

Determination of the Antibacterial Activity of *Clitoria ternatea* (Blue ternatea) Flower Ethanolic Extract Against *Citrobacter koseri*

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Abstract. *Citrobacter koseri* is a growing pathogenic concern, leading to increased antibiotic resistance and posing a public health threat as standard antibiotics become less effective in treating infections caused by this bacterium. Hence, there is a need to investigate alternative antibacterial agents to mitigate the emergence of resistant strains of *Citrobacter koseri*. This study utilized *Clitoria ternatea*, also known as Blue ternatea and Butterfly pea flower, a medicinal plant recognized for its broad-spectrum antimicrobial properties and for containing significant levels of phenolic compounds, flavonoids, and anthocyanins. The study evaluated the antibacterial activity of *Clitoria ternatea* flower ethanolic extraction in four different concentrations (25%, 50%, 75%, and 100%) against *Citrobacter koseri*, assessed through the Kirby-Bauer Disk Diffusion Method, comparing the antibacterial activity to the antibiotic Cefazolin. Data were analyzed using zone-of-inhibition measurements and the Kruskal-Wallis test to determine significance. The findings suggested that the 100% concentration of the extract exhibited the largest zone of inhibition, showing a proximate inhibition zone of 22mm, as compared to the antibiotic Cefazolin, which had an average zone of inhibition of 24mm. However, the extract demonstrated resistance to *Citrobacter koseri* at the lower concentrations. In conclusion, the ethanolic extract of *Clitoria ternatea* exhibits antibacterial activity against *Citrobacter koseri*. However, when this plant is combined with antibiotics or other plants with potent antibacterial activity, it can be more effective at combating resistance in *Citrobacter koseri*.

Keywords: Antibacterial activity of *Clitoria ternatea*; Blue ternatea antimicrobial activity; *Citrobacter koseri*; *Clitoria ternatea* ethanolic extracts

1.0 Introduction

The clinical significance of emerging nosocomial and community-acquired pathogenic diseases is becoming a global phenomenon (Kumar et al., 2021b), including bacteria, viruses, and fungi (Sikora, A., & Zahra, F., 2023), acknowledged as a source of multiple human infections in prevailing localizations, including the urinary tract, respiratory tract, bloodstream, and central nervous system (Kumar et al., 2021b), in neonates and immunocompromised individuals (Sharma et al., 2024). Evidence shows that infections caused by *Citrobacter* species are increasing by about 3-6% among hospitalized patients (Jabeen et al., 2023b), some of which are multidrug-resistant (Fonton et al., 2024). Often isolated, *Citrobacter koseri* is a gram-negative, non-spore-

forming, rod-shaped, facultatively anaerobic bacterium (Alhaidhal et al., 2024). This bacterium is commonly isolated as *Citrobacter* sp. and is generally associated with nosocomial infections (Jabeen et al., 2023b). Various research groups have elucidated findings on this pathogen, establishing a sustained increase in multidrug antibacterial resistance driven by overexpression of chromosomal and plasmid-mediated β -lactamases (Kumar et al., 2021b). The pathogen's high pathogenicity island (HPI) gene cluster contributes to its ability to cause severe infections in high-risk populations, including infants and immunocompromised individuals (Emery et al., 2019b).

