Asian Plant Research Journal

Volume 12, Issue 2, Page 14-21, 2024; Article no.APRJ.112925
ISSN: 2581-9992

Exploring the *In-vitro* Antibacterial Efficacy of Aqueous Extracts from Leaves, Bark, and Stem of *Celtis timorensis* Span: A Comprehensive Study

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/APRJ/2024/v12i2244

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/112925

Original Research Article

Received: 15/12/2023
Accepted: 20/02/2024
Published: 24/02/2024

ABSTRACT

The current study evaluated the in-vitro antibacterial activity of *Celtis timorensis* Span. (*C. timorensis*) against Escherichia coli (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and *Staphylococcus aureus* (ATCC 25923) in response to the global healthcare challenge of infectious diseases caused by Multidrug Resistance organisms. The study was motivated by the inappropriate use of existing antimicrobials and the insufficient discovery of new agents contributing to this crisis. Drawing inspiration from historical evidence in plant-based Ayurveda and traditional medicine, the study focused on *C. timorensis*, a plant known for its historical use in treating infectious diseases.
Crude extracts from the leaves, bark, and stem of *C. timorensis* were prepared using the cold

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maceration process, and their antibacterial activity was assessed against the aforementioned bacterial strains. The cylinder plate method was employed to measure zones of inhibition at various concentrations (250 µg/ml, 500 µg/ml, 750 µg/ml, and 1000 µg/ml). Positive and negative controls included Gentamicin and distilled water respectively. The diameter of inhibition zones was measured after 24 hours of incubation.

Results indicated positive antibacterial effects of all three extracts against *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853). Notably, aqueous extraction from the stem exhibited the highest inhibitory zones against E. coli and *P. aeruginosa*. However, none of the concentrations of the extracts showed positive antibacterial effects against *S. aureus* (ATCC 25923). Statistical analysis confirmed the significance of the observed antibacterial activity against E. coli and *P. aeruginosa*.

Dose-response study results highlighted the efficacy and potency of aqueous extractions from bark and leaves against Escherichia coli. In contrast, leaves and bark demonstrated the highest efficacy and potency, respectively against *Pseudomonas aeruginosa*. In conclusion, the study scientifically validated the hypotheses formulated at the study's outset, utilizing evidence from plant-based Ayurveda and traditional medicine. The findings underscore the potential of *C. timorensis* as a source of antibacterial agents in the context of addressing multi-drug resistance-related infectious diseases.

Keywords: Celtis timorensis; Escherichia coli; Pseudomonas aeruginosa; Staphylococcus aureus; antibacterial activity.

1. INTRODUCTION

Microbial diseases, constituting a substantial percentage exceeding 20% of global mortality, persist as a formidable challenge despite the advancements in antimicrobial chemotherapy [1]. The World Health Organization (WHO) actively addresses this challenge through initiatives like the Global Antibiotic Research and Development Partnership (GARDP), which advocates for innovative antibiotic treatments and alternative approaches. Despite the routine application of plant-derived remedies by Ayurvedic practitioners for therapeutic interventions, there exists a notable underutilization of numerous plant-derived antimicrobial agents within Western medical practices. This discrepancy is ascribed, in part, to the prevailing inclination toward using plant-derived medicine, attributed to their perceived diminished side effects compared to their synthetic counterparts. Consequently, the scope of research findings on plant-derived antimicrobial agents has experienced a notable surge. These agents exhibit both bactericidal and bacteriostatic effects, with the diverse array of secondary metabolites in medicinal plants serving as viable alternatives to resistance-modifying agents, thereby impeding the adaptive response of bacteria, viruses, and fungi [2].

The previous studies evaluated the unexplored antibacterial properties of alcoholic extractions of *C.timorensis*, renowned in Sinhala Ayurveda for its strong antimicrobial effects [3]. Despite recognition, the antibacterial activity of aqueous macerations from specific plant parts remains scientifically untested. Notably, Ayurvedic literature underscores *C. timorensis*'s traditional use in treating various skin infections, corroborated by recent studies emphasizing its wound healing attributes due to antimicrobial properties [4]. The primary aim of this study was to conduct an antibacterial assay against wound-infecting bacteria, focusing on *Escherichia coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), and *Staphylococcus aureus* (ATCC 25923).

