



Antibacterial Activity of Combined *Clitoria ternatea* Flower and *Citrus aurantifolia* Fruit Extracts Against *Shigella dysenteriae* and *Escherichia coli*

^{1*}Fitriyah, ²Rizki Sri Lestari, ³Ziskino Qurota A'yun, ⁴Badzilatul Nada

^{1,2,3,4}Department of Biology Education, Faculty of Science and Technology, Universitas Islam Negeri Maulana Malik Ibrahim Malang, Malang, Indonesia.

*Corresponding Author e-mail: fitriyahfaizin@bio.uin-malang.ac.id

Received: August 2026; Revised: September 2026; Accepted: September 2026; Published: September 2026

Abstract: This study aimed to evaluate the antibacterial activity of combinations of *Clitoria ternatea* flower and *Citrus aurantifolia* extracts against *Escherichia coli* ATCC 25922 and *Shigella dysenteriae* and to determine the minimum inhibitory concentration (MIC) of the most effective combination. An experimental study was conducted using five treatments with three replicates: P1 (25% *C. ternatea* : 75% *C. aurantifolia*), P2 (50% : 50%), P3 (75% : 25%), P4 (100% *C. ternatea*), and P5 (100% *C. aurantifolia*). Antibacterial activity was assessed using the disk diffusion method based on inhibition-zone diameters, and the most effective combination was subsequently evaluated using a microdilution assay to determine its MIC against both bacterial species. Among the combination treatments, P1 exhibited the greatest antibacterial activity, producing mean inhibition-zone diameters of 10.17 mm against *E. coli* and 7.50 mm against *S. dysenteriae*. The MIC assay indicated that the P1 combination inhibited the growth of both bacterial species at a concentration of 3.125%, based on visual turbidity assessment and microplate-reader measurements. These findings demonstrate that the combination of *C. ternatea* flower and *C. aurantifolia* extracts possesses antibacterial activity against both Gram-negative bacterial species, with the 25% *C. ternatea* : 75% *C. aurantifolia* formulation showing the strongest activity among the tested combinations. Further studies are needed to confirm its bactericidal activity and elucidate the underlying antibacterial mechanisms.

Keywords: Antibacteria; *Escherichia coli*; minimum inhibitory concentration; *Shigella dysenteriae*

How to Cite: Fitriyah, Lestari, R. S., A'yun, Z. Q., & Nada, B. (2026). Antibacterial Activity of Combined *Clitoria ternatea* Flower and *Citrus aurantifolia* Fruit Extracts Against *Shigella dysenteriae* and *Escherichia coli*. *Bioscientist: Jurnal Ilmiah Biologi*, 14(3), 1625–1639. <https://doi.org/10.33394/bioscientist.v14i3.22363>



<https://doi.org/10.33394/bioscientist.v14i3.22363>

Copyright© 2026, Fitriyah et al

This is an open-access article under the [CC-BY-SA](#) License.



INTRODUCTION

Diarrhea is one of the most common infectious diseases affecting the gastrointestinal tract and remains an important public health problem worldwide (Surapati & Gautama, 2022). According to the World Health Organization (WHO) (2024), diarrhea is defined as the passage of three or more loose or watery stools per day. The disease is commonly associated with enteric pathogens, including *Escherichia coli* and *Shigella dysenteriae*, which can enter the human gastrointestinal tract through contaminated food or water (Mayanti et al., 2023). *Escherichia coli* is among the bacterial pathogens frequently associated with diarrhea and food contamination (Mayanti et al., 2023), whereas *S. dysenteriae* is considered one of the most virulent species within the genus *Shigella*. This bacterium produces Shiga toxin, which can damage red blood cells and vascular endothelial cells, particularly in the kidneys, and may cause bloody diarrhea, vomiting, fever, abdominal cramps, and complications such as hemolytic uremic syndrome (HUS). In addition, *S. dysenteriae* can form biofilms and may cause infection at a very low infectious dose of approximately 10–100 cells (Khairunnisa et al., 2024).

Bacterial diarrhea is commonly treated with antibiotics, including ciprofloxacin, pivmecillinam, and ceftriaxone. In cases of *S. dysenteriae* infection, these antibiotics have been reported to eliminate susceptible bacterial populations in most cases

(Williams & Berkley, 2018). However, inappropriate or excessive use of antibiotics can promote the development and spread of antimicrobial resistance (Syafridah, 2022). *Shigella dysenteriae* has been reported to exhibit resistance to several antimicrobial agents, including ampicillin, tetracycline, cefixime, penicillin, vancomycin, aztreonam, ertapenem, meropenem, ciprofloxacin, imipenem, and fluoroquinolones (Cao, 2021; Raihan & Hossain, 2022). Although antibiotics remain effective against susceptible bacterial pathogens, they do not directly address other infection-associated processes, including inflammation and oxidative stress. Antimicrobial resistance has therefore become a major global public health concern, with an estimated 1.27 million deaths directly attributable to bacterial antimicrobial resistance and 4.95 million deaths associated with bacterial antimicrobial resistance worldwide in 2019 (Antimicrobial Resistance Collaborators, 2022). These concerns have encouraged the search for alternative antibacterial agents, particularly those derived from medicinal plants.

One plant with potential antibacterial activity is *Clitoria ternatea*. Pratap et al. (2012) demonstrated that *C. ternatea* flower extract exhibited antibacterial activity at concentrations of 5%, 10%, 25%, and 50% against Gram-positive bacteria. Similarly, Febrianti et al. (2022) reported that ethanolic extraction of *C. ternatea* flowers yielded several secondary metabolites with antibacterial properties, particularly against pathogenic bacteria such as *E. coli*. These compounds include tannins, flavonoids, alkaloids, saponins, terpenoids, and phenolic compounds.

Clitoria ternatea flowers are also rich in anthocyanins, and anthocyanin-rich fractions have demonstrated antibacterial activity (Jeyaraj et al., 2023). However, anthocyanins are relatively unstable because their chemical structure is sensitive to changes in temperature and pH. Anthocyanins are generally more stable under acidic conditions, particularly at a pH of approximately 5 (Charurungsipong et al., 2020). Under acidic conditions, anthocyanins predominantly occur in the flavylium form, which has been reported to possess stronger antibacterial activity than the forms that predominate under neutral or alkaline conditions, such as chalcone (Lin et al., 2023). Therefore, maintaining an acidic environment may help preserve anthocyanin stability and potentially support its antibacterial activity.

