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Research Article

Extraction and Activity Test of Antibacterial Compounds from Limberry Leaves (*Triphasia Trifolia*) Against *Vibrio* Sp.

Novita Herdiana*, Yosua Musada Sagala, Samsul Rizal, and Maria Erna Kustyawati

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Abstract
Limberry *(Triphasia trifolia)* is one of traditional medicinal plants whereas its leaves are known for various uses, one of which is as an antibacterial agent. The most common bacteria found in marine waters is *Vibrio sp.* This work seeks to distinguish antibacterial compounds inside the ethanol extract of limberry leaves and assess its repressive effect on *Vibrio sp.* The research was conducted with three repetitions and five different concentration levels: D1 (5%), D2 (10%), D3 (15%), D4 (20%), and D5 (25%). The observational data were analyzed using a completely randomized design (CRD) analysis of variance, followed by the least significant difference (LSD) test at a 5% significance level. Phytochemical screening revealed that the ethanol extract of limberry leaves contained antibacterial compounds namely flavonoids, tannins, steroids, alkaloids, and phenolics. UV-Vis spectrophotometry analysis indicated that the ethanol extract of limberry leaves had absorption at wavelengths of 202, 211, 228, 253, 270, and 323 nm. FTIR analysis showed that the ethanol extract of limberry leaves contained some functional groups, i.e., O–H, C–H alkanes, C–H alkenes C≡N, C=O, C=C, C–O esters, and C–O ethers. The inhibitory test also demonstrated that the ethanol extract of limberry leaves could inhibit the growth of *Vibrio sp.,* with inhibition zone diameters formed at each concentration of 5%, 10%, 15%, 20%, and 25% being 7.280, 7.640, 7.713, 8.027, and 8.070 mm, respectively.

Keywords: inhibitory, limberry, leaf extract, Vibrio sp

1. INTRODUCTION

The limberry (*Triphasia trifolia*) is one of the several types of shrub plants with stems less than 5 cm tall, cylindrical, vertical, and has thorns on its surface. Its fruit is round with smooth and thin red skin [1]. This plant is known for its benefits as a traditional medicine for cough and diarrhea. Additionally, limberry is often used in beauty treatments, such as nail care, and also functions as an ornamental plant [2]. In addition to being an ornamental plant, limberry is one of several plant groups that have various uses, one of which is as an antibacterial agent. As noted in the previous research [3], it is known that the extracts of the stems and leaves of the limberry contain secondary metabolites that can be used as natural antibacterial agents. The study also revealed that the leaf and stem extracts of limberry have high antibacterial activity against *M. luteus* bacteria. In another experiment, it was mentioned that the essential oil from limberry leaves has inhibitory capabilities against *Escherichia coli* bacteria [4].

In contrast, *Vibrio* sp. is a pathogenic bacterium commonly found in marine waters. *Vibrio* sp. is a bacterium that is more resistant to extreme temperatures compared to *E. coli*, and it can optimally develop at higher temperatures [5]. *Vibrio* sp. also can survive at lower pH levels compared to *E. coli* and can cause more severe symptoms, such as wound infections and septicemia. The *Vibrio* sp. bacteria group that often infects humans includes *Vibrio parahaemolyticus* and *Vibrio cholera*, which cause health disorders such as vibriosis [6]. Therefore, infections by pathogenic *Vibrio* sp. bacteria must be handled properly, and one of the most common methods is the use of antibiotics.

Antibiotics are types of synthetic or natural compounds that can inhibit or halt the biochemical reactions of a living organism, particularly during bacterial infections [7]. However, inaccurate and continuous use of antibiotics can lead to health problems. According to the previous works regarding general guidelines for antibiotic use, it is mentioned that the high frequency of antibiotic use can lead to various health complications, especially refusal of antibiotics by bacteria [8][9]. Thus, alternatives are required to minimize the usage of antibiotics in handling *Vibrio* sp. bacterial infections. One alternative that can be used to

