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In Vitro Evaluation of the Antibacterial Potential of Beetroot (*Beta vulgaris*) Leaf Extract Against *Escherichia Coli*

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Abstract: This study aimed to evaluate the antibacterial activity of *Beetroot* (*Beta vulgaris*) leaf extract against *Escherichia coli*. Leaves were collected from Lantapan, Bukidnon, and extracted using 70% ethanol through rotary evaporation. The antibacterial activity of the extract was assessed at different concentrations (100%, 75%, 50%, and 25%) using the Kirby–Bauer disc diffusion method. Distilled water served as the negative control, while chloramphenicol was used as the positive control. Qualitative phytochemical screening revealed the presence of flavonoids, saponins, phenols, tannins, alkaloids, terpenoids, and steroids, indicating the existence of potentially bioactive compounds. However, results demonstrated that all tested concentrations of the beetroot leaf extract exhibited no antibacterial activity against *E. coli*, as evidenced by the absence of zones of inhibition comparable to the negative control and significantly lower than the positive control. These findings suggest that, under the conditions employed in this study, *Beta vulgaris* leaf extract does not exhibit effective antibacterial properties against *E. coli*. Nevertheless, the presence of phytochemicals indicates the potential for further investigation using alternative extraction methods or applications.

Keywords: *Beta Vulgaris, Escherichia Coli, Distilled Water, Chloramphenicol, Flavonoids, Tannins, Saponins, Phenols, Steroids, Alkaloids, Terpenoids, Rotary Evaporator, Antibacterial Activity.*

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I. INTRODUCTION

In the field of bacteriology and microbiology, microorganisms play a crucial role in maintaining environmental and biological balance. They exist as part of the normal microflora in both biotic and abiotic environments, where they may exhibit commensal, mutualistic, or pathogenic relationships with their hosts. While many microorganisms contribute positively to host function, some are pathogenic and can disrupt normal body homeostasis.

One of the most widely studied microorganisms is *Escherichia coli* (*E. coli*), a Gram-negative bacillus commonly found as part of the normal intestinal flora. It is also present in various environments, including hospital settings and long-term care facilities. Although generally harmless in its commensal form, *E. coli* includes numerous strains capable of causing both intestinal and extraintestinal diseases. These range from mild, self-limiting gastroenteritis to severe conditions such as renal failure and septic shock. Its pathogenicity is largely attributed to its ability to evade host immune responses and develop resistance to commonly used antibiotics (Mueller et al., 2023).

In both human and animal ecology, *E. coli* functions as an early colonizer of the gastrointestinal tract shortly after birth, while also serving as a significant pathogen. It is the leading cause of urinary tract infections in humans and has been implicated in infections across nearly all anatomical sites, including the gastrointestinal tract, bloodstream, respiratory system, skin, and central nervous system. Additionally, it is associated with conditions such as appendicitis, pneumonia, meningitis, endocarditis, intra-amniotic infections, and puerperal infections in pregnant women. These infections may be either community-acquired or healthcare-associated, affecting individuals across all age groups (Galindo-Méndez, 2020).

The emergence and accumulation of multidrug-resistant *E. coli* strains have become a major global concern in both human and veterinary medicine. Although *E. coli* is naturally susceptible to many antimicrobial agents, it has a remarkable ability to acquire resistance genes, particularly through horizontal gene transfer (Poirel et al., 2018). This adaptability contributes to the growing challenge of antibiotic resistance, highlighting the need for alternative therapeutic approaches.

In response to this issue, there has been increasing interest in the use of medicinal plants as potential antibacterial agents. Numerous plants have been traditionally used to treat bacterial infections, and some have undergone in vitro screening to evaluate their efficacy. However, many herbal remedies have not yet been thoroughly validated through controlled clinical studies. While conventional antibiotics remain effective, the rise of resistance underscores the need for new and complementary treatment options. Although natural products are not inherently safer than synthetic drugs, they are often preferred by patients, making it important for healthcare professionals to understand their potential benefits and limitations (Martin, 2003).

Beetroot (*Beta vulgaris*), commonly known as red beet, table beet, or garden beet, is a nutrient-rich root vegetable recognized for its health-promoting properties. It is a valuable source of fiber, folate (vitamin B9), manganese, potassium, iron, and vitamin C. Beetroot and its derivatives have been associated with various health benefits, including improved blood circulation, reduced blood pressure, and enhanced physical performance. Given its phytochemical composition and potential biological activity, beetroot has been considered a candidate for antimicrobial investigation.

Thus, this study aimed to determine the effectiveness of beetroot (*Beta vulgaris*) leaf extract as an antibacterial agent against *Escherichia coli* through in vitro application.

II. METHODOLOGY AND MATERIALS

This chapter presented the description of the study's research design, research locale, experimental procedure, and the inclusion of instruments used in the procedure.

➢ *Research Design*

The study employed an experimental research design to evaluate the activity of *Beetroot* (*Beta vulgaris*) leaf extract against the test organism *Escherichia coli* (*E. coli*). Distilled water was used as the negative control, as it does not inhibit bacterial growth and allows verification that any observed effects are due solely to the compounds being tested (Abdelkader et al., 2018). Chloramphenicol served as the positive control.