Citrus aurantifolia is another plant that has been widely investigated for its antibacterial properties. Sari et al. (2021) demonstrated that *C. aurantifolia* peel extract inhibited the growth of *E. coli*, with antibacterial activity observed at concentrations starting from 20%. *C. aurantifolia* contains several bioactive compounds, including citric acid, flavonoids such as hesperidin, naringenin, eriocitrin, and quercetin, as well as essential oils, tannins, and saponins, which may contribute to its antibacterial activity. Citric acid can chelate divalent ions such as Ca^{2+} and Mg^{2+} , which play important roles in maintaining the stability of the lipopolysaccharide (LPS) layer of Gram-negative bacteria. This chelation may destabilize the outer membrane and increase its permeability (Burel et al., 2021). In addition, naringenin and hesperidin have been reported to increase membrane permeability, disrupt cellular integrity, and inhibit bacterial biofilm formation.

The complementary properties of the bioactive compounds present in *C. ternatea* and *C. aurantifolia* provide a rationale for evaluating their combined antibacterial activity. Hydrophobic compounds and citric acid in *C. aurantifolia* may alter the permeability of the outer membrane of Gram-negative bacteria, including *E. coli* and *S. dysenteriae*. Hydrophobic constituents may interact with membrane lipids, whereas citric acid may interfere with the interactions between LPS and divalent ions such as Mg^{2+} and Ca^{2+} that contribute to membrane stability (Burel et al., 2021). Such changes may facilitate the penetration or activity of bioactive compounds from *C. ternatea*,

including flavonoids and anthocyanins, against bacterial cellular targets. Therefore, combining *C. ternatea* flower and *C. aurantifolia* extracts may enhance antibacterial activity through complementary mechanisms. However, this proposed interaction should be interpreted as a mechanistic rationale rather than evidence of true synergism, because synergistic effects require specific quantitative evaluation.

The antibacterial potential of *C. aurantifolia* is further supported by previous research. Umar and Susan (2024) reported that crude *C. aurantifolia* extract at a concentration of 25% inhibited the growth of *E. coli*, producing an inhibition zone of 14 mm. Based on this previous evidence, and because both *E. coli* and *S. dysenteriae* are Gram-negative bacteria characterized by an outer membrane containing lipopolysaccharide, a concentration of 25% was selected as the minimum proportion used in the combination treatments in the present study. This concentration was used as the basis for comparing formulations containing lower, equal, or higher proportions of *C. aurantifolia* relative to *C. ternatea*.

Furthermore, Waisnawi et al. (2022) reported that the addition of *C. aurantifolia* influenced the pH, total anthocyanin content, and antioxidant activity of *C. ternatea* flower beverages. These findings suggest that *C. aurantifolia* may modify the physicochemical environment of *C. ternatea*-based preparations and potentially influence the stability or activity of their bioactive compounds. Nevertheless, previous studies have predominantly investigated *C. ternatea* and *C. aurantifolia* separately, while evidence regarding the antibacterial effects of different combinations of these extracts against Gram-negative enteric pathogens remains limited. In particular, the influence of different extract ratios on antibacterial activity against both *E. coli* and *S. dysenteriae* has not been sufficiently characterized. We therefore hypothesized that increasing the proportion of *C. aurantifolia* in combination with *C. ternatea* would enhance antibacterial activity against both bacterial species. Accordingly, this study aimed to evaluate the antibacterial activity of different combinations of *C. ternatea* flower and *C. aurantifolia* extracts against *E. coli* and *S. dysenteriae* and to identify the combination exhibiting the greatest antibacterial activity.

METHOD

Experimental Design

This study employed an experimental design to evaluate the antibacterial activity of combinations of *Clitoria ternatea* flower and *Citrus aurantifolia* extracts against *Escherichia coli* and *Shigella dysenteriae*. The independent variable was the proportion of *C. ternatea* and *C. aurantifolia* extracts in the treatment combinations, whereas the dependent variable was bacterial growth, as assessed by antibacterial activity. The experimental controls consisted of ciprofloxacin at 1 mg/mL as the positive control [K(+)], 1% dimethyl sulfoxide (DMSO) as the negative control [K(-)], and ascorbic acid adjusted to pH 4 as the pH control. Each treatment was performed in triplicate.

The extract formulations were prepared in 1% DMSO using different proportions of *C. ternatea* flower and *C. aurantifolia* extracts. The treatment and control groups were as follows:

K(+) = ciprofloxacin (1 mg/mL)

K(-) = 1% DMSO

pH control = ascorbic acid (pH 4)

P1 = 25% *C. ternatea* extract : 75% *C. aurantifolia* extract

P2 = 50% *C. ternatea* extract : 50% *C. aurantifolia* extract

P3 = 75% *C. ternatea* extract : 25% *C. aurantifolia* extract

P4 = 100% *C. ternatea* flower extract

P5 = 100% *C. aurantifolia* extract

The selection of the treatment concentrations was based on previous evidence and the objective of comparing different proportions of the two extracts. Febrianti et al. (2022) reported that a 25% *C. ternatea* flower extract prepared in 1% DMSO produced an inhibition zone of approximately 7 mm against *E. coli*, which was classified as moderate antibacterial activity. Accordingly, 25% was used as the minimum proportion of *C. ternatea* in the combination treatments. The formulations were designed to compare combinations containing lower, equal, or higher proportions of *C. aurantifolia* relative to *C. ternatea*. A minimum proportion of 25% *C. aurantifolia* was therefore included in the combination treatments. An ascorbic acid control at pH 4 was also included to evaluate whether acidic conditions contributed to the observed antibacterial activity.

Study Location and Period

The study was conducted from April to July 2026 at the Microbiology Laboratory, Department of Biology, Faculty of Science and Technology, Maulana Malik Ibrahim State Islamic University of Malang, and the Microbiology Laboratory, Islamic University of Malang, Indonesia.