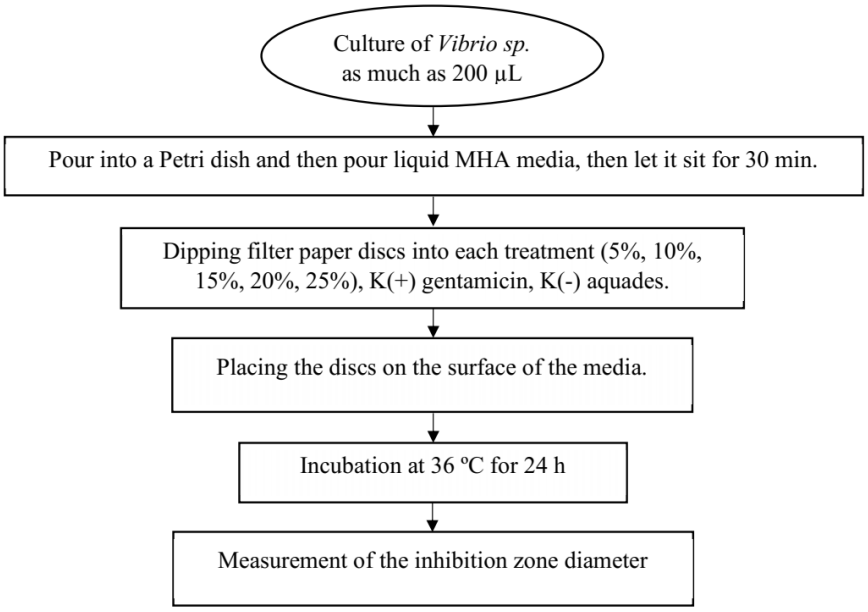

Figure 1. Flow diagram of bacterial suspension.

prevent *Vibrio* sp. bacteria is utilizing the secondary metabolites incorporated ethanol extract within limberry leaves.

The choice of solvent used in the extraction process certainly affects the secondary metabolite content produced. The variation in solvent polarity will affect the amount of extract and the composition of the phytochemicals produced [10]. The use of ethanol solvent in the extraction process can be an optimal choice because ethanol solvent has effective absorption capabilities and maximum extraction power, allowing it to filter non-polar, semi-polar, and polar compounds [10]. The use of antibacterial agents from ethanol extract of limberry leaves (*T. trifolia*) has positive environmental impacts. This is due to its natural properties and biodegradability, which prevent harmful residues from being left in the environment. The utilization of this plant as an antibacterial agent also has the potential to promote conservation and enhance appreciation for biodiversity [11]. Additionally, from an economic perspective, the use of natural antibacterial agents provides positive benefits to the fisheries sector, as its application can reduce fish mortality rates caused by *Vibrio* sp. infections, thus increasing fish farmers' productivity [12]. The research aims to determine the antibacterial compounds present in the ethanol extract of limberry leaves and to ascertain the inhibitory power of the ethanol extract of klimberry leaves as a natural antibacterial in inhibiting the growth of *Vibrio sp.*

2. MATERIALS AND METHODS

2.1. Materials

Ingredients utilized in this observation included limberry leaves obtained from Bandar Lampung, cultures obtained from the Lampung Veterinary Center, *Vibrio* sp., 70% alcohol, 96% ethanol, distilled water, NaCl, and Mullen Hilton Agar (MHA). The tools used in this study consisted of ovens, pans, analytical balances, desiccatators, porcelain dishes, clamping scissors, choppers, grinders, jars, ose needles, disc paper (6 mm), vacuum rotary evaporator, 36 ºC incubator, hotplate, densicheck, test tube, test tube rack, beaker glass, petri dish, vortex, lab shaker, filter paper, aluminum foil, measuring cup, bunsen, autoclave, micropipette, drip pipette, erlenmeyer, glass bottles, tweezers, and calipers.

2.2. Methods

This study was designed using a completely randomized design (CRD) with a single factor and three repetitions. The test factor is the concentration of limberry leaf extract, consisting of five concentration levels: 5%, 10%, 15%, 20%, and 25% (v/v). After collecting the data, Bartlett's test was used to check the homogeneity of variance, and

Tukey's test was applied to assess data additivity. The data from the previous tests were then analyzed for variance to determine the effect between treatments, followed by further testing using the least significant difference (LSD) test at a 5% significance level.

2.2.1. Sample Preparation and Extract Making

Sample Preparation

Sample preparation is carried out by preparing 2 kg of limberry leaves, then continuing the sorting process to get good-quality limberry leaves. After that, the moisture content of fresh limberry leaves is calculated. Additionally, the limberry leaves are air-dried for 8 h and then oven-dried at 50 °C for 24 h. After drying, orange leaves are reduced or mashed using a chopper and powder grinder. After obtaining a fine powder of leaves, the moisture content is calculated. The fine powder of limberry leaves is ready to macerate.