The analysis was based on the zones of inhibition exhibited by varying concentrations of the beetroot leaf extract, which were compared with the negative control to determine any clinically significant differences. The Kirby–Bauer disc diffusion method was utilized, as it is widely employed in evaluating the activity of plant and microbial extracts (Mounyr Balouiri et al., 2016).

Sterile filter paper discs impregnated with 50 µL of beetroot leaf extract at concentrations of 100%, 75%, 50%, and 25% were placed on the surface of Mueller–Hinton Agar (MHA) plates previously inoculated with *E. coli*. The plates were allowed to dry briefly and then incubated at 37°C. After incubation, the diameters of the zones of inhibition surrounding each disc were measured to assess the activity of the extract.

➢ *Research Materials*

Beetroot (*Beta vulgaris*) leaves used as the experimental variable in this study were collected from Lantapan, Bukidnon. The study utilized a range of instruments and materials essential for microbiological and analytical procedures. MacConkey agar was employed as a selective and differential medium to isolate and differentiate organisms within the colon–typhoid–dysentery group based on lactose fermentation.

Graduated cylinders were used to measure precise volumes of liquids, while an autoclave was utilized for sterilization to eliminate potential contaminants and denature proteins. Sterile inoculating loops were used for the aseptic transfer of microbial cultures. A digital caliper was employed to accurately measure the diameters of zones of inhibition during antibacterial testing.

Additional materials included personal protective equipment (PPE), beakers, forceps, and a stove, all of which supported the preparation and execution of experimental procedures throughout the study.

➢ *Experimental Protocol*

This section outlined the experimental protocol, providing a comprehensive and structured plan that detailed the steps and procedures for conducting the study. It served as a guide to help the researchers maintain consistency and accuracy throughout their experiments.

➢ *Beetroot Collection and Experimental Preparation*

The experiment was conducted in the Medical Technology Laboratory located on the 4th floor of Adventist Medical Center College (AMCC), San Miguel, Iligan City. The untreated strain of *Escherichia coli* (*E. coli*) used in this study was obtained from Central Mindanao University. *Beetroot* (*Beta vulgaris*) samples were purchased and collected from Lantapan, Bukidnon.

Prior to the experiment, all laboratory materials and equipment were properly prepared and disinfected to prevent contamination. Glassware, including beakers and Erlenmeyer flasks, as well as consumables such as yellow and blue pipette tips, were sterilized through autoclaving at 121°C for 15–20 minutes. All equipment was thoroughly dried before use to prevent dilution of the extracts and minimize the risk of contamination.

➢ *Sterilization and Autoclave Procedure*

To maintain sterility and ensure researcher safety throughout the study, appropriate personal protective equipment (PPE), including lab coats, bouffant caps, masks, and gloves, was worn at all times. All materials used in the experiment, such as glassware, metal instruments, culture media, and heat-resistant plastics, were sterilized through autoclaving to eliminate contaminants.

A total of 500 mL of distilled water was placed in the autoclave to ensure adequate steam generation. The prepared materials were arranged properly within the chamber to allow efficient steam circulation. The autoclave was then securely closed and operated at 121°C and 15 pounds per square inch (psi) of pressure for 20 minutes. After completion, the materials were allowed to remain inside the autoclave until the internal pressure returned to zero and the temperature decreased to a safe level. Sterilized items were then carefully removed using heat-resistant gloves to prevent contamination and thermal injury (Rutala et al., 2019).

During inoculation, aseptic techniques were strictly followed. The inoculating loop was sterilized by passing it through the flame of a gas burner at an angle until the wire became red-hot, ensuring the destruction of any residual microorganisms (The Australian Wine Research Institute, 2011). All sterilized materials were handled with caution to avoid exposure to non-sterile surfaces. Proper hand hygiene was consistently practiced, and aseptic procedures were rigorously observed throughout all experimental processes.

- *Airdrying of Beetroot*

A total of 1000 grams of fresh *Beta vulgaris* leaves (Azwanida, 2015) were collected from Lantapan, Bukidnon and used in this study. The leaves were thoroughly washed with distilled water to remove dirt and other contaminants, followed by an additional rinsing step to ensure cleanliness. After washing, the leaves were suspended in a well-ventilated area at room temperature and protected from direct sunlight to preserve their phytochemical components. The plant materials were then air-dried for approximately one month (Altavas et al., 2024). Air-drying is a natural method of moisture removal that does not involve artificial heat sources such as ovens or dryers. Instead, it relies on ambient airflow and natural evaporation to gradually reduce moisture content. This gentle process helps maintain the integrity of the plant's bioactive compounds, including essential oils and other phytochemicals with potential antibacterial properties.

- *Ethanolic Extraction of Sample Using Rotary Evaporator*

The extraction process utilized dried *Beta vulgaris* (beetroot) leaves and 70% ethanol as the primary solvent. A total of 1000 mL of 70% ethanol was measured and used for the extraction. Ethanol extraction served as both a means of obtaining botanical extracts for experimental application and as a preparation method for subsequent phytochemical analysis. The extraction aimed to isolate bioactive compounds from the plant matrix while minimizing the presence of unwanted substances that could interfere with analytical or biological evaluation.