Equipment and Materials

The principal equipment used in this study included an oven, blender, rotary evaporator, vacuum pump, analytical balance, hot plate, magnetic stirrer, Laminar Air Flow (LAF) cabinet, autoclave, incubator, vortex mixer, spectrophotometer, colony counter, 96-well microplate, Diatek DR-200BC microplate reader, micropipettes, Petri dishes, test tubes, inoculating needles, sterile cotton swabs, and 6-mm-diameter filter-paper discs. Standard laboratory glassware and other supporting materials were also used as required.

The materials included *C. ternatea* flowers, *C. aurantifolia* material, *E. coli* ATCC 25922, *S. dysenteriae*, 70% analytical-grade ethanol, ciprofloxacin (1 mg/mL), distilled water, Nutrient Agar (NA), Nutrient Broth (NB), Mueller–Hinton Agar (MHA), Mueller–Hinton Broth (MHB), ascorbic acid, sterile 0.9% NaCl, a 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL), 1% DMSO, and 70% alcohol.

Preparation of *Clitoria ternatea* Flower and *Citrus aurantifolia* Extracts

The *C. ternatea* flowers used in this study were obtained from Benih Nusantara Farm, Blora, Indonesia, whereas the *C. aurantifolia* material was obtained in crude-drug form from Gubuk Herbal Solo, Indonesia. Extraction was performed by maceration using 70% ethanol as the solvent. The samples were macerated for 24 h, consisting of 6 h of agitation on a shaker followed by 18 h of standing at room temperature. For *C. ternatea* flowers, a sample-to-solvent ratio of 1:10 was used.

Following maceration, the mixture was filtered through filter paper to obtain the filtrate. The filtrate was subsequently concentrated using a rotary evaporator at 40°C until most of the solvent had evaporated and a viscous extract was obtained. The concentrated extract was diluted with 1% DMSO to prepare the required treatment concentrations for antibacterial testing. The remaining concentrated extract was stored as a stock preparation at –20°C until further use.

Bacterial Cultures of *Escherichia coli* ATCC 25922 and *Shigella dysenteriae*

The *E. coli* and *S. dysenteriae* bacteria were obtained from pure isolates sourced from AgaviLab; they were not the result of personal isolation or cultivation. The bacteria were revived on NA (Nutrient Agar) medium. The NA medium was prepared at a ratio

of 28g : 1000 mL distilled water; thus, to prepare 3 petri dishes with a total of 60 mL of medium, 1.68 g of medium was required, which was then sterilized in an autoclave. The bacteria were aseptically inoculated onto the NA medium inside a LAF using a lit Bunsen burner. Bacterial inoculation was performed using the streak plate method, followed by incubation for 1 × 24 hours at 37°C in an incubator.

Preparation of Mueller-Hinton Agar (MHA) Test Medium

The medium used in the antibacterial activity test, which measures inhibition zones, is Mueller-Hinton Agar (MHA). This medium is prepared by dissolving 5.7 grams of the medium in 150 mL of distilled water, then homogenizing the mixture and heating it on a hot plate until the solution becomes clear, followed by sterilization in an autoclave. The sterile MHA medium to be used is poured into sterile Petri dishes in 20 mL portions.

Preparation of *Escherichia coli* and *Shigella dysenteriae* Suspensions

The solution used for the *Escherichia coli* and *Shigella dysenteriae* suspensions is 0.9% NaCl. Take 10 mL of the 0.9% NaCl solution and pour it into a test tube. Take 1 loop or an appropriate amount of the recultured *Escherichia coli* and *Shigella dysenteriae* and add them to the previously prepared NaCl solution in separate test tubes. The turbidity of the suspensions was standardized using a McFarland 0.5 solution corresponding to approximately 1.5×10^8 CFU/mL, and the absorbance was measured using a spectrophotometer at a wavelength of 600 nm until a standard McFarland turbidity value of 0.08–0.1 was obtained. The bacterial suspensions were then used in the antibacterial activity test.

Antibacterial Inhibition Zone Test of the Combination of *Clitoria ternatea* Flower and *Citrus aurantifolia* Extracts

Antibacterial activity tests of *C. ternatea* flower extract, *C. aurantifolia* extract, and a combination of *C. ternatea* flower and *C. aurantifolia* extracts were conducted using the disk diffusion method with sterile filter paper disks under aseptic conditions in a Laminar Air Flow (LAF) cabinet. A total of 20 µL of each treatment *Clitoria ternatea* flower ethanol extract and the combined *C. ternatea* and *C. aurantifolia* extract was prepared. 6-mm sterile filter paper disk were soaked in the three types of extract combinations prepared earlier, and the soaked sterile paper disk filters were then placed on MHA medium that had been previously inoculated with *E. coli* or *Shigella dysenteriae*.

The paper discs were also soaked in a positive control (antibiotic) and a negative control (1% DMSO solution), then placed in Petri dishes containing the test bacteria. Incubation was then carried out at 37°C for 24 hours. After 24 hours of incubation, the inhibition zones formed were measured using a vernier caliper (mm) by calculating the average of the horizontal and vertical diameters. The inhibition categories were then analyzed based on established literature.

Minimum Inhibitory Concentration (MIC) Test

The Minimum Inhibitory Concentration (MIC) test was performed using the microdilution method in a 96-well plate. The total volume used in each well of the plate is 200 µL. The best extract combinations from the zone inhibition test were prepared for further MIC testing. The 25% *Clitoria ternatea* : 75% *Citrus aurantifolia* extract combination was prepared using a ratio of 0.125 g : 0.375 g, respectively, and then dissolved in 1% DMSO to a final volume of 2 mL. *Escherichia coli* or *Shigella dysenteriae* bacteria were first suspended to a turbidity equivalent to McFarland 0.5 (approximately 1.5×10^8 CFU/mL) to ensure a uniform inoculum concentration. The

MIC test used a more dilute bacterial suspension of 5×10^5 CFU/mL in accordance with inoculum standards. This resulted in an OD (600) of ± 0.008 – 0.01 , which was suitable for use in the subsequent MIC test. Dilution was performed by taking 10 μ L and adding 90 μ L of MHB medium to obtain an OD (600) equivalent to ± 0.008 – 0.01 (Kadeřábková *et al.*, 2024).