Plants combat bacterial resistance by producing bioactive compounds, including phenols, flavonoids, and alkaloids, that target multiple bacterial pathways and disrupt their defense mechanisms (Suganya et al., 2022). One potential plant with antimicrobial properties is *Clitoria ternatea*, known as butterfly pea. This plant is commonly used as a natural food colorant and is found in the Philippines, Asian countries, and South and Central America (Jeyaraj, Lim, et al., 2022b). More than a food colorant, it is also renowned for its potent antibacterial properties, due to various bioactive compounds, including phenols, flavonoids, anthocyanins, and cyclotides, which are also abundant in its flowers (Oguis et al., 2019). These bioactive compounds demonstrate mechanisms to disrupt bacterial cell membranes, inhibit critical enzyme functions, prevent protein synthesis, and block biofilm formation (Kumar et al., 2021b). Anthocyanins are responsible for the vibrant blue color of *Clitoria ternatea* flowers (Multisona et al., 2023). It plays a crucial role in its antibacterial action, increasing the permeability of bacterial cell membranes by altering the lipid bilayer structure. This results in the leakage of essential intracellular contents, such as ions and proteins, which leads to bacterial cell death (Jaafar et al., 2020b). Flavonoids in the flower disrupt bacterial metabolic pathways by inhibiting enzymes critical for bacterial survival. Further, flavonoids have several mechanisms to inhibit bacterial growth, including disruption of cell wall synthesis, biofilm formation, and membrane integrity (Liu et al., 2025). It also interferes with bacterial DNA gyrase and topoisomerase IV, which are essential for DNA replication and transcription, thus hindering the bacteria's ability to reproduce and function (Tan et al., 2022). Phenolic acids are potent antioxidants and disrupt bacterial cell membranes by targeting the lipid bilayer, increasing fluidity and permeability. This damage causes the release of intracellular materials, leading to bacterial death (Jaafar et al., 2020). Cyclotides are small, cyclic peptides that are highly stable due to their unique structural features, including a cyclic cystine knot. According to a study by Oguis et al. (2019), *Clitoria ternatea* has shown antibacterial activity against a range of Gram-negative bacteria, including *Escherichia coli*. Notably, ethanolic extracts of *Clitoria ternatea* have demonstrated significant antibacterial activity, positioning it as a potential natural alternative to conventional antibiotics, particularly against infections caused by drug-resistant strains such as *Citrobacter koseri* (Kumar et al., 2021b). Hence, the *Clitoria ternatea* flower's high concentration of bioactive molecules, particularly in its petals, contributes to its potent antibacterial properties, effectively targeting bacterial cells, disrupting their growth and defense mechanisms (Aslam et al., 2021).

Given the plant's potent bioactive compounds and the limited applications of *Clitoria ternatea*, this study aims to determine the antimicrobial activity of its ethanolic flower extract against *Citrobacter koseri*. Further, this study aims to determine the concentrations that best combat *Citrobacter koseri*.

2.0 Methodology

2.1 Research Design

This study employed an experimental design to investigate the antibacterial activity of ethanolic extraction from the *Clitoria ternatea* (Blue *ternatea*) flower against *Citrobacter koseri*. The extraction procedures for the ethanolic extract were adapted from the methodology of Dhanasekaran et al. (2019). The experimental approach involved preparing various ethanolic extracts from *Clitoria ternatea* flowers and testing their antibacterial activity against *Citrobacter koseri* in a controlled laboratory setting. This study utilized antibacterial susceptibility testing, including zone diameter measurements and determination of the minimum inhibitory concentration (MIC), to assess antimicrobial activity.

2.2 Research Locale

The experimental processes, such as extract preparation, culture preparation, antibacterial susceptibility testing, and autoclaving, were conducted under controlled conditions at the National University - MOA and the National University - Manila.

2.3 Statistical Analysis

This study used descriptive statistics to measure the zone of inhibition of *Clitoria ternatea* (Blue ternatea) flower ethanolic extract at varying concentrations against *Citrobacter koseri* in an experimental setup comprising three trials, each with three replicates. The Kruskal-Wallis Test is used as a nonparametric alternative to determine whether the antibacterial activity of *Clitoria ternatea* (Blue ternatea) flowers with different ethanolic extracts differs significantly from the study's controls. Post-hoc tests, specifically Dunn's test, were used to identify which concentrations differ significantly following a Kruskal-Wallis test.

2.4 Data Gathering Procedure

The *Clitoria ternatea* (Blue ternatea) 300g dried flower was obtained and blended into a fine powder. The pulverized, 25g *Clitoria ternatea* (Blue ternatea) flower was extracted with 300mL of 95% ethanol using the Soxhlet apparatus for 4 hours. Then, the mixture was filtered using No. 1 Whatman filter paper. Lastly, the filtered *Clitoria ternatea* (Blue ternatea) extract was subjected to rotary evaporation at 40°C for 5 minutes. The samples were stored in a refrigerator maintained at 4°C (39°F) to ensure their integrity, avoid contamination, and uphold their purity until the time of testing.

The crude ethanolic extract of *Clitoria ternatea* (Blue Ternatea) flowers (100g of solute) was added to 300mL of distilled water. To gain the concentration of 100% of the *Clitoria ternatea* (Blue ternatea) flower ethanolic extract, 100mL of the crude extract was also used. To acquire a concentration of 75% of the *Clitoria ternatea* (Blue ternatea) flower ethanolic extract, 75mL of the crude extract was diluted using 25mL of distilled water. Additionally, the 50% concentration of the *Clitoria ternatea* (Blue ternatea) flower ethanolic extract was diluted using 50mL of distilled water. Moreover, for a 25% concentration of the *Clitoria ternatea* (Blue ternatea) flower ethanolic extract, 25mL of the crude extract was diluted using 75mL of distilled water.