A solid–liquid extraction (SLE) method was employed, wherein the dried plant material was soaked directly in ethanol. This traditional technique has been widely used for botanical extraction due to its simplicity and effectiveness (Lab Alley, 2022). Following extraction, the solution was subjected to qualitative phytochemical screening to identify the major classes of compounds present, including betalains, flavonoids, polyphenols, saponins, and inorganic nitrates (Kairupan et al., 2019).

The use of 70% ethanol was considered essential due to its enhanced extraction efficiency and antimicrobial properties. The presence of water in the solvent system improves the penetration of ethanol into plant tissues and facilitates the denaturation of proteins in microbial cell membranes. At this concentration, ethanol is more effective in disrupting microbial cell structures, leading to protein coagulation and eventual cell death (Origin, 2020).

Subsequently, the extract was concentrated using a rotary evaporator (ROTAVAP) to remove the ethanol solvent under reduced pressure. This method allows the solvent to evaporate at a lower temperature than under normal atmospheric conditions, thereby preserving heat-sensitive phytochemicals and maintaining the integrity of the extracted compounds.

- *Preparation of Culture Media (MacConkey Agar and Mueller–Hinton Agar)*

The preparation of MacConkey and Mueller-Hinton Agar medium has specific preparation depending on the manufacturer. In preparing MacConkey agar, it involves 54 mixing the powder with distilled water. Gently stir the mixture until it dissolves. Once the mixture fully dissolves, gently heat the mixture and sterilize the solution by placing it in an autoclave for 121°C for 20 minutes. After sterilization, let the solution cool down for about 45°C-50°C and then carefully pour it into a clean and sterilized petri dish. In McConkey agar it suspends 49.53 of powder in 1000 ml. of Distilled water then dissolves by heating with frequent agitation until boiling. Autoclave for 20 mins. Then cool and pour in petri dishes. (HiMedia Laboratories Pvt.Ltd.) If we need (14 dishes) instead of the entire 1000 mL the volume of media we need to prepare: Calculated as (14x20=280 mL) of media 49.53g/1000 mL = x/280 mL x=280 x 49.53/1000 =13.86 g So, 13.86 g powder should be dissolved in 280 mL of Distilled water. In Eosin Methylene Blue (EMB) it suspends 39 g of powder in 1000 mL of Distilled water then dissolves by heating with frequent agitation until boiling. Autoclave for 20 mins. Then cool and pour in petri dishes. (Sisco Research Laboratories Pvt.Ltd.) If we need (14 dishes) instead of the entire 1000 mL the volume of media we need to prepare: Calculated as (14x20=280 mL) of media 39g/1000 ml. = x/280 mL x=280 x 39/1000 =10.92 g So, 10.92 g powder should be dissolved in 280 mL of Distilled water.

- *Bacterial Cultivation and Preparation of the Test Organism*

The test microorganism used in this study was an untreated strain of *Escherichia coli* (*E. coli*), obtained from

Central Mindanao University, while the culture media were sourced from the Adventist Medical Center College (AMCC) Medical Technology Laboratory. Isolation of *E. coli* was performed by inoculating the organism onto MacConkey agar plates and incubating them at 37°C for 18–24 hours. The preparation of bacterial colonies was conducted using the streak plate method to obtain well-isolated colonies. The selective and differential properties of MacConkey agar enabled efficient isolation, as Gram-positive bacteria were inhibited while Gram-negative bacteria were allowed to grow (Martinson & Walk, 2020). As a lactose-fermenting organism, *E. coli* produced pink colonies due to the formation of acidic byproducts during lactose fermentation, which lowered the pH and caused the pH indicator to turn pink (Jung et al., 2022), thereby allowing clear differentiation from non-lactose-fermenting bacteria.

- *Preparation of Inoculum*

The bacterial suspension of the test organism was prepared using *Escherichia coli* isolates. A sterile inoculating loop was used to aseptically collect four isolates, which were then suspended in a tube containing sterile saline solution.

Standardization of the inoculum was performed using the McFarland turbidity standard method, with the aid of a Wickerham card for visual comparison to ensure uniform bacterial density. The McFarland standard is a reference scale used to estimate bacterial concentration based on turbidity. In this study, a 0.5 McFarland standard was employed, corresponding to an approximate concentration of 1.5×10^8 colony-forming units (CFU) per milliliter (Wiegand et al., 2008). This standardization ensured consistency and reliability in the preparation of the bacterial inoculum for subsequent antimicrobial susceptibility testing.

- *Inoculation of the Bacterial Suspension on MHA*

The prepared bacterial suspension was inoculated onto Mueller-Hinton agar (MHA) plates using a sterile cotton swab, spreading it uniformly over the agar surface. For this study, six MHA plates were used to assess the antibacterial activity, with three replicates for each concentration of the Beetroot (*Beta vulgaris*) leaf extract. Each plate included a 6 mm filter paper disc impregnated with the tested concentrations of the *Beta vulgaris* extract, which were 25%, 50%, 75%, and 100%. All the plates were incubated at 37°C for 18–24 hours (Francline, 2021). Following incubation, the inhibition zones around the discs were measured using digital calipers in millimeters. The measurements were recorded, and the test was replicated three times to ensure reliability.