MHB medium was aseptically pipetted into each well in the LAF at a volume of 100 μ L. The previously prepared combination of *C. ternatea* flower and *C. aurantifolia* extract was added after the MHB using a different micropipette at a volume of 100 μ L into the first well, then homogenized. A 100 μ L sample of the MHB and extract mixture from the first well was then transferred to the second well and homogenized again (this step was repeated up to the seventh well). A 100 μ L sample of the diluted solution from the seventh well was then discarded. The treatment was repeated three times.

Four controls were prepared for the MIC test: an MHB medium only control, a medium and extract control, a medium and bacteria control, and a medium bacteria and antibiotic control. The medium control was filled with 200 μ L of MHB medium. The medium and extract control was filled with 100 μ L of MHB medium and 100 μ L of the extract mixture. The “media and bacteria” control was filled with 100 μ L of MHB medium and 100 μ L of bacterial suspension in MHB. Next, the “MHB medium-bacterial-suspension antibiotic” control was filled sequentially with 100 μ L, 33 μ L, and 67 μ L, respectively.

The microplates were incubated at 37°C for approximately 20 hours. The turbidity of the incubation results was observed qualitatively and documented. Quantitatively, the results were read using an ELISA reader (Diatek Dr-200BC) at a wavelength of 575 nm to obtain the OD (Optical Density) values. The calculated OD results were analyzed, and the true turbidity (excluding false turbidity caused by the extract's color) was determined using the following formula:

$$\% \text{ of inhibition} = \frac{OD \text{ control} - OD \text{ treatment} * 100}{OD \text{ control}}$$

Data Analysis

The data were tested by SPSS test for normality using the Shapiro-Wilk test and for homogeneity using Levene's test to determine the homogeneity of the data distribution. The data were then analyzed using the Kruskal-Wallis test (non-parametric) (Sari *et al.*, 2021). A Kruskal-Wallis test was conducted to determine whether there were differences among the treatment groups in the data obtained. A post hoc multiple comparison test using the Mann-Whitney method was conducted to determine whether there were differences between treatments or compared to the control group. The calculated data are presented in tabular form to facilitate interpretation of the effects of extract administration on the growth of *Escherichia coli* and *Shigella dysenteriae*.

RESULTS AND DISCUSSION

Antibacterial Activity of a Combination of *Clitoria ternatea* and *Citrus aurantifolia* Extracts Against *Escherichia coli* and *Shigella dysenteriae*

The results of the antibacterial activity test of the combination of *Clitoria ternatea* and *Citrus aurantifolia* extracts against *Escherichia coli* and *Shigella dysenteriae* showed that treatment P1 (25% *C. ternatea* flower extract : 75% *C. aurantifolia* extract) exhibited the highest antibacterial activity against both test bacteria. The treatments consisted of five concentration combinations (P1–P5), namely 25%:75%, 50%:50%, 75%:25%, 100% *C. ternatea* flower extract, and 100% *C. aurantifolia* extract,

respectively. Antibacterial activity was assessed based on the diameter of the inhibition zone (clear zone) formed around the filter paper discs soaked in the extract combinations.

The largest inhibition zone against *E. coli* was observed in treatment P1, with a diameter of 10.17 mm, which was categorized as strong antibacterial activity. Similarly, P1 produced the largest inhibition zone against *S. dysenteriae*, with a diameter of 7.50 mm, which was categorized as moderate antibacterial activity. The inhibition zone diameters observed for treatments P2–P5 and the control are presented in Table 1.

Table 1. Results of antibacterial tests on a combination of *Clitoria ternatea* and *Citrus aurantifolia* extracts against *Escherichia coli* and *Shigella dysenteriae*

Bacteria	Treatments (%)	Average Diameter (mm)	Description
<i>Escherichia coli</i>	P1 (T 25% : J 75%)	10.17	Strong
	P2 (T 50 : J 50%)	2.67	Moderate
	P3 (T 75% : J 25%)	1.00	Moderate
	P4 (T 100%)	0	-
	P5 (J 100%)	10.83	Strong
	K (-)	0	-
	K (+)	25.83	Very strong
	Kontrol Asam Askorbat	2.67	Moderate
<i>Shigella dysenteriae</i>	P1 (T 25% : J 75%)	7.5	Moderate
	P2 (T 50 : J 50%)	2.8	Weak
	P3 (T 75% : J 25%)	3.00	Weak
	P4 (T 100%)	3.8	Weak
	P5 (J 100%)	4.8	Weak
	K (-)	0	-
	K (+)	25	Strong
	Kontrol Asam Askorbat	0	-

The results presented in Table 1 show that treatments containing a higher proportion of *C. aurantifolia* extract exhibited greater antibacterial activity than those containing an equal or higher proportion of *C. ternatea* flower extract. This trend was observed against both *Escherichia coli* and *Shigella dysenteriae*. This finding may be attributed to several factors, including the proportion of each extract in the combination, the secondary metabolites present in the extracts and their chemical properties and mechanisms of action, extract stability, the bacterial strains used, and other experimental factors.

The initial concentration of *C. aurantifolia* extract was set at the same concentration as that of *C. ternatea* flower extract, namely 25%, to evaluate their antibacterial activity when combined in equal proportions. This initial concentration may have contributed to the greater antibacterial activity observed for the *C. aurantifolia* extract. Research by Sari et al. (2021) showed that *C. aurantifolia* peel extract at a concentration of 25% produced an average inhibition zone of 19.43 ± 1.288 mm against *E. coli*, which was classified as strong antibacterial activity. Similarly, Prasetya et al. (2023) reported that *C. aurantifolia* peel extract at a concentration of 20% exhibited antibacterial activity, with an average inhibition zone of 13.7 ± 1.5 mm. These findings suggest that the antibacterial activity of *C. aurantifolia* may be associated with the presence of secondary metabolites with antibacterial properties. In the present study, the initial concentrations were not determined based on the minimum concentration required to inhibit bacterial growth but were selected based on concentrations previously reported to exhibit strong antibacterial activity against bacteria such as *E. coli*. In addition to the antibacterial compounds present in *C.*

aurantifolia, *C. ternatea* flower extract also contains secondary metabolites that may contribute to its antibacterial activity.