After the process of plant collection and bacterial isolation, this study prepared four different ethanolic extracts of *Clitoria ternatea* (Blue ternatea) flowers (25%, 50%, 75%, and 100%). The extracts were prepared following the computed measurements presented in Table 1. Following the procedure, the *Citrobacter koseri* was exposed to the various *Clitoria ternatea* (Blue ternatea) flowers' ethanolic extracts (CTFEE) in triplicate. The zone of inhibition was then measured using a caliper for each exposure to determine the antimicrobial activity of CTFEE against *Citrobacter koseri*. The Minimum Inhibitory Concentration was determined as the inverse of the zone of inhibition. In the antimicrobial activity comparison, cefazolin was used as the positive control.

Table 1. Preparation of the Various Concentrations of *Clitoria ternatea* (Blue ternatea) Flowers Ethanolic Extracts (CTFEE)

Volume of Solute	Solvent (Distilled Water)	Volume of Solution	Percentage
25 mL	75 mL	100 mL	25 %
50 mL	50 mL	100 mL	50 %
75 mL	25 mL	100 mL	75 %
100 mL		100 mL	100 %

2.5 Ethical Considerations

In the study of the antibacterial activity of the ethanolic extract of *Clitoria ternatea* (Blue ternatea) flowers against *Citrobacter koseri*, it is imperative to prioritize ethical and safety considerations. Adherence to local regulations governing plant and microbial research is crucial, necessitating the acquisition of relevant permits for collection, handling, and experimentation. This study has strictly followed the general guidelines for conducting laboratory procedures and maintained ethical standards throughout. It has adopted Biosafety Level 2, as defined by the Centers for Disease Control and Prevention (2019), which encompasses all laboratories that work with agents associated with pathogenic human diseases. Regarding safety, the researchers were informed about handling and disposing of biological materials, the potential health risks, and the need to wear proper personal protective equipment.

3.0 Results and Discussion

The antibacterial activity of *Clitoria ternatea* (Blue ternatea) flower ethanolic extract against *Citrobacter koseri*: zone of inhibition (triplicate) and minimum inhibitory concentration. The results for minimum inhibitory concentration are highlighted below.

Table 2. The Mean Value of Each Trial in the Repetition of Different Concentrations of *Clitoria ternatea* (Blue ternatea) Ethanolic Extract Against *Citrobacter koseri*

Concentration	Trial 1			T2			T3			Mean
	R1	R2	R3	R1	R2	R3	R1	R2	R3	
25%	8.0	6.0	6.0	6.0	2.0	6.0	6.0	12.0	6.0	6.8
50%	14.0	8.0	6.0	14.0	3.8	14.0	16.0	14.0	6.0	11.6
75%	18.0	16.0	18.0	16.0	1.2	18.0	18.0	20.0	18.0	17.8
100%	23.0	22.0	22.0	16.0	2.7	22.0	26.0	23.0	20.0	21.3
Cefazolin	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0

Note: The values measured are expressed in millimeters (mm).

Table 2 presents the results of antibacterial activity, showing the zone of inhibition for the 25%, 50%, 75%, and 100% concentrations of the ethanolic extract from *Clitoria ternatea* (Blue ternatea) flowers against *Citrobacter koseri*. The experiment was conducted using triplicate to ensure the accuracy and reliability of the data obtained. The ethanolic extract of *Clitoria ternatea* (Blue ternatea) flower shows a mean zone of inhibition (ZOI) with consistently similar results across three experimental groups. The zone of inhibition (ZOI) mean values were 6.89 mm for the 25% concentration, 11.6 mm for the 50% concentration, 17.8 mm for the 75% concentration, and 21.3 mm for the 100% concentration. The findings indicate limited antibacterial activity against *Citrobacter koseri*, as the *Clitoria ternatea* (Blue ternatea) flower ethanolic extract demonstrates a minimal zone of inhibition (ZOI) when compared to the positive Control (Cefazolin), as evidenced by the clear zone around the impregnated discs.