- *Dispensing of Beta vulgaris Leaf Extract Using the Kirby–Bauer Disc Diffusion Method*

The activity of *Beetroot* (*Beta vulgaris*) leaf extract was evaluated using the Kirby–Bauer disc diffusion method. A 0.5 McFarland standardized suspension of *Escherichia coli* (*E. coli*) was uniformly inoculated onto sterile Mueller–Hinton Agar (MHA) plates using a sterile cotton swab. Sterile 6 mm filter paper discs were impregnated with 50 µL of the extract at concentrations of 25%, 50%, 75%, and 100%, then aseptically placed on the agar surface with adequate spacing. Each concentration was tested in triplicate. Distilled water and chloramphenicol served as the negative and positive controls, respectively. Plates were allowed to stand for 15–20 minutes to permit diffusion and then incubated at 37°C for 18–24 hours. Zones of inhibition were measured in millimeters using a digital caliper, and mean values were calculated (Pangeni et al., 2021). Extract concentrations were prepared using the dilution formula ($C_1V_1 = C_2V_2$) (BYJU'S, 2024), resulting in the following formulations: 25% (13 µL extract + 37 µL distilled water), 50% (25 µL + 25 µL), 75% (37–38 µL + 13 µL), and 100% (50 µL pure extract). A constant solvent system of 70% ethanol was maintained across all samples to ensure consistency and reliability of results.

- *Preparation of the Controls*

To ensure the reliability and accuracy of the antibacterial susceptibility tests, both positive and negative controls were incorporated (Andrews, 2001). These controls served as reference standards for evaluating the effectiveness of the tested substances and for validating the experimental results, thereby reducing the likelihood of false-positive or false-negative outcomes (Olivi et al., 2014).

Chloramphenicol, a broad-spectrum antibiotic with a well-established mechanism of action, was used as the positive control. Its inclusion confirmed that the test system was capable of detecting antibacterial activity under the given experimental conditions (Salam et al., 2023).

Distilled water was employed as the negative control, as it does not exhibit antibacterial properties and therefore should not inhibit bacterial growth. This allowed for comparison with the test samples to determine whether any observed inhibition was attributable to the extract being evaluated.

- *Testing the Anti-bacterial Activity and Data Gathering Procedure*

The methods used to evaluate the ability of Beetroot (*Beta vulgaris*) extract to inhibit bacterial growth involved measuring the zone of inhibition (ZOI) using the Kirby-Bauer disc diffusion method. The effectiveness of the extract was assessed by calculating the mean ZOI. In this study, a digital caliper was employed to measure the diameter of the ZOI for each well in millimeters. The ZOIs from three replicates at the same concentration were summed and then divided by the number of replicates to calculate the mean ZOI. The concentration that produced the highest mean ZOI indicated greater antibacterial activity compared to the other concentrations.

- *One Way Analysis of Variants*

The data obtained from the experiment, particularly the measurements of the zones of inhibition (ZOI) for each concentration of *Beta vulgaris* leaf extract (25%, 50%, 75%, and 100%), were analyzed using One-Way Analysis of Variance (ANOVA). This statistical method was employed to determine whether there were statistically significant differences among the mean ZOI values of the different treatment groups tested against *Escherichia coli*.

Each concentration was tested in triplicate, and the mean ZOI for each group was calculated prior to analysis. One-Way ANOVA was selected as it is appropriate for comparing the means of more than two independent groups, making it suitable for evaluating the effect of varying extract concentrations on bacterial inhibition.

III. RESULTS AND DISCUSSIONS

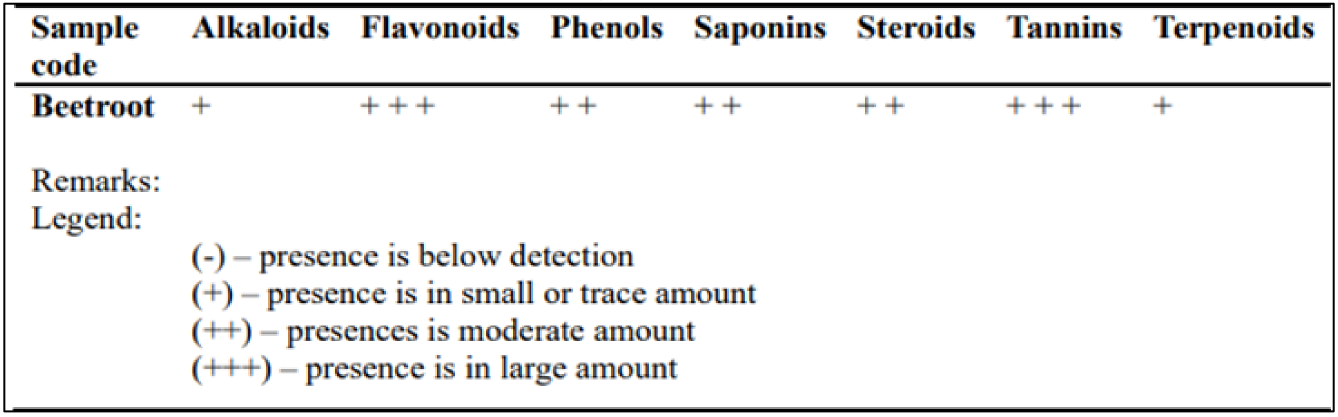
The findings of the in-vitro study evaluating the antibacterial activity of Beetroot (*Beta vulgaris*) leaf extract against *Escherichia coli* were presented in this chapter.