The present study focuses on *Celtis timorensi*s, colloquially known as "Gurenda" [5] a flowering plant extensively distributed in humid regions encompassing India, Sri Lanka, Burma, and the Malay Islands (https://indiabiodiversity.org*, 2023*). *C. timorensis* belongs to the Cannabaceae family and is recognized for its medicinal properties, notably in wound healing [6]. Despite extensive global studies on its therapeutic attributes, limited information is available concerning its antimicrobial effects. *C. timorensis* comprises various phytoconstituents, including alkaloids, carbohydrates, proteins, sterols, phenols, flavonoids, gums, mucilage, and saponins. Sinhala Ayurveda literature attests to the historical use of *C. timorensis* as a remedy for various epidemic diseases, a fact reinforced by explicit acknowledgment from the Institute of Ayurveda in Sri Lanka (Ayurvedic Medicinal Plants of Sri Lanka Compendium Version 3, 2003). This study endeavors to comprehensively evaluate the antibacterial efficacy of aqueous extracts derived from the leaves, bark, and stem of *C. timorensis* Span, thereby contributing valuable insights into its potential therapeutic applications.

Fig. 1. *Celtis timorensis* plant

Fig. 2. *Celtis timorensis* leaves

2. MATERIALS AND METHODS

2.1 Sample Collection

Well-grown and fully expanded mature fresh leaves stem, and bark parts of *Celtis timorensis* were collected during daytime within the natural flowering months of the plant from an estate in Peradeniya district in Central Province, Sri Lanka (latitude of 7° 15' 9.00" N and longitude of 80° 35' 28.79" E).

2.2 Identification and Authentication of the Plant

Properly rinsed, dried, and pressed specimens were sent to the National Herbarium, Peradeniya, Sri Lanka for authentication.

2.3 Preparation of Crude Plant Material Extracts

20g of each part of *C. timorensis* was ground into a fine powder and added to 100 ml of solvent. The prepared samples were filtered, and the filtrate was considered the stock crude solution. The extracts were dissolved in distilled water and a dilution series was prepared (200 mg/ml, 150 mg/ml, 100 mg/ml, and 50 mg/ml).

Fig. 3. Maceration of Bark, stem and root extracts of *C. timorensi*

2.4 Evaluation of *In vitro* antibacterial activity

2.4.1 Collection and sub-culturing of test microorganisms

Pathogenic strains of *Escherichia coli* (*E. coli)* (ATCC 25922), *Pseudomonas aeruginosa (P. aeruginosa*) (ATCC 27853), and *Staphylococcus aureus* (*S. aureus)* (ATCC 25923) were acquired from the Medical Research Institute, Colombo 08, Sri Lanka [7]. Subsequently, these strains were subcultured on nutrition agar and stored at a temperature of 4°C for maintenance purposes.

2.4.2 Preparation of test solutions

The test solutions underwent the preparation of a concentration gradient, comprising concentrations of 1000 µg/ml, 750 µg/ml, 500 µg/ml, and 250 µg/ml

2.4.3 Preparation of gentamicin antibiotic for positive control

Gentamicin was used as a positive control in the study. A 125 µl volume of Gentamicin was measured and transferred into a volumetric flask. The solution was replenished to a final volume of 100 ml with sterile distilled water, resulting in a final concentration of 50 µg/ml [8].

2.4.4 Preparation of mcfarland standards

A 0.5 McFarland standard is a bacterial suspension containing 1x108 to 2x108 CFU/ml. It was prepared in the lab by adding $BaCl_2$ to H_2SO_4 with constant stirring [9].

2.4.5 Preparation of bacterial broth

Bacterial broths were prepared using subcultures 24 hours prior. Saline was used as the medium and bacterial colonies were acquired using an inoculating loop and dissolved in saline under aseptic conditions [10].

2.4.6 Preparation of culture media

38.04 grams of Mueller-Hinton Agar were dissolved in distilled water, heated, and aliquoted into 100 ml conical flasks. The flasks were autoclaved at 121°C for 15 minutes, and the glassware was sterilized in a hot air oven at 170°C for 60 minutes [11]. These steps collectively produce a microbiologically pristine Mueller-Hinton Agar medium suitable for various microbiological applications, particularly in antibiotic susceptibility testing [12].