In comparison to the study of Jeyaraj et al. (2022), a gram-negative bacterium, *Pseudomonas aeruginosa*, was inoculated in Mueller-Hinton Agar (MHA) media with the addition of discs with 50%, 60%, 70%, 80%, 90% and 100% concentrations of *Clitoria ternatea* (Blue ternatea) flower ethanolic extract. The highest inhibition was observed at 100% concentration. The antibacterial effectiveness of the concentrations was categorized as weak and strong. For gram-positive bacteria, Deorankar et al. (2020) conducted a study using the ethanolic extract of *Clitoria ternatea* (Blue ternatea) flowers against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans*, and *Aspergillus niger*. The bacteria do not have a different concentration, but it has been shown that *Clitoria ternatea* (Blue ternatea) is potentially effective. It has a zone of inhibition for each bacterium. For *Escherichia coli* (16mm), for *Pseudomonas aeruginosa* (18mm), for *Staphylococcus aureus* (17mm), for *Candida albicans* (12mm), and for *Aspergillus niger* (13mm). Comparing the two studies, *Clitoria ternatea* (Blue ternatea) showed a potent inhibitory activity against gram-positive bacteria, with *Pseudomonas aeruginosa* showing the most significant inhibition. It only showed potent inhibitory activity at 100% concentration against a gram-negative bacterium. However, in this study, dried flowers were used to make them easier to powder. Both studies examined the antibacterial properties of *Clitoria ternatea* (Blue ternatea). This study needs to focus on the antibacterial activity of *Clitoria ternatea* (Blue ternatea), specifically against *Citrobacter koseri*. Finally, it can be observed that there are varying degrees of antibacterial activity against different bacterial species.

Table 3. The Minimum Inhibitory Concentration of Varying Concentrations of *Clitoria ternatea* (Blue ternatea) Flower Ethanolic Extract

Concentration	Trial 1			T2			T3			Mean
	R1	R2	R3	R1	R2	R3	R1	R2	R3	
25%	8.0	6.0	6.0	6.0	2.0	6.0	6.0	12.0	6.0	6.8
50%	14.0	8.0	6.0	14.0	3.8	14.0	16.0	14.0	6.0	11.6
75%	18.0	16.0	18.0	16.0	1.2	18.0	18.0	20.0	18.0	17.8
100%	23.0	22.0	22.0	16.0	2.7	22.0	26.0	23.0	20.0	21.3
Cefazolin	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0	24.0

Note: The values measured are expressed in millimeters (mm).

The observed average of the Zone of Inhibition (ZOI) values for the ethanolic extract of *Clitoria ternatea* (Blue ternatea) at different concentrations (6.89mm for 25%, 11.6mm for 50%, 17.8mm for 75%, and 21.3mm for 100%) presented in Table 3, the concentration of 100% exhibits the largest Zone of Inhibition (ZOI). As the zone of inhibition is inversely related to the Minimum Inhibitory Concentration (MIC), in this study, the MIC is determined to be 100% of the CTFFEE concentration.

Table 4. Significance Difference in Zone of Inhibition of Different Concentrations in *Clitoria ternatea* (Blue ternatea) Flower Ethanolic Extract and Positive Control

ZOI	Kruskal-Wallis				
	Kruskal-Wallis	df	ϵ^2	p	Significance
	36.5	5	0.89	<.001	Significant

The data in Table 4 illustrate the zone of inhibition at various concentrations of the ethanolic extract of *Clitoria ternatea* (Blue ternatea) flowers, along with the control group. This analysis was conducted using the Kruskal-Wallis Test, yielding a p-value of <0.001, which is below the 0.05 significance threshold. Thus, there is a significant difference in the distribution of zone-of-inhibition values among the groups. Based on the result, it is recommended to conduct a post-hoc test to identify which pairwise comparison of means accounts for the overall significant difference observed in the computation.

Table 5. Post-hoc Test for Pairwise Comparison in Different Concentrations of *Clitoria ternatea* (Blue ternatea) Flower Ethanolic Extract and Positive Control Against *Citrobacter koseri*

Pairwise Comparisons of Groups	Post-hoc Test		
	W	P	Significance
25%-50%	3.7	0.094	Not Significant
25%-75%	5.32	0.002	Significant
25%-100%	5.23	0.003	Significant
25%-Positive Control	3.97	0.056	Not Significant
25%-Negative Control	-1.21	0.957	Not Significant
50%-75%	5.07	0.005	Significant
50%-100%	5.04	0.005	Significant
50%-Positive Control	3.63	0.106	Not Significant
50%-Negative Control	-2.9	0.313	Not Significant
75%-100%	3.98	0.056	Not Significant
75%-Positive Control	3.81	0.077	Not Significant
75%-Negative Control	-3.81	0.077	Not Significant
100%-Positive Control	2.79	0.356	Not Significant
100%-Negative Control	-3.59	0.113	Not Significant
Positive Control - Negative control	-3.16	0.221	Not Significant