Clitoria ternatea flowers are reported to contain anthocyanins, flavonoids, tannins, and saponins, which have been associated with antimicrobial activity. Jeyaraj et al. (2022) reported that the anthocyanin-rich fraction of *C. ternatea* exhibited stronger antibacterial activity than the crude extract and was capable of inhibiting the growth of *E. coli*. This finding suggests that the anthocyanin-rich fraction may exhibit greater antibacterial activity than the crude extract, potentially because of the higher concentration of anthocyanin compounds in the fraction. Anthocyanins have been reported to exert antibacterial effects through several mechanisms, including disruption of bacterial cell membrane integrity, interference with cellular metabolism, and disturbance of energy metabolism, which may ultimately inhibit the growth of *Escherichia coli*. Although *E. coli* possesses an outer membrane rich in lipopolysaccharides (LPS) that provides a protective barrier, exposure to anthocyanins may disrupt membrane stability and reduce the bacterium's ability to maintain cellular integrity under environmental stress.

Jeyaraj et al. (2023) demonstrated that anthocyanins do not directly bind to and damage cell membranes; rather, exposure to anthocyanins induces changes in metabolites associated with glycerophospholipids, amino acids, and cellular energy. Anthocyanins from the *C. ternatea* flower do not act like antibiotics that can directly kill bacterial cells; rather, these compounds disrupt key metabolic processes in bacteria, including *Escherichia coli*. These compounds alter lipid metabolism, which plays a role in building cell membranes. This can result in abnormal phospholipid formation, membrane instability, and increased membrane permeability, leading to membrane leakage.

Jeyaraj et al. (2023) demonstrated that anthocyanins do not directly bind to or damage bacterial cell membranes. Instead, exposure to anthocyanins induces changes in metabolites associated with glycerophospholipids, amino acids, and cellular energy metabolism. Thus, anthocyanins from *C. ternatea* flowers may exert their antibacterial effects differently from conventional antibiotics that directly target and kill bacterial cells. Rather than acting through a single direct mechanism, anthocyanins may interfere with several key metabolic processes in bacteria, including *Escherichia coli*. These metabolic alterations may affect lipid metabolism, which is essential for maintaining bacterial cell membrane structure. Consequently, abnormal phospholipid formation may occur, leading to membrane instability and increased membrane permeability, which may ultimately result in the leakage of intracellular contents.

Clitoria ternatea contains flavonoids capable of forming soluble extracellular proteins that bind to bacterial cell walls and membranes; these proteins then dissolve, causing intracellular compounds (ions, proteins, and nucleic acids) to leak out, which damages the bacterial cell membrane by disrupting bacterial cell metabolism. The saponins in the *C. ternatea* flower reduce the surface tension of bacterial cells, increasing their permeability; as a result, intracellular substances leak out of the cells, leading to bacterial death (PH, Dian Pramana et al., 2025). In addition to the role of compounds from *C. ternatea* flower, the secondary metabolites in *C. aurantifolia* extract are believed to synergize with those in the *C. ternatea* flower extract, thereby enhancing the antibacterial efficacy of the *C. ternatea* flower.

Citrus aurantifolia contains secondary metabolites that are also found in *C. ternatea* flowers, such as flavonoids, saponins, tannins, and alkaloids. Therefore, the combination of these two extracts may provide complementary antibacterial effects

through the interaction of their bioactive compounds. According to Wong (2017), *C. aurantifolia* peel contains other active compounds that may contribute to its antibacterial activity, including limonene, linalool, and myrcene, which have been reported to disrupt bacterial cell membranes. In addition, *C. aurantifolia* contains essential oils and citric acid, which may also contribute to its antibacterial activity. Citric acid has a relatively small molecular size, high water solubility, and good diffusion capacity. The presence of citric acid in *C. aurantifolia* may lower the pH of the surrounding environment, potentially disrupting the stability and permeability of the outer membrane of *Escherichia coli* and resulting in the leakage of intracellular components.

This finding suggests that treatment P1 exhibited the highest antibacterial activity, indicating that a higher proportion of *C. aurantifolia* may contribute to greater antibacterial activity. The larger inhibition zone observed in treatment P1 (25% *C. ternatea* flowers:75% *C. aurantifolia* extract) may be associated with the higher proportion of bioactive compounds in *C. aurantifolia* that can contribute to increased permeability of the outer membrane of *Escherichia coli* (Alakomi et al., 2000). Citric acid in *C. aurantifolia* may create acidic conditions that disrupt the stability of the lipopolysaccharide (LPS) layer in the outer membrane of Gram-negative bacteria. Disruption of the LPS structure may increase membrane permeability, potentially facilitating the entry of other bioactive compounds into bacterial cells. In addition, flavonoids and essential oils in *C. aurantifolia* may interfere with membrane integrity and metabolic enzyme activity. These effects may facilitate the interaction of anthocyanins from *C. ternatea* flowers with bacterial metabolic processes, including lipid and energy metabolism, as reported by Jeyaraj et al. (2023). The combined effects of these mechanisms may ultimately inhibit the growth of *E. coli*, resulting in a larger inhibition zone in P1 than in the other treatments.

The combination treatments of extracts applied to *Shigella dysenteriae* also showed that P1 was the most effective treatment, with an inhibition zone diameter of 7.5 mm. *Shigella dysenteriae* is a Gram-negative bacterium, similar to *Escherichia coli*, but differs in its level of pathogenicity. The dominance of *C. aurantifolia* extract concentration demonstrated the best antibacterial efficacy compared to other combined extract treatments. This is generally related to the presence of a membrane in Gram-negative bacteria, such as *Escherichia coli*; the outer membrane of Gram-negative bacteria acts as the primary barrier to the penetration of flavonoid compounds. This is why citric acid from *C. aurantifolia* plays a crucial role in facilitating the entry of antibacterial compounds into the *C. ternatea* flower.

Khadse et al. (2008) also explained that *S. dysenteriae*'s resistance to various antimicrobial agents is closely related to modifications in the outer membrane porins OmpF and OmpC; clinical isolates of *Shigella* with mutations in the OmpF porin were found to be resistant to several antibiotics because porins serve as the primary pathway for compounds to penetrate the outer membrane. The dominance of *C. aurantifolia* in treatment P1 was a key factor in the success of this combination in producing the largest inhibition zone. *Citrus aurantifolia* contains high concentrations of citric acid; the citric acid concentration in *C. aurantifolia* can reach 8% of dry weight (Gozali et al., 2023). Citric acid is a triprotic organic acid with three pKa values, namely approximately 3.1, 4.7, and 6.4. Under certain pH conditions, citric acid ionizes to form negatively charged species capable of chelating divalent ions such as Ca^{2+} and Mg^{2+} on the outer membrane of Gram-negative bacteria. The antibacterial mechanism of citric acid occurs through a combination of the acidification of the environment and the sequestration of divalent ions in the lipopolysaccharide (LPS) layer, which causes

destabilization of the outer membrane and ultimately leads to bacterial cell death (Burel et al., 2021). It is through this disruption of the outer membrane of *S. dysenteriae* that the bioactive compounds from the buttercup flower gain significantly better access to penetrate the bacterial cell and act on their intracellular targets.