➢ *Organoleptic Evaluation of the Beetroot Extract*

Assessing the sensory qualities of Beetroot *(Beta vulgaris)* extract through human perception was part of the organoleptic evaluation process. The *Beta vulgaris (Beetroot)* leaf extract exhibited a dark green hue and semi liquid consistency. Its aroma had a unique grassy scent.

Phytochemicals have a great antioxidant potential and their beneficial effects on human health, and they give greater health benefits to the consumers (Thakur et al., 2020). The phytochemical analysis showed Beetroot (*Beta vulgaris*) leaf contained some secondary metabolites. In this analysis the presence of alkaloids, flavonoids, phenols, saponins, steroids, tannins and terpenoids was obtained.

Table 1 Phytochemical Test Result



Sample code	Alkaloids	Flavonoids	Phenols	Saponins	Steroids	Tannins	Terpenoids
Beetroot	+	+ + +	+ +	+ +	+ +	+ + +	+

Remarks:
Legend:
(-) – presence is below detection
(+) – presence is in small or trace amount
(++) – presences is moderate amount
(+++) – presence is in large amount

➢ *Phytochemical Test Result*

The table presents the results of the phytochemical screening of *Beta vulgaris* (beetroot) leaf extract, indicating the presence and relative abundance of various bioactive compounds. The extract exhibited a high concentration of flavonoids and tannins, as indicated by the "+++" notation. Phenols, saponins, and steroids were present in moderate amounts, denoted by "++". Alkaloids and terpenoids were detected in trace amounts, represented by "+".

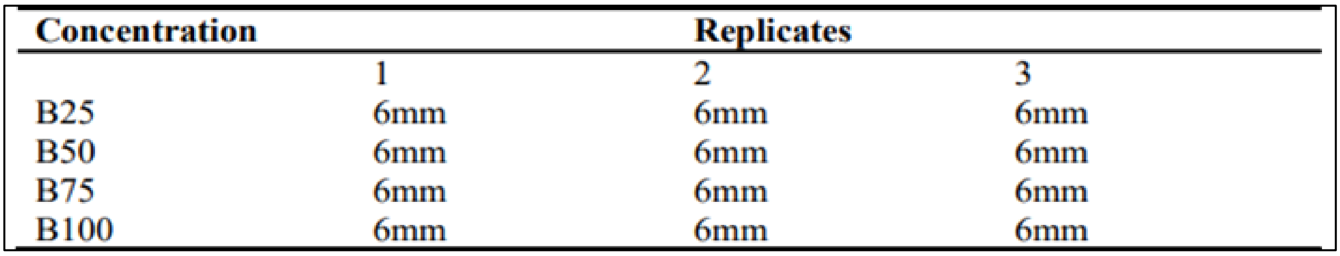
These findings suggest that beetroot leaf extract contains a diverse range of phytochemicals, particularly flavonoids and tannins, which are known for their antioxidant and potential therapeutic properties. Overall, the results indicate that the extract is a rich source of biologically active compounds that may contribute to its medicinal potential.

The high levels of flavonoids and tannins may help explain the traditional medicinal applications of beetroot and support further investigation into its pharmacological properties. Future studies may focus on the isolation and characterization of these compounds to better understand their mechanisms of action and explore their potential applications in nutraceutical and pharmaceutical development.

➢ *Determination of Zone of Inhibition*

The effectiveness of an antibiotic or plant extract is demonstrated by the presence of a clear zone of growth inhibition surrounding the disc on an agar plate, commonly referred to as the zone of inhibition (Singh et al., 2017). This clear circular area indicates suppression of bacterial growth due to the antimicrobial activity of the tested compound. The zone of inhibition (ZOI) forms as the antimicrobial agent diffuses outward from the impregnated disc and inhibits the growth of surrounding bacteria. Its size is influenced by several factors, including the concentration and diffusion rate of the agent, bacterial growth rate, and the composition of the agar medium. In this study, ZOI was measured in millimeters using a digital caliper to ensure accuracy. Each concentration of *Beetroot* (*Beta vulgaris*) leaf extract (25%, 50%, 75%, and 100%) was tested in triplicate, and the diameters of the inhibition zones were recorded. The mean ZOI for each concentration was computed by averaging the three replicates and compared to determine which concentration exhibited the greatest inhibitory effect against *Escherichia coli*. The presence of a measurable zone around the disc was considered a positive result, indicating susceptibility of the microorganism to the extract (Bhargav, 2016).