2.5 Determination of Antimicrobial Assay

2.5.1 Inoculum standardization

All bacterial strains were inoculated in Müeller-Hinton broth for about 24 hours [13]. Bacterial strains were cultured at 37◦C on slopes of nutrient agar. All the procedures were conducted under aseptic conditions. The glassware that was used underwent sterilization.

2.5.2 Assay of antibacterial activity using agar well diffusion method

Agar was applied to petri plates, followed by a bacterial-seeded agar layer. The plates were treated with plant extract concentrations and positive/negative controls. After incubation, the inhibition zones were measured using a digital vernier caliper, and the data was averaged from three independent trials for precision [14,15].

2.6 Statistical Analysis

The acquired data underwent statistical analysis using SPSS version 23, and dose-response curves were generated using GraphPad Prism 8.0.1 software. All data were analyzed by an independent sample t-test. It was considered to be statistically significant when the P value was less than 0.05.

3. RESULTS

3.1 Evaluation of Levels of Antimicrobial Efficacy of Celtis Timorensis Span. Plant Parts

3.1.1 Antibacterial effect of celtis timorensis span. Against escherichia coli (ATCC 25922)

As depicted in Table 1, the highest antibacterial efficacy against *E. coli* was observed in the aqueous stem extract at a concentration of 1000 µg/mL, resulting in a substantial inhibitory zone of 14.85 mm. In contrast, the aqueous leaf extract exhibited the lowest antibacterial activity, while the bark extract demonstrated a moderate effect. Mean inhibition zone measurements for the bark and leaf extracts at a concentration of 1000 µg/mL were recorded as 14.20 mm and 14.05 mm, respectively.

At a concentration of 1000 µg/mL, all three extracts—leaves, bark, and stem—exhibited their most significant antibacterial effects. Furthermore, these extracts demonstrated consistent antibacterial activity across all tested concentrations (1000 µg/mL, 750 µg/mL, 500 µg/mL, 250 µg/mL), underscoring their efficacy against *E. coli*.

3.1.2 Antibacterial effect of Celtis timorensis Span. against *Pseudomonas aeruginosa* (ATCC 27853)

As depicted in Table 3, the evaluation of antibacterial activity against *Pseudomonas aeruginosa*, the aqueous extraction from the stem of the plant displayed the highest efficacy, producing a notable 11.91 mm zone of inhibition at a concentration of 1000 µg/ml. In contrast, the bark extraction exhibited the least substantial effect, with the leaf extract falling in between at 11.76 mm and 11.80 mm, respectively. Interestingly, all three extracts demonstrated discernible antibacterial effects at concentrations of 750 µg/ml and 500 µg/ml, yet none exhibited activity at the lower concentration of 250 µg/ml.

3.1.3 Antibacterial effect of aqueous extracts of *Celtis timorensis* leaves, bark and stem against *Staphylococcus aureus*

None of the extracts of leaves or bark or stem exhibited any discernible antimicrobial effect against *S. aureus*.

Table 1. Antibacterial effect of aqueous extracts of *C. timorensis* leaves, bark, and stem against *E. coli*

Antimicrobial-al effect of *Celtis timorensis* Span.	Zone of inhibition (mm)					
	1000 µg/ml	750µg/ml	500 µg/ml	250 µg/ml	Positive control	Negative control
Leaves	14.05±0.13	13.10±0.09	10.96±0.04	9.75±0.10	17.06±0.15	8.00±0.0
Bark	14.20±0.07	12.16±0.08	10.82±0.02	9.38±0.08	16.98±0.23	8.00±0.0
Stem	14.85±0.13	13.55±0.13	11.00±0.05	10.05±0.09	17.18±0.14	8.00±0.0

Table 2. Antibacterial effect of aqueous extracts of Celtis timorensis leaves, bark and stem against *P. aeruginosa*

Antimicrobial effect of *Celtis timorensis* Span.	Zone of inhibition (mm)					
	1000 µg/ml	750µg/ml	500 µg/ml	250 µg/ml	Positive control	Negative control
Leaves	11.80±0.33	10.70±0.35	9.62±0.51	8.00±0.0	15.12±0.42	8.00±0.0
Bark	11.76±0.20	10.25±0.07	8.46±0.21	8.00±0.0	15.91±0.15	8.00±0.0
Stem	11.91±0.39	10.38±0.15	8.38±0.14	8.00±0.0	16.08±0.30	8.00±0.0