Clitoria ternatea flowers (*Clitoria ternatea* L.) contribute through their quercetin glycoside content, the primary flavonol that targets intracellular components of *S. dysenteriae* after its membrane has been destabilized by *C. aurantifolia* compounds. Quercetin exhibits antimicrobial activity through several mechanisms, including disrupting cell membrane integrity, increasing bacterial membrane permeability, and inhibiting bacterial growth by interacting with key components of microbial cells (Herslambang et al., 2020).

In addition, quercetin has also been reported to exhibit antibacterial activity against both Gram-positive and Gram-negative bacteria by inhibiting cell wall biosynthesis, disrupting membrane permeability, inhibiting vital enzymes, and suppressing the expression of virulence genes. In *Staphylococcus aureus*, quercetin has the potential to inhibit the formation of the virulence protein ClfB (clumping factor B) a surface protein that plays a crucial role in the initial attachment of bacteria to host tissues and biofilm formation thereby reducing adhesion and biofilm formation, even at sub-MIC concentrations, with a reduction in biofilm formation exceeding 60% (Lesbatta et al., 2026). This inhibition is highly relevant to *S. dysenteriae* because lpa (invasion plasmid antigen) expression is controlled by virulence regulation, and quorum sensing coordinates the expression of invasion proteins required by the bacterium to infect intestinal epithelial cells.

The acidic pH conditions created by the dominance of *C. aurantifolia* acid also enhance anthocyanin stability. This aligns with the chemical properties of anthocyanins described by Vidana Gamage et al. (2021), who noted that anthocyanins *C. ternatea* flowers exhibit an intense blue color and optimal stability at acidic pH levels ranging from 3.2 to 5.2. Thus, the dominance of *C. aurantifolia* in P1 not only disrupts the LPS membrane but also indirectly stabilizes and enhances the bioavailability of the anthocyanins from the *C. ternatea* flower, resulting in a synergistic reinforcement between the two. The results of this optimal combination treatment were then subjected to concentration optimization to potentially yield significantly better antibacterial activity.

Minimum Inhibitory Concentration (MIC) Test

The results of the microdilution test, obtained through visual observation, showed changes in turbidity intensity at each concentration of the combination of *C. ternatea* flower and *C. aurantifolia* extracts against *Escherichia coli* and *Shigella dysenteriae*. There were five well concentrations: 25%, 12.5%, 6.25%, 3.125%, and 1.5625%. Based on the results obtained, it was found that both bacteria exhibited the same pattern: at concentrations ranging from 25% to 3.125%, the solution appeared clear, whereas at the lowest concentration of 1.5625%, the solution appeared turbid, indicating bacterial growth (Table 2).

Table 2. Turbidity levels based on visual or qualitative MIC test results

Bacteria	Concentrations	Turbidity Levels	Description
<i>Escherichia coli</i>	Well 1 (25%)	–	Clear
	Well 2 (12.5%)	–	Clear
	Well 3 (6.25%)	–	Clear
	Well 4 (3.125%)	–	Clear

Bacteria	Concentrations	Turbidity Levels	Description
	Well 5 (1.5625%)	+	Turbid
	Medium control	-	Clear
	Medium + extract control	-	Clear
	Medium + bacteria control	+++	Very turbid
	Medium + bacteria + ciprofloxacin control	-	Clear
<i>Shigella dysenteriae</i>	Well 1 (25%)	-	Clear
	Well 2 (12.5%)	-	Clear
	Well 3 (6.25%)	-	Clear
	Well 4 (3.125%)	-	Clear
	Well 5 (1.5625%)	++	Turbid
	Medium control	-	Clear
	Medium + extract control	-	Clear
	Medium + bacteria control	+++	Very turbid
	Medium + bacteria + ciprofloxacin control	-	Clear

After the qualitative test, it was found that the extract solution in the fourth well was no longer turbid, indicating that the fourth well was likely the KHM value. However, this finding must be confirmed through a quantitative test using an ELISA reader to determine the KHM value based on its absorbance.

Table 3. Results of the quantitative minimum inhibitory concentration (MIC) assay using a microplate reader

Bacteria	Treatments	OD Average	OD Correction	%Inhibition	Description
<i>Escherichia coli</i>	A1 (25%)	3.437	2.947	-87.0%	Color correction
	A2 (12,5%)	2.084	1.594	-1.1%	Color correction
	A3 (6,25%)	1.274	0.784	50.3%	Inhibited
	A4 (3,125%)	0.990	0.500	68.3%	Inhibited
	A5 (1,563%)	0.679	0.189	88.0%	Inhibited
	K1	0.490	0,000 (baseline)	-	-
	K2	3.893	-	-	-
	K3	2.066	-	0% (reference for 100% bacterial growth)	-
	K4	0.777	-	81.789	-
<i>Shigella dysenteriae</i>	A1 (25%)	3,734	-0,133	111,17	Color correction
	A2 (12,5%)	1,922	-0,011	100,21	Color correction
	A3 (6,25%)	1,060	0,094	92,57	Inhibited
	A4 (3,125%)	0,624	0,141	88,27	Inhibited
	A5 (1,563%)	0,971	0,729	42,58	Inhibited
	K1	0,125	-	-	-
	K2	3,867	-	-	-
	K3	1,399	-	-	-
	K4	0,000	-	-	-

The MIC value is determined based on the lowest antibacterial concentration capable of inhibiting bacteria, as indicated by a clear solution after overnight incubation (Andrews, 2001; Jubair et al., 2022). Based on the analytical data obtained from the study shown in Table 3, it is suspected that the 3.125% concentration (fourth well) represents the MIC value. These results indicate that the extract combination remains effective in inhibiting the growth of *Escherichia coli* and *Shigella dysenteriae* even after being serially diluted from the initial concentration of 25%. However, further testing is required for *Escherichia coli* using the KBM assay to determine whether the 3.125%

concentration of the extract combination is indeed the minimum concentration capable of inhibiting growth.