The Post-hoc Test reveals that the pairwise comparisons between the groups 25%-75%, 25%-100%, 50%-75%, and 50%-100% are "significant" with p-values less than 0.05. On the other hand, the comparisons for the following groups are "not significant" with computed p-values greater than 0.05: 25%-50%, 25%-Positive Control, 25%-Negative, 50%-Positive Control, 50%-Negative, 75%-100%, 75%-Positive Control, 75% Negative, 100%-Positive Control, 100%-Negative, and Positive Control-Negative. In the study by Jeyaraj et al. (2022), it was found that *Clitoria ternatea* (Blue ternatea) at 80% and 100% concentrations exhibited vigorous antibacterial activity, with dose-dependent activity against *Citrobacter koseri*. Bioactive compounds such as flavonoids, anthocyanins, and cyclotides were linked to their antibacterial properties. Meanwhile, the post-hoc test for pairwise comparisons in Table 5 revealed that the 75% and 100% concentrations of *Clitoria ternatea* (Blue ternatea) flower ethanolic extract showed antibacterial activity against *Citrobacter koseri*.

4.0 Conclusion

The study indicates that the ethanolic extract of *Clitoria ternatea* (Blue ternatea) flower exhibits minimal antibacterial activity against *Citrobacter koseri*, with an average zone of inhibition of 6.89 mm at 25%, 11.6 mm at 50%, 17.8 mm at 75%, and 21.3 mm at 100%. The findings confirm the plant extract's antibacterial activity. The 100% ethanolic extract demonstrates a significantly greater ability to inhibit bacterial growth than the 25% concentration. In addition, the significant activity indicates that the 100% ethanolic extract of *Clitoria ternatea* (Blue ternatea) exhibits the largest zone of inhibition, reflecting the most potent antibacterial activity among the concentrations evaluated. Furthermore, the study indicated a significant difference in the activity of the ethanolic extract of *Clitoria ternatea* (Blue ternatea) flower compared to Cefazolin and standard saline solution NSS. The p-value of *Clitoria ternatea* (Blue ternatea) flower ethanolic extract against *Citrobacter koseri* is $p < 0.001$, indicating a statistically significant difference between the positive Control. Although the *Clitoria ternatea* (Blue ternatea) extract shows significant antibacterial activity, Cefazolin remains more effective, demonstrating a significant difference in the outcome. Nevertheless, the study indicated that the extract demonstrated higher activity than the negative control, standard saline solution. Despite the minimal antibacterial activity exhibited by *Clitoria ternatea* (Blue Ternatea) flower ethanolic extract, its potential remains undiminished. The study

indicates that higher concentrations of the extract (100%) exhibit significant antibacterial activity, suggesting that plant-derived compounds could be developed to be effective against resistant bacterial infections. The study opens up further research avenues by investigating the specific bioactive compounds in *Clitoria ternatea* (Blue ternatea), such as flavonoids, anthocyanins, and cyclotides, which could lead to the development of novel antibacterial agents. It encourages future studies to examine its efficacy against a broader range of pathogens beyond *Citrobacter koseri* and to evaluate its safety and potential side effects in clinical applications. Ultimately, this research contributes to the broader efforts to combat antibiotic resistance and highlights the role of medicinal plants in modern Medicine. Although some studies have documented the efficacy of *Clitoria ternatea* (Blue ternatea) against specific bacterial strains, this research underscores the necessity for further investigation, given its limited inhibitory effect on *Citrobacter koseri*. Expanding the scope of bacterial targets in future studies will enable a more comprehensive evaluation of *Clitoria ternatea's* antibacterial capabilities.

5.0 Contributions of Authors

Raymund J. Capagas, RMT, MPH – Writing – Review & Editing, Visualization, Validation, Supervision, Methodology, Investigation, Data Curation, Conceptualization.

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Joey Flores - Validation, Supervision, Methodology.

Chelsea Delos Reyes - Validation, Supervision, Methodology.

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7.0 Conflict of Interests

The authors declare no conflict of interest.

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