2.2.2. Limberry Leaf Extraction

Limberry leaf extract is obtained using the maceration method. A total of 300 g of limberry leaf powder were weighed and then dissolved using a 500 mL of 96% ethanol solvent. After that, it is stored by placing the container containing the extract on the shaker tool (120 rpm). The maceration process lasts for 3 × 24 h with three screenings. After the filtering process, the extract evaporation stage continues using a vacuum rotary evavorator at a temperature of 30 °C to produce limberry leaf extract. After the extract was obtained, the moisture content of the limberry leaf extract was calculated.

2.2.3. Preparation of Test Bacterial Suspension

Bacterial Rejuvenation

Bacterial rejuvenation is carried out using MHA media. A total of 2 ose of pure cultures of *Vibrio* sp. were taken using sterile ose needles, then etched zigzaggingly MHA media evenly, and then incubated at 36 °C for 24 h.

2.2.4. Preparation of Test Suspension

The manufacture of the test bacterial suspension was carried out by taking a colony of *Vibrio* sp. bacteria, which has been rejuvenated on MHA media as much as 3 ose, then dissolved into NaCl as much as 5 mL aseptically and vortex for 30 s. After that, measure the turbidity level obtained using a densichek tool with a standard of 0.5 McFarland. If the suspension of the test bacteria is too turbid, then the addition of a NaCl solution is carried out. If the suspension of the test bacteria is less turbid, then the addition of some rejuvenated bacterial ose is carried out. Test bacterial suspensions that have complied with the McFarland 0.5 standard are then used for antibacterial activity tests.

2.2.5. Phytochemical Screening

To test for phenolic compounds, 1 mL of the sample extract is placed in a test tube, and 1% $FeCl_3$ reagent is added. A black color indicates the presence of phenolic compounds [2]. To test the flavonoid with Wilstater reagent, 1 mL of the extract is mixed with a few drops of concentrated HCl and a small amount of powdered magnesium. The appearance of color yellow indicates a positive reaction [2]. To test the alkaloid with Mayer's reagent, 1 mL of the extract is combined with 2 drops of Mayer's reagent solution. An accumulation of thick and white or yellow residue is a strong indication the sample reacts positively [2]. To test

Table 1. Phytochemical screening test results.

No	Types of Phytochemical Qualitative Tests	Phytochemical Test Results	Description
1	Saponin	-	Negative
2	Steroid	+++	Strong positive
3	Terpenoid	-	Negative
4	Tanin	+++	Strong positive
5	Alkaloid	+	Positive
6	Flavonoid	+++	Strong positive
7	Phenolic	+++	Strong positive

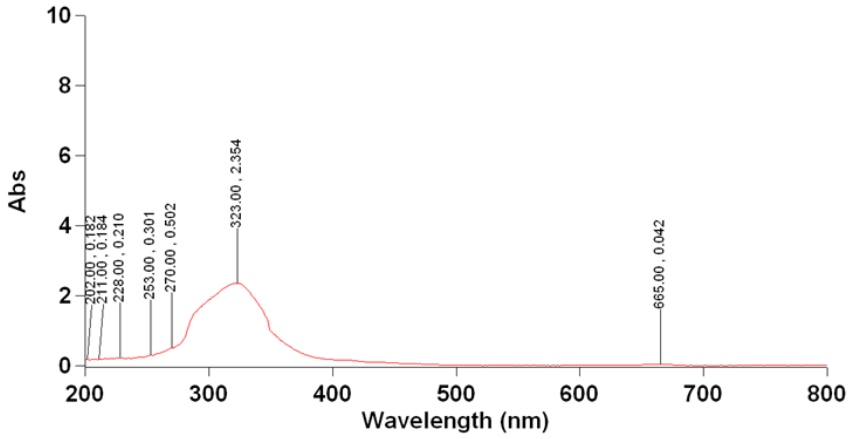

Figure 2. UV-Vis spectrum of limberry leaf ethanol extract.

saponins, the sample is first heated to boiling in a water bath with 20 mL of water. The filtrate is then shaken and left to sit for 15 min. The presence of stable foam indicates a positive result for saponins [2]. To test steroids or terpenoids, anhydrous acetate and concentrated H_2SO_4 were added to the sample. A green-blue discoloration indicates the presence of steroids, while a red-purple color change signifies the presence of triterpenoids [2]. To test tannin, the sample was boiled with 20 mL of water then it went through a filtration. After filtering, a few drops of 1% $FeCl_3$ are added to the filtrate. A greenish-brown or blue-black color is an indication of tannins' presence [2].