Table 2 Zones of Inhibition of the Discs Containing the Beetroot (B) Leaf Extract in Triplicate Against *E. coli* Using Kirby-Bauer Test

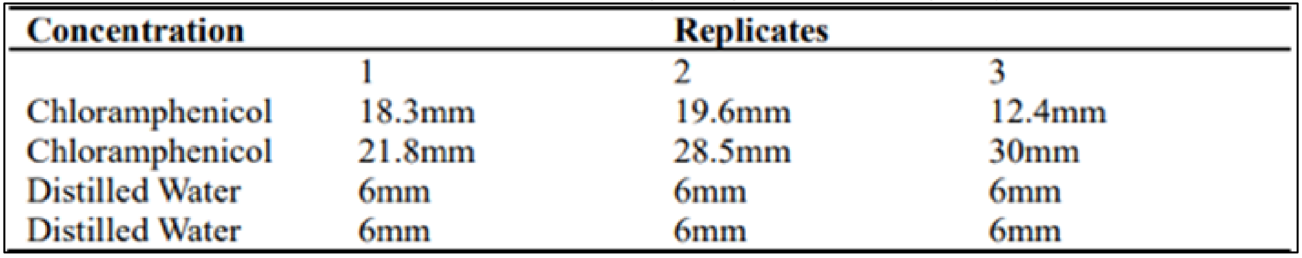


Concentration	**Replicates**		
	1	2	3
B25	6mm	6mm	6mm
B50	6mm	6mm	6mm
B75	6mm	6mm	6mm
B100	6mm	6mm	6mm

The results showed that all discs containing varying concentrations of *Beetroot* (*Beta vulgaris*) leaf extract (100%, 75%, 50%, and 25%) produced no significant antibacterial effect, with uniform zones of inhibition measuring 6 mm observed across all three replicates. This indicates that the beetroot leaf extract did not exhibit antibacterial activity against *Escherichia coli* under the conditions tested. Based on the Clinical and Laboratory Standards Institute (CLSI) interpretive criteria, a zone of inhibition of less than 12 mm is categorized as resistant, 13–17 mm as intermediate, and greater than 16 mm as susceptible. Accordingly, all observed values fall within the resistant category, indicating no detectable inhibitory effect of the extract against *E. coli*. These findings suggest that, under the specific experimental conditions employed—including extract concentrations, exposure time, incubation temperature, and diffusion method—the beetroot leaf extract did not inhibit bacterial growth. Therefore, it can be concluded that *Beta vulgaris* leaf extract, in the forms and concentrations used in this study, does not demonstrate effective antibacterial activity against *E. coli*.

According to the Clinical and Laboratory Standards Institute (CLSI), a zone of inhibition with a diameter of less than 12 mm fell into the resistant category, 13–17 mm was considered intermediate, and greater than 16 mm was classified as susceptible. Based on these criteria, the beetroot leaf extract falls into the resistant category and did not exhibit any clear antibacterial effect against E. coli. In simpler terms, using the specific setup of this study including the concentration of extract applied, duration of bacterial exposure, temperature, and the method used the extract did not prevent the growth of the bacteria. Based on these observations, it could be concluded that, at least in the form and concentrations used in this experiment, beetroot leaf extract did not appear to have effective antibacterial properties against *E. coli.*

Table 3 Zones of Inhibition of the Control Agents Against E. coli Using Kirby-Bauer Test



Concentration	**Replicates**		
	1	2	3
Chloramphenicol	18.3mm	19.6mm	12.4mm
Chloramphenicol	21.8mm	28.5mm	30mm
Distilled Water	6mm	6mm	6mm
Distilled Water	6mm	6mm	6mm

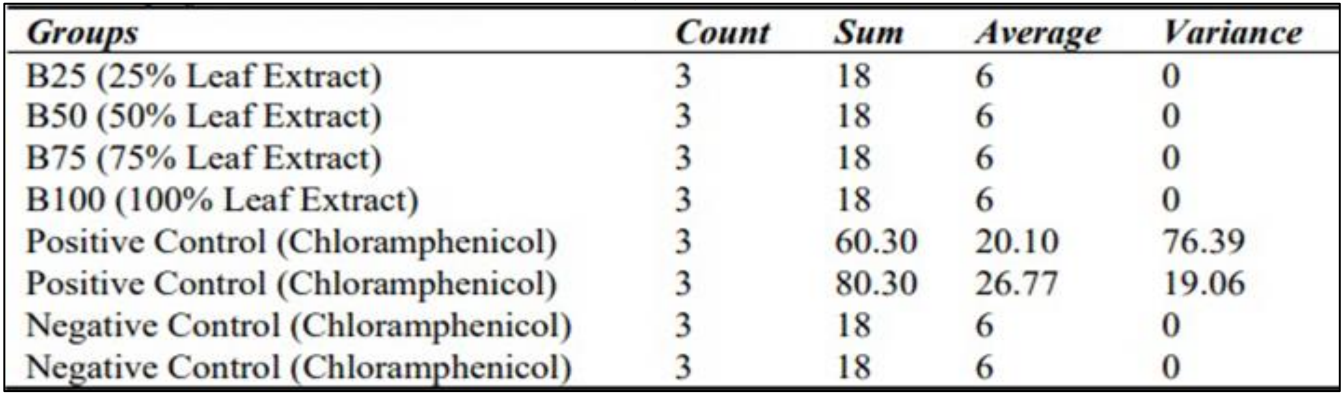
In the process of applying antibacterial susceptibility testing, control agents played a crucial role and were vital for validating the accuracy and reliability of the results. According to CLSI guidelines, a zone of inhibition with a diameter of less than 12 mm was classified as resistant, 13–17 mm as intermediate, and greater than 16 mm as susceptible. Based on these criteria, the positive control confirmed the reliability of the test conditions and the efficacy of the antibiotic used. The Kirby-Bauer disk diffusion method, which was employed in this study, is a widely accepted technique for assessing the effectiveness of antimicrobial agents against specific bacterial strains.