Table 3. Antibacterial effect of aqueous extracts of *C. timorensis* leaves, bark and stem against *S. aureus*

Antimicrobial effect of *Celtis timorensis* Span	Zone of inhibition (mm)					
	1000 µg/ml	750 µg/ml	500 µg/ml	250 µg/ ml	Positive control	Negative control
Leaves	8.00±0.0	8.00±0.0	8.00±0.0	8.00±0.0	19.58±0.37	8.00±0.0
Bark	8.00±0.0	8.00±0.0	8.00±0.0	8.00±0.0	19.08±0.15	8.00±0.0
Stem	8.00±0.0	8.00±0.0	8.00±0.0	8.00±0.0	19.75±0.14	8.00±0.0

3.2 Dose-Response Study

3.2.1 Dose-response curves and EC_{50} values of aqueous extractions of *Celtis timorensis Span.* plant parts against *Escherichia coli* (ATCC 25922)

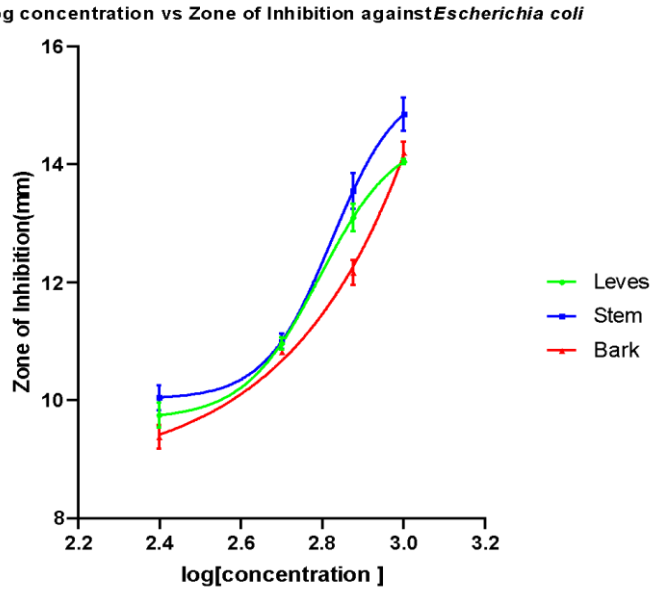

Fig. 4. Dose-response curves of *C. timorensis* leaves, bark and stem extracts against *E. coli*.

Fig. 4 demonstrates that with the increasing log concentrations of *C. timorensis* extracts, zone of inhibitions was also increased.

3.2.2 Dose-response curves and EC_{50} values of aqueous extractions of *Celtis timorensis* Span. plant parts against *Pseudomonas aeruginosa* (ATCC 27853)

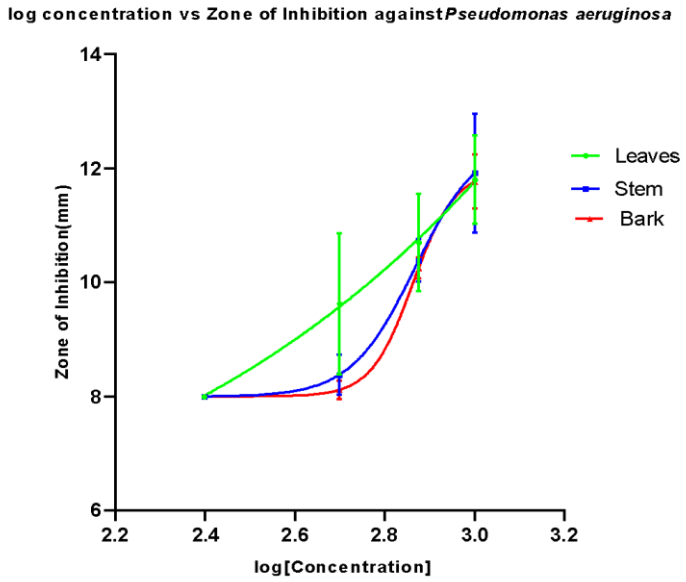

Fig. 5. Dose-response curves of *C. timorensis* leaves, bark and stem extractions against *P. aeruginosa*

Fig. 5 depicts that with the increasing log concentrations of *C. timorensis* extracts, the zone of inhibitions was also increased.