Escherichia coli and *Shigella dysenteriae* are Gram-negative bacteria that share the same membrane components, specifically an outer membrane containing lipopolysaccharide (LPS). However, these two bacteria are suspected to have different types of O antigens, which may account for differences in their responses to the antibacterial extract combination. *Escherichia coli* and *Shigella dysenteriae* are thought to have different O-antigens, even though they belong to the same genus as *Salmonella* and *Shigella* (Mphande et al., 2022). Antigens have been reported to stimulate or activate the bacterial immune system and protect the bacteria from the bactericidal effects of drugs. These differences suggest that specific interactions may occur between the bioactive compounds in the extract combinations and the cellular structures of each bacterium, resulting in different inhibition zones even within the same group of Gram-negative bacteria.

The data from this study were analyzed using SPSS to test for normality and homogeneity, thereby determining the appropriate statistical tests to be conducted subsequently. The Shapiro-Wilk test was used to determine whether the data were normally distributed. The p-value from the Shapiro-Wilk test was $p > 0.05$; however, some data points had a significance value of 0.00, indicating that the data were not entirely normally distributed, as the significance values for the control (+) and ascorbic acid control treatments were < 0.05 . The 100% *C. ternatea* flower extract and 1% DMSO groups, marked with a (–), are suspected to be non-normally distributed because each replicate had identical values, indicating that the data are not entirely normally distributed.

The homogeneity test was conducted using Levene's test to assess the homogeneity of the data. The homogeneity test yielded a significance value of 0.004 with a p-value < 0.05 , indicating that the data are not homogeneous. Based on the results of the normality and homogeneity tests, it was concluded that the data could not be analyzed using ANOVA (parametric); therefore, a post-hoc test was conducted using the Kruskal-Wallis test (non-parametric) (Sari et al., 2021).

Sari et al. (2021) stated that the Kruskal-Wallis test was conducted to determine whether the data obtained showed differences among treatment groups. The results of the Kruskal-Wallis test yielded a sig. value of 0.003; with $p < 0.05$, it can be concluded that there are significant differences in the inhibition zone variance on MHA medium among the groups. However, to determine whether the differences between each treatment group were statistically significant, a Mann-Whitney post-hoc test was conducted. The results of the Mann-Whitney test showed that some treatment groups exhibited significant differences ($p < 0.05$), while others did not show significant differences ($p > 0.05$).

CONCLUSION

The combination of *Clitoria ternatea* flower and *Citrus aurantifolia* extracts exhibited antibacterial activity against both *Escherichia coli* and *Shigella dysenteriae*, with the relative proportion of each extract influencing the inhibitory response. Among the combination treatments, P1 (25% *C. ternatea* : 75% *C. aurantifolia*) showed the greatest antibacterial activity against both bacterial species. The MIC assay indicated that this formulation retained growth-inhibitory activity at a concentration of 3.125%. However, this MIC value should be interpreted with caution because the color of the extract may have interfered with optical density measurements. Overall, these findings indicate that the combination of *C. ternatea* flower and *C. aurantifolia* extracts has

potential as a natural antibacterial agent against Gram-negative bacteria under *in vitro* conditions. Further studies are required to confirm the MIC, determine bactericidal activity, and clarify the mechanisms underlying the observed antibacterial effects.

RECOMMENDATION

Recommendations for future research include extending the antibacterial assay to determine the Minimum Bactericidal Concentration (MBC), which would provide information on the minimum concentration required for the extract combination to kill the test bacteria. Test materials, including the extracts, bacterial cultures, and test solutions, should be properly maintained by controlling storage temperature, handling procedures, and storage duration to minimize the risk of material degradation. During the extract evaporation process, it should be ensured that the solvents are completely removed to minimize potential interference from residual solvents in the antibacterial assay. In addition, the methodology for MIC testing of colored plant extracts should be carefully reviewed, including the preparation of appropriate controls for each extract dilution, such as medium controls and extract controls, as well as the use of colorimetric indicators such as resazurin or TTC to facilitate more reliable assessment of differences in optical density (OD).

ACKNOWLEDGEMENT

The authors would like to express their sincere gratitude to the Faculty of Science and Technology, the Biology Study Program, the research group supervisors, and all members of the research team for their support, guidance, and valuable contributions, as well as for providing the opportunity to conduct this research. The authors acknowledge that this article is not yet perfect and may still require further improvement. Therefore, the authors greatly appreciate constructive feedback, suggestions, and contributions from readers and other interested parties to improve the quality of this article. Any feedback provided will be highly valued and considered for future improvements.

The authors used AI-assisted technologies solely to improve the readability and language quality of the manuscript. The authors reviewed and edited the final content and take full responsibility for the manuscript.

REFERENCES

- Antimicrobial Resistance Collaborators. (2022). Global burden of bacterial antimicrobial resistance in 2019: A systematic analysis. *The Lancet*, 399(10325), 629–655. [https://doi.org/10.1016/S0140-6736\(21\)02724-0](https://doi.org/10.1016/S0140-6736(21)02724-0)
- Burel, C., Kala, A., & Purevdorj-Gage, L. (2021). Impact of pH on citric acid antimicrobial activity against Gram-negative bacteria. *Letters in Applied Microbiology*, 72(3), 332–340.
- Cao, M., Wang, W., Zhang, L., Liu, G., Zhou, X., Li, B., Shi, Y., Zhu, Z., & Zhang, J. (2021). Epidemic and molecular characterization of fluoroquinolone-resistant *Shigella dysenteriae* 1 isolates from calves with diarrhea. *BMC Microbiology*, 21(1), 6. <https://doi.org/10.1186/s12866-020-02050-9>
- Charurungsipong, P., Tangduangdee, C., Amornraksa, S., Asavasanti, S., & Lin, J. (2020). Improvement of anthocyanin stability in butterfly pea flower extract by co-pigmentation with catechin. *E3S Web of Conferences*, 141, 03008. <https://doi.org/10.1051/e3sconf/202014103008>
- Febrianti, F., Widyasanti, A., & Nurhasanah, S. (2022). Aktivitas antibakteri ekstrak bunga telang (*Clitoria ternatea* L.) terhadap bakteri patogen. *ALCHEMY Jurnal*