2.2.6. UV-Vis Spectrophotometer Analysis

Analysis of limberry leaf extract using a UV-Vis spectrophotometer begins with the preparation of blanks and samples. Then the blank absorbance measurement is carried out at a wavelength of 200–800 nm, and the blank absorbance value will be used as a control or as data representing the solvent. After that, absorbance measurements are carried out on samples with the same wavelength (200–800 nm). After that, identification and analysis of the data obtained are carried out.

2.2.7. FTIR Analysis

Fourier transform infrared (FTIR) is a good analytical technique for identifying and structurally analyzing chemical compounds. The sample is placed on the ATR plate, and then the ATR lever is rotated until pressing the sample on the ATR plate. Background measurements are carried out every scanning, and it has been connected to a computer that has been equipped with OPUS software used to control the work of the spectrophotometer in the range of 4000-650 cm^{-1} with a resolution of 16 cm^{-1} and scans 32 times.

2.2.8. Antibacterial Activity Test

This study used five concentration levels: D1 (5%), D2 (10%), D3 (15%), D4 (20%), and D5 (25%). The selection of these concentrations is used to evaluate the dose effects of ethanol extract of limberry leaves on the inhibition of *Vibrio* sp. bacteria, as well as to identify the minimum effective concentration and its potential toxicity. Test the antibacterial activity of limberry leaf extract using a culture of *Vibrio* sp. bacteria. First, test the inhibitory power of 96% ethanol extract, Gentamicin antibiotics as a positive control, and distilled water as a negative control. Testing is carried out by means of bacterial culture (*Vibrio* sp.). A total of 200 µL is poured into a sterile petri dish that has been prepared, and then MHA media liquid is poured into a petri dish that contains test bacteria. The petri dish is then flattened by gently shaking the petri dish on the surface of the workbench and let sit for 30 min. After that, the disc paper was dipped in each concentration of limberry leaf extract. Disc paper that has been dipped in each treatment (5%, 10%, 15%, 20%, and 25%) is then placed on the surface of 3 discs of media. Petri dishes that have been given disc paper are then incubated at 36 °C for 24 h). After the incubation period, the formed clear zone diameter is

measured utilizing a digital caliper. The flow diagram of the antibacterial testing process is presented in Figure 1.

3. RESULTS AND DISCUSSIONS

3.1. Phytochemical Screening

Phytochemical screening is a qualitative test conducted to determine the group of active compounds or secondary metabolites contained in the ethanol extract of limberry leaves. The groups of compounds tested include saponins, steroids, terpenoids, tannins, alkaloids, flavonoids, and phenolics. The results of the phytochemical screening analysis are shown in Table 1.

Based on Table 1, it is known that the ethanol extract of limberry leaves contains active compounds such as steroids, tannins, alkaloids, flavonoids, and phenolics. In the active steroid compound test, the addition of glacial acetic acid and H_2SO_4 to the ethanol extract of limberry leaves showed a color change to green, indicating the presence of steroids in the sample. Positive results for tannin tests were obtained by reacting the sample with $FeCl_3$ reagent. The $FeCl_3$ reagent interacts with one of the hydroxyl groups present in tannins, resulting in a color change to bluish-black. Condensed tannins can cause a color change to blackish blue [13].

In the alkaloid test, positive results were obtained by adding chloroform and Mayer's reagent to the extract. The alkaloid test principle relies on a precipitation reaction caused by metal replacement. In Mayer's test for alkaloids, the indication of a positive reaction is the formation of a white precipitate. This precipitate is a potassium-alkaloid complex formed from the reaction between the nitrogen compounds in alkaloids and the K^+ ions from potassium tetraiodomercurate(II) or Mayer's reagent [2]. In the flavonoid test, the sample experienced a color change after being reacted with magnesium powder and concentrated HCl, resulting in a color change of the solution to red. As claimed, adding magnesium powder and HCl into the flavonoid test leads to flavonoid compounds reduction in the sample, producing a reddish color response which indicates the presence of flavonoids [14].