In the experimental treatments, beetroot extract at varying concentrations was tested alongside chloramphenicol (denoted as “C”) as the positive control, and distilled water (DW) as the negative control, to evaluate their efficacy against the test microorganism, Escherichia coli (E. coli). Effectiveness was determined by measuring zones of inhibition, which represent areas where bacterial growth was suppressed. According to Table 4.3, chloramphenicol demonstrated a clear inhibitory effect against E. coli, with measurable zones of inhibition recorded in all replicates. In the first set of replicates using chloramphenicol, the zones of inhibition were 18.3 mm, 29.6 mm, and 12.4 mm. The second set yielded slightly higher and more consistent results, with zones measuring 21.8 mm, 28.5 mm, and 30 mm. These results confirmed that chloramphenicol was effective in inhibiting E. coli growth. However, the variation in inhibition zone diameters, particularly in the first set, suggests a degree of inconsistency in the antibacterial response. This variability might be attributed to several experimental factors. One possible explanation was variation in the application technique, such as uneven distribution of the antibiotic or inconsistencies in the amount applied. Additionally, differences in the thickness or composition of the Mueller-Hinton agar used in the Kirby-Bauer test could have influenced the diffusion rate of the antibiotic, thus affecting the zone sizes. The density of the bacterial lawn, if not applied uniformly, might also impact results, as a denser bacterial field might require more antibiotics to produce a comparable inhibitory effect. Furthermore, external factors such as incubation time, temperature, or humidity might be contributed to the observed variability.

In contrast, the distilled water control showed no zones of inhibition across all replicates, each recording a diameter of 6 mm. This outcome was expected, as distilled water lacks antimicrobial properties. Its use as a negative control was essential in demonstrating that the inhibition observed was solely due to chloramphenicol and not due to any extraneous effects. In conclusion, the data from the Kirby-Bauer test confirmed the antibacterial efficacy of chloramphenicol against E. coli, despite some inconsistencies likely caused by technical or environmental factors. The observed inhibition still placed it within the susceptible category according to CLSI guidelines. The absence of any inhibition by distilled water supported the validity of the results, reinforcing that the antibacterial effects were indeed due to chloramphenicol. For future studies, ensuring strict consistency in methodology and environmental conditions is vital to reduce variability and enhance the reliability of experimental outcomes.

- *Results of the Statistical Treatment Data (ONE-WAY ANOVA)*

Table 4 Summary of the Treatment Data



Groups	Count	Sum	Average	Variance
B25 (25% Leaf Extract)	3	18	6	0
B50 (50% Leaf Extract)	3	18	6	0
B75 (75% Leaf Extract)	3	18	6	0
B100 (100% Leaf Extract)	3	18	6	0
Positive Control (Chloramphenicol)	3	60.30	20.10	76.39
Positive Control (Chloramphenicol)	3	80.30	26.77	19.06
Negative Control (Chloramphenicol)	3	18	6	0
Negative Control (Chloramphenicol)	3	18	6	0

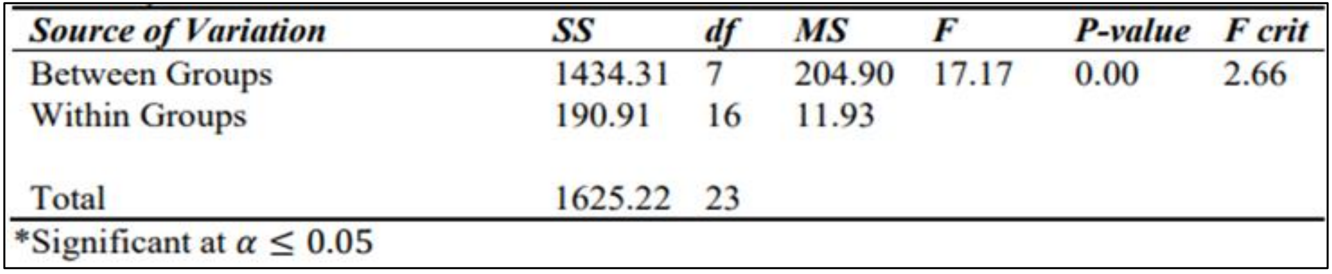
The results from the table indicate that the antibacterial activity of *Beta vulgaris* leaf extract against *Escherichia coli* remained consistent across all tested concentrations (25%, 50%, 75%, and 100%), each producing an average zone of inhibition of 6 mm with no observed variance. This suggests that increasing the concentration of the extract did not enhance its inhibitory effect on *E. coli*, indicating that its antibacterial activity remained minimal and unaffected by concentration changes under the conditions of this study.