According to the data obtained by EC_{50} values and Dose-Response curves, aqueous extract of leaves exhibited the highest efficacy and lowest potency and the aqueous extract of bark exhibited the highest potency and lowest efficacy against *P. aeruginosa*.

4. DISCUSSION

The current study represents the inaugural investigation into the antibacterial properties of aqueous extracts from various components (leaves, stem, and bark) of *Celtis timorensis.* Grounded in existing literature, this research endeavors to assess the *in vitro* antibacterial activity of these aqueous extracts, marking the first-of-its-kind exploration in the scientific domain.

According to the previous studies ethanol and water extracts of leaf samples of *Celtis timorensis* strongly inhibited the gram-negative bacterial species *Pseudomonas aeruginosa* and *Salmonella enteric*. And gram-positive species i.e. *Bacillus megatherium Artherobacter protophormiae* and *P. aeruginosa* were moderately inhibited by chloroform, ethanol and water extracts of C. tomorensis leaf. (Mallika and Shailaja, 2023) And also the methanolic leaf extract of *Celtis australis* had the highest activity against *S. aureus* at 200mg/ml concentration with 10.5±0.57mm zone of inhibition. Also increasing the concentrations of methanolic and aquoeus leaf extracts of *C. australis* resulted in increased antibacterial potential against *S. aureus* and *P. aeruginosa* [14].

As per the current study Zone of inhibition of aqueous leaf extracts of *C. timorensis* showed zone of inhibition as 8.00±0.0 at concentrations of 1000, 750, 500, and 250 µg/ml concentrations. It is below the zone of inhibition of methanolic leaf extract of *Celtis australis.*

As depicted in Table 1, the antibacterial assay against *E. coli* revealed that the 1000 µg/ml concentration of all three plant extracts (Leaves, Bark, and Stem) exhibited the highest zone of inhibition, demonstrating a direct proportional relationship between plant extract concentration and inhibition zone size. The aqueous extraction of the stem displays the maximum zone of inhibition (14.85 mm), while bark and leaves show zones of 14.20 mm and 14.05 mm, respectively. All three parts exhibit statistically significant differences ($p<0.05$) in their mean zones of inhibition against E. coli, although all are lower than the positive control.

Similarly, the antibacterial assay against *P. aeruginosa* indicates that the aqueous extraction of the stem achieves the highest zone of inhibition (11.91 mm), while bark and leaves display zones of 11.80 mm and 11.76 mm, respectively (Table 2). All three parts exhibit statistically significant differences ($p<0.05$) in their mean zones of inhibition against *P. aeruginosa*, with values below the positive control.

As delineated in Table 3 against *S. aureus*, none of the concentrations for any plant part show positive antibacterial responses, in contrast to the positive control (approximately 19.4 mm). Consequently, aqueous extractions of leaves, bark, and stem of *C. timorensis* exhibit positive antibacterial responses against gram-negative bacteria but lack efficacy against gram-positive *S. aureus*.

In summary, the study concludes that aqueous extractions of *C. timorensis* leaves, bark, and stem demonstrate positive antibacterial responses against gram-negative bacteria (*E. coli* and *P. aeruginosa*) but lack efficacy against gram-positive *S. aureus*. The potency and biological effect vary among plant parts, with leaves displaying the highest potency against *E. coli* and bark against *P. aeruginosa* [16].

5. CONCLUSION

The current study investigated the in-vitro antimicrobial activity of *C. timorensis* Span. plant components (leaves, bark, and stem) against wound infecting bacteria—*Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. While gram-negative bacteria were susceptible to aqueous extracts, Staphylococcus aureus, gram-positive bacteria showed resistance. The cylinder plate method revealed the highest inhibition zones against Escherichia coli and *Pseudomonas aeruginosa* with 1000 g/ml concentrated aqueous extraction of stem and the inhibition zones were 14.85 mm and 11.91 mm respectively. Concentration and the inhibition zones are directly correlated with all three plant sections. Statistical analysis indicated significant differences among plant components against *E. coli* and *P. aeruginosa*. Dose-response data identified aqueous extraction of bark as most efficacious against *E. coli* and

aqueous extraction of leaves against *P. aeruginosa*. Overall, *C. timorensis* leaves and bark displayed robust antibiotic activity against gram-negative bacteria, particularly *E. coli* and *P. aeruginosa*.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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The peer review history for this paper can be accessed here:
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