The phenolic test also yielded positive results, demonstrated by the sample turning blackish-blue after reacting with a 2% $FeCl_3$ solution. Meanwhile, different results were obtained in the saponin and terpenoid compound tests. The saponin test yielded negative results because no foam was produced after reacting distilled water with the sample. Similarly, the terpenoid test also yielded negative results when reacting with glacial acetic acid and H_2SO_4 did not show any color change in the sample, indicating that the ethanol extract of limberry leaves tested negative for terpenoids.

3.2. UV-Vis Spectrophotometer

The results of the UV-Vis spectrophotometry analysis of the limberry leaf ethanol extract are presented in Figure 2. Based on Figure 2, the absorption wave patterns observed indicate the

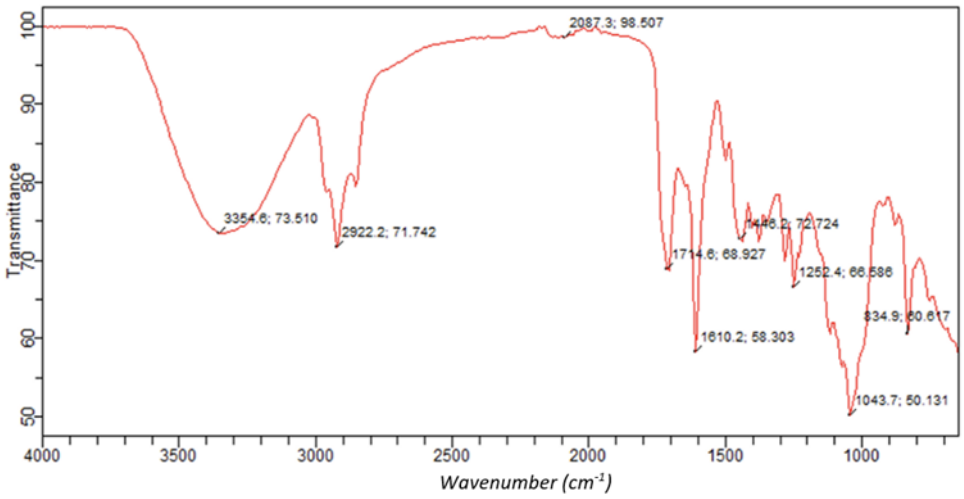

Figure 3. FTIR spectrum of ethanol extract of leaf limberry.

Table 2. Functional group of limberry leaf ethanol extract.

Functional Group	Functional Group (cm^{-1})	
	Extract	Literature
O–H	3354.6	3200–3600
C–H alkane	2922.2	2850–2970
C≡N	2087.3	2000–2300
C=O	1714.6	1690–1760
C=C	1610.2	1610–1680
C–H alkane	1446.2	1340–1470
C–O ester	1252.4	1050–1300
C–O eter	1043.7	1000–1300
C–H alkene	834.9	675–995

Source : Nandianto et al. [16]

presence of specific compounds in the sample. The spectrum that appears at wavelengths of 202, 211, 228, 253, 270, and 323 nm shows maximum absorption, indicating the presence of conjugated double bonds in the sample. This occurs because chromophores (the pigment parts most sensitive to light stimuli) transition from π to π when the conjugated system absorbs light at λ_{max} > 200 nm. Meanwhile, when the conjugated system absorbs light at λ_{max} > 300 nm, the chromophore transitions from n to π [15]. This indicates that the absorption patterns observed in this analysis suggest the presence of conjugated double bonds in the sample.

3.3. FTIR Analysis

FTIR analysis was performed on the ethanol extract of limberry leaves to identify the functional groups present in the extract. FTIR analysis of the ethanol extract of limberry leaves results are shown in Figure 3 and detailed in Table 2.

Based on FTIR analysis, the signal that appears at wavenumber 3354.6 cm^{-1} is the vibration of the O –H group. This group comes from flavonoids, tannins, terpenoids, saponins, and polyphenols [17]. The signal at 2992.2 cm^{-1} is the C–H vibration of alkane compounds, and the signal at 2087.3 cm^{-1} is a C≡N group. Absorption at 1714.6 cm^{-1} indicates the presence of a C=O group, and signal at 1610.2 cm^{-1} a spectrum is obtained, which is a C=O group, and at 1446.2 cm^{-1} a vibration of the CH_3 group is obtained. Vibrations at 1252 and 1043 cm^{-1} are vibrations of C–O ester and ether, and vibrations at 834.9 cm^{-1} indicate the presence of C–H groups. These absorptions are characteristic for flavonoid compounds, namely the presence of O–H, C–H, C=O, and C–O groups [18].