Notably, the negative control yielded identical results, reinforcing that the observed measurements in the extract groups did not reflect any meaningful antibacterial activity beyond baseline conditions.

In contrast, the positive control, chloramphenicol, demonstrated significantly higher antibacterial activity, with mean zones of inhibition of 20.10 mm and 26.77 mm across separate measurements. This marked difference highlights the strong inhibitory effect of chloramphenicol against *E. coli* compared to the beetroot leaf extract. The recorded variance values for chloramphenicol (76.39 and 19.06) suggest some variability in its observed effectiveness, which may be attributed to experimental conditions or differences in bacterial response.

Overall, the findings demonstrate that *Beta vulgaris* leaf extract exhibited minimal antibacterial activity against *E. coli* when compared to the positive control. These results indicate that, under the conditions of this experiment, beetroot leaf extract is not an effective antibacterial agent against *E. coli*.

Table 5 One-Way ANOVA Results



Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1434.31	7	204.90	17.17	0.00	2.66
Within Groups	190.91	16	11.93			
Total	1625.22	23				

*Significant at $\alpha \leq 0.05$

The results of the One-Way Analysis of Variance (ANOVA) indicated that differences in antibacterial activity among the treatment groups of *Beta vulgaris* leaf extract against *Escherichia coli* were statistically significant. The total variation in the data was partitioned into Between Groups (1434.31) and Within Groups (190.91), with corresponding mean square values of 204.90 and 11.93, respectively.

The computed F-value of 17.17 exceeded the critical F-value of 2.66, while the obtained p-value of 0.00 was below the significance level ($\alpha \leq 0.05$). Since the F-value surpassed the critical threshold and the p-value indicated statistical significance, the null hypothesis (H_0) was rejected. This suggests that there were statistically significant differences among the treatment groups.

However, despite the statistical significance observed in the ANOVA results, further interpretation of the raw experimental data suggests that this significance may be largely attributed to the strong effect of the positive control (chloramphenicol), rather than the antibacterial activity of the beetroot leaf extract itself. All concentrations of beetroot extract consistently produced a zone of inhibition measuring 6 mm, which falls under the resistant category based on CLSI standards, indicating no meaningful antibacterial effect against *E. coli*.

Thus, while ANOVA results indicate significant variation among groups, practical interpretation reveals that this variation is driven primarily by the effectiveness of the positive control rather than any measurable antibacterial activity from *Beta vulgaris* leaf extract. Therefore, under the conditions of this study, the extract did not demonstrate effective antibacterial properties against *E. coli*.

IV. CONCLUSIONS

The concentrations of *Beta vulgaris* (beetroot) leaf extract tested against *Escherichia coli* did not exhibit any antibacterial activity. All tested concentrations (100%, 75%, 50%, and 25%) produced uniform zones of inhibition measuring 6 mm, which fall within the resistant category as defined by CLSI standards.

The absence of antibacterial activity across all concentrations suggests that the bioactive compounds present in the beetroot leaf extract, at the levels tested, were insufficient to disrupt bacterial cell wall integrity, interfere with essential metabolic processes, or inhibit bacterial replication.

Therefore, further studies are recommended to explore alternative extraction techniques, higher concentrations, or different microbial targets to better evaluate the potential antibacterial properties of beetroot leaf extract. In conclusion, *Beta vulgaris* leaf extract, under the conditions of this study, demonstrated no observable antibacterial activity against *Escherichia coli*.

RECOMMENDATIONS

According to the results and experimentation conducted throughout the test, the specimen extract did not demonstrate a significant impact on the elimination of the Gram-negative bacterium *E.coli*.

As researchers, gathered data and identified methods that could be explored for further research of this very subject.

- Explore the inoculation of different species of microorganisms, including other Gram-negative and Gram-positive bacteria. 76
- Consider extending the incubation period to 48–72 hours to observe any delayed antimicrobial effects.
- Conduct synergistic studies by combining *Beta vulgaris* leaf extract with another medicinal plant extract.
- Use alternative extraction methods and solvents, e.g., Soxhlet extraction, ultrasonic-assisted extraction, or maceration with methanol, acetone, or water.
- Investigate different drying techniques for beetroot leaves, e.g., freeze-drying and oven-drying at controlled temperatures.
- Pursue expanded research approaches, including in vivo studies, clinical trials, and enhanced experimental methods.

Implementing these recommendations may lead to more reliable and meaningful outcomes, ultimately contributing to a deeper understanding of the antimicrobial potential of *Beta vulgaris* leaf extract and its possible applications in future therapeutic developments.

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