- Penelitian Kimia, 18(2), 234–241. <https://doi.org/10.20961/alchemy.18.2.52508.234-241>
- Gozali, T., Assalam, S., Ikrawan, Y., & Nurfalia, I. (2023). Optimalisasi formula minuman olahan jeruk nipis (*Citrus aurantifolia*) dengan parameter karakteristik produk. *Jurnal Penelitian Pertanian Terapan*, 23(2), 288–301. <https://doi.org/10.25181/jppt.v23i2.2923>
- Jeyaraj, E. J., Lim, Y. Y., & Choo, W. S. (2022). Antioxidant, cytotoxic, and antibacterial activities of *Clitoria ternatea* flower extracts and anthocyanin-rich fraction. *Scientific Reports*, 12(1), 15321. <https://doi.org/10.1038/s41598-022-19146-z>
- Jeyaraj, E. J., Han, M. L., Li, J., & Choo, W. S. (2023). Metabolic perturbations and key pathways associated with the bacteriostatic activity of *Clitoria ternatea* flower anthocyanin fraction against *Escherichia coli*. *Access Microbiology*, 5(6), 000535-v5. <https://doi.org/10.26180/21687398.v1>
- Jubair, N., R. M., Fatima, A., Mahdi, Y. K., & Abdullah, N. H. (2022). Evaluation of catechin synergistic and antibacterial efficacy on biofilm formation and *acrA* gene expression of uropathogenic *E. coli* clinical isolates. *Antibiotics*, 11(9), 1223. <https://doi.org/10.3390/antibiotics11091223>
- Kadeřábková, N., Mahmood, A. J., & Mavridou, D. A. (2024). Antibiotic susceptibility testing using minimum inhibitory concentration (MIC) assays. *npj Antimicrobials and Resistance*, 2(1), 37. <https://doi.org/10.1038/s44259-024-00051-6>
- Khadse, V., Patwardhan, M., & Joshi, C. (2008). Differential expression of *ompC* and *ompF* in multidrug-resistant *Shigella dysenteriae* and *Shigella flexneri* by aqueous extract of *Aegle marmelos*, altering its susceptibility toward β -lactam antibiotics. *Diagnostic Microbiology and Infectious Disease*, 61(3), 321–328.
- Khairunnisa, K., Ramadhani, Y., & Sapada, I. E. (2025). Studi efektivitas antibakteri ekstrak etanol rimpang bangle (*Zingiber purpureum* Roxb.) terhadap patogen *Shigella dysenteriae*. *Generics: Journal of Research in Pharmacy*, 5(1).
- Lin, Y., Li, C., Shi, L., & Wang, L. (2023). Anthocyanins: Modified new technologies and challenges. *Foods*, 12(7), 1368. <https://doi.org/10.3390/foods12071368>
- Mayanti, L., Rahayu, Y. P., Lubis, M. S., & Yuniarti, R. (2023). Analisis cemaran bakteri coliform pada saus jajanan di sekitar sekolah menengah kejuruan di Kota Medan. *Journal of Pharmaceutical and Sciences*, 1282–1289.
- Patel, D. K., Kumar, R., & Laloo, D. (2016). Natural medicines from plant source used for therapy of dysentery. *Pharmacognosy Reviews*, 10(20), 118–126. <https://phcogrev.com/sites/default/files/PhcogRev-10-20-118.pdf>
- Prasetya, I. A. W., Aziz, A., Ekawati, E. R., Pradana, M. S., Al Batati, N., & Pratiwi, E. R. (2023). Uji potensi antibakteri ekstrak kulit jeruk nipis *Citrus aurantifolia* (Christm.) Swingle terhadap *Vibrio* sp. *Jurnal Sainhealth*, 7(1), 9–14.
- Pratap, G. M., Manoj, K. M., Sai, S. A., Sujatha, B., & Sreedevi, E. (2012). Evaluation of three medicinal plants for anti-microbial activity. *Ayu*, 33(3), 423. <https://doi.org/10.4103/0974-8520.108859>
- Rahmah, A. F., Arma, U., Lestari, C., Edrizal, E., & Zia, H. K. (2024). Uji zona hambat ekstrak metanol teripang putih (*Holothuria scabra*) Mentawai terhadap *Streptococcus sanguinis* pada stomatitis aftosa rekuren secara *in vitro*: Studi eksperimental. *Padjadjaran Journal of Dental Researchers and Students*, 8(1), 71–79. <https://doi.org/10.24198/pjdrs.v8i1.52551>
- Raihan, M. M., & Hossain, M. S. (2022). Isolation, identification and evaluation of antibiotic sensitivity and resistance of *Shigella dysenteriae* isolated from food, water and soil. *Journal of Microbiology and Sustainability*, 2(1), 1–10. <https://isciencepress.com/journals/index.php/jms/article/view/4/6>

- Sari, D. I., Wahjuni, R. S., Praja, R. N., Utomo, B., & Wibawati, P. A. (2021). Lime peel liquid (*Citrus aurantifolia* Swingle) inhibit *Escherichia coli* in vitro. *Jurnal Medik Veteriner*, 4(1), 63–71.
- Syafridah, A. (2022). Hubungan tingkat pengetahuan ibu dengan perilaku penggunaan antibiotik pada balita usia 0–2 tahun di Puskesmas Dewantara Kabupaten Aceh Utara. *AVERROUS: Jurnal Kedokteran dan Kesehatan Malikussaleh*, 8(2), 51–58.
- Waisnawi, P. A. G., Puspawati, G. A. K. D., & Wrasianti, L. P. (2022). Pengaruh penambahan jeruk nipis terhadap pH, total antosianin dan aktivitas antioksidan pada minuman bunga telang. *Jurnal Ilmiah Teknologi Pertanian Agrotechno*, 7(1), 89.
- Wong, M. L. (2017). *Uji aktivitas antibakteri minyak atsiri kulit buah jeruk nipis (Citrus aurantifolia Swingle) terhadap Streptococcus pyogenes* [Doctoral dissertation, Widya Mandala Catholic University Surabaya].