3.4. Antibacterial Activity Test

An antibacterial activity test of the ethanol extract of limberry leaves against *Vibrio* sp. bacteria carried out at concentrations of 5%, 10%, 15%, 20%, and 25% with gentamicin antibiotics as a positive control produced a clear zone while distilled water as a negative control did not produce a clear zone. The clear zone forms after incubating for 24 h at 36 °C. The formed clear zones are visible in Figure 4.

Based on the inhibition test of the ethanol extract of limberry leaves against *Vibrio* sp. bacteria, it is known that each concentration has an inhibitory effect. The concentrations of the ethanol extract of limberry leaves used were D1 (5%), D2 (10%), D3 (15%), D4 (20%), D5 (25%), as well as gentamicin (positive control) and distilled water (negative control). The inhibition measurement results were obtained by measuring the clear zone that appeared on the side of the formed disc paper, then averaging all repetitions and performing the LSD test at a 5% significance level. The average diameter of the inhibition zones formed and the LSD test results at a 5% significance level for the ethanol extract of limberry leaves can be seen in Table 3.

Based on the analysis results of the ethanol extract of limberry leaves in Table 3, it is known

that there are significant differences in each treatment. According to the results of the 5% LSD test, it is known that treatment D1 has a different notation from all treatments, indicating that treatment D1 is significantly different from all treatments, namely D2, D3, D4, and D5. Treatments D2 and D3 have the same notation, indicating that treatments D2 and D3 are not significantly different in the diameter of the inhibition zone formed by the ethanol extract of limberry leaves but are significantly different from treatments D1, D4, and D5. The subsequent treatments, D4 and D5, also have the same notation, meaning that treatments D4 and D5 are not significantly different in the diameter of the inhibition zone formed but are significantly different from treatments D1, D2, and D3.

The average measurement results of the inhibition zone for the ethanol extract from limberry leaves reveal that the diameter of the inhibition zone increases in direct proportion to the concentration used. In other words, a higher concentration results in a larger inhibition zone diameter. The diameter of the inhibition zone measurements showed that treatment D1 (5%) as the lowest concentration had the smallest inhibition zone diameter of 7.280 mm. In treatment D2, the inhibition zone diameter formed was 7.640 mm, in treatment D3, the inhibition zone diameter formed was 7.713 mm, in treatment D4, the inhibition zone diameter formed was 8.027 mm, and in treatment D5, which was the highest concentration, the average largest inhibition zone diameter formed was 8.070 mm. Based on the average diameter of the inhibition zone obtained, all treatments of ethanol extract from limberry leaves have a moderate inhibition category. This finding aligns with the previous observation [19], which noted that in the agar diffusion test, a clear zone diameter of less than 5 mm is classified as weak inhibition, 5–10 mm as moderate, 10–19 mm as strong, and greater than 20 mm as very strong.

Accordant with the inhibition zone test conducted using ethanol extract of limberry leaves and gentamicin as a positive control, it is known that the inhibition zones formed by the two have significant differences. The inhibition zone formed by gentamicin was 15.34 mm, whereas the inhibition zone formed by the limberry leaf extract at concentration D (5) 25% was 8.070 mm. This is due to the stronger bacteriostatic nature of gentamicin. Gentamicin is an antibiotic belonging to the aminoglycoside group (antibiotics for bacterial infections by gram-negative bacteria). This antibiotic is effective in inhibiting bacterial growth, including *Vibrio* sp. bacteria, which often cause infections. Its mechanism of action mainly involves inhibiting bacterial protein synthesis, thus stopping bacterial growth and reproduction. In addition, gentamicin is known to have advantages over other antibiotics in antibacterial tests against *Vibrio* sp. because it has a broad spectrum effective against various types of bacteria, including Gram-negative bacteria. Gentamicin also shows a relatively low resistance level against many strains of *Vibrio* sp. [20].

Entrenched in the analysis, it is determined that the ethanol extract of limberry leaves contains

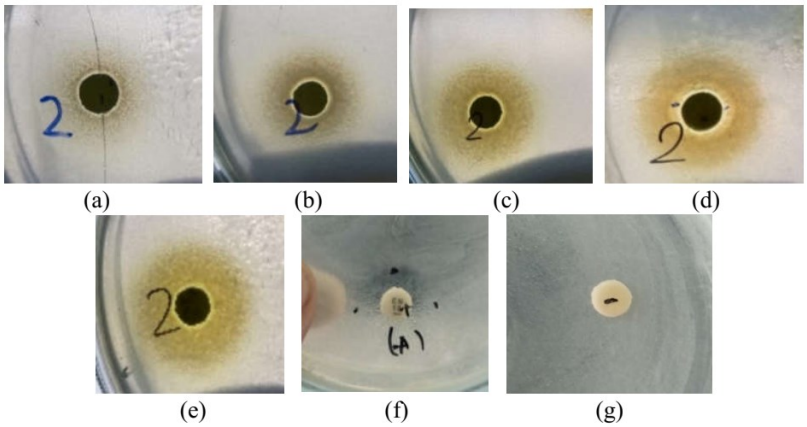

Figure 4. Clear zone diameter against *Vibrio* sp. by the addition of (a) 5%, (b) 10%, (c) 15%, (d) 20%, and (e) 25% leaf ethanol extract, (f) Gentamicin and (g) distilled water.

Table 3. Results of the inhibition zone diameter and LSD test at 1% and 5% significance levels for the ethanol extract of limberry leaves against *Vibrio* sp.

Treatment	Inhibition zone diameter ± sd (mm)	Category
D1	7.280± 0.0200[a]	Medium
D2	7.640± 0.2007[b]	Medium
D3	7.713± 0.0757[b]	Medium
D4	8.027± 0.0611[c]	Medium
D5	8.070± 0.0557[c]	Medium
K0(+)	15.340	Strong
K0(-)	-	-

Note: Numbers followed by the same letter indicate no significant difference in the LSD test at the 5% significance level (LSD 0.05 = 0.1879). D1 = 5%, D2 = 10%, D3 = 15%, D4 = 20%, D5 = 25% concentration of ethanol extract of limberry leaves, K0(+) = Gentamicin (Positive control); K0(-) = Distilled water (Negative control).

antibacterial compounds, including steroids, tannins, alkaloids, flavonoids, and phenolics. These antibacterial compounds are known to prevent bacterial growth through various mechanisms. The antibacterial mechanism of steroids is closely linked to their interaction with bacterial lipid membranes. Steroids can interact with phospholipids in the bacterial cell membrane, allowing lipophilic compounds to enter the cell. As a result, this causes a collapse in membrane integrity, disrupting its normal function, and ultimately leading to bacterial cell lysis. This process occurs due to the sensitivity of bacteria to steroid components, resulting in bacterial liposome leakage, changing the cell membrane morphology to become more fragile [21].

The mechanism of action of tannins as antibacterial agents involves several stages. Tannins act by blocking the assembly of bacterial cell walls, which consist of polypeptides, causing the cell wall to become incomplete. Consequently, the integrity of the bacterial cell is disrupted, and it may undergo lysis, leading to bacterial cell death. Additionally, tannins can inactivate bacterial enzymes and inhibit protein processes within bacterial cells [22]. Alkaloid compounds also work as antibacterial agents by inhibiting peptidoglycan components that make up the bacterial cell wall. Peptidoglycan is an essential component that provides structural strength to the bacterial cell wall. Disruption of peptidoglycan by alkaloids results in an improperly formed cell wall, leading to structural weakness in the bacterial cell and eventually causing cell death [23].

Flavonoids function as antibacterial agents by disrupting bacterial cell membrane integrity and interfering with energy metabolism. Specifically, flavonoids form complexes with extracellular proteins, resulting in damage to the bacterial cell membrane, which ultimately allows intracellular bacterial compounds to leak out of the cell. Additionally, flavonoids inhibit the use of oxygen by bacteria, which is part of the inhibition of bacterial energy metabolism [21]. Phenolic compounds also exhibit antibacterial effects by damaging bacterial membranes. They achieve this by inhibiting the synthesis of the bacterial membrane lipid bilayer and disrupting membrane permeability, which ultimately leads to bacterial cell damage [24].

Based on the research and previous studies, it is established that the ethanol extract of limberry leaves has inhibitory effects against both Gram-positive and Gram-negative bacteria. Previous studies described that the ethanol extract of limberry leaves can inhibit the growth of *Salmonella* sp. and *Staphylococcus aureus* bacteria [25][26]. The inhibition zones formed in the antibacterial activity test against *Vibrio* sp. and *Salmonella* sp. as Gram-negative bