg/ml)	Subculture
S. aureus	30	No growth	20	No growth	1.5	No growth
E. coli	60	No growth	40	No growth	1	No growth
P. aeruginosa	75	No growth	40	No growth	1.5	No growth

Discussion

This study provides compelling in vitro evidence supporting the traditional use of thankuni (*Centella asiatica*) for managing infections. Our findings demonstrate that both aqueous and ethanolic leaf extracts possess significant and broad-spectrum antibacterial activity against the tested Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*, and *Pseudomonas aeruginosa)* pathogens. The consistent results from the disc diffusion and broth dilution methods affirm the reliability of these findings. The superior efficacy of the ethanolic extract (ETLE) over the aqueous extract (ATLE), evidenced by larger zones of inhibition and

lower MIC values, is a critical observation. This is consistent with phytochemical principles, as ethanol is a more versatile solvent than water, efficiently extracting a wider range of antimicrobial compounds, including alkaloids, flavonoids, terpenoids, and saponins.[14] The presence of such bioactive compounds in *C. asiatica*, particularly triterpenoid saponins (asiaticoside, madecassoside) and various flavonoids, has been well-documented.[8,15] These compounds are known to disrupt bacterial cell membranes, inhibit enzyme activity, and interfere with cell wall synthesis.[16] The stronger activity of ETLE suggests that the antimicrobial principles in Thankuni are more effectively solubilized in ethanol. The variation in susceptibility among the bacterial species aligns with known microbiological characteristics. *Staphylococcus aureus* was the most susceptible, a finding reported in other studies on medicinal plant extracts.[17] This can be attributed to the structural simplicity of the Gram-positive cell wall, which is more permeable to external agents compared to the complex outer membrane of Gram-negative bacteria.[18] Among the Gram-negative bacteria, *P. aeruginosa* exhibited the highest resistance, requiring the highest MIC values from both extracts. This intrinsic resistance is a hallmark of *P. aeruginosa*, attributed to its low-permeability outer membrane and efficient efflux pump systems.[19] Nonetheless, the significant activity of ETLE against this notoriously resilient pathogen is particularly promising. While the antibacterial activity of the crude extracts was substantial, it was, as expected, orders of magnitude less potent than the standard antibiotic, Ceftriaxone. This is not a shortcoming but rather a reflection of the difference between a complex crude extract and a purified, targeted pharmaceutical. The clinical relevance of plant extracts often lies in their multi-component, synergistic action, which can potentially reduce the likelihood of resistance development.[6] The subculture studies, which confirmed the bactericidal (MBC) nature of the extracts, further strengthen their therapeutic potential, indicating they kill bacteria rather than merely their growth. Our results are in agreement with several previous studies.[19,20] Our study also found that ethanolic extracts of *C. asiatica* to be more effective than aqueous ones. Research has attributed the plant's bioactivity to its triterpenoid and flavonoid content, which correlates with our phytochemical reasoning.[8] However, some studies have reported varying degrees of efficacy, which can be influenced by factors such as plant geography, harvest time, and extraction methodology.[8,15] This research validates the ethnomedicinal use of *Centella asiatica* and scientifically confirms its potent, broad-spectrum, and bactericidal activity. The ethanolic extract shows particular promise as a source of potential antibacterial agents. In an era of escalating antimicrobial resistance,[21] such readily available medicinal plants offer a valuable reservoir for developing new therapeutic strategies or complementary alternatives.

A key limitation of this study is the use of crude extracts, which preclude the identification of the specific active compound(s) responsible for the observed antibacterial activity. Future research should focus on compound isolation and purification.

Conclusion

This study conclusively demonstrates that both aqueous and ethanolic extracts of *Centella asiatica* possess significant, broad-spectrum, and bactericidal activity against *Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa*. The ethanolic extract showed superior potency. These findings scientifically validate the plant's traditional use and

position it as a promising source for developing novel antibacterial agents to combat the growing threat of antimicrobial resistance. Further investigation to isolate the active compounds is warranted. Moreover, future research should focus on the bioassay-guided fractionation and identification of the specific active compounds. Subsequent in vivo studies and investigations into the precise mechanism of action are also highly recommended to advance these promising findings towards therapeutic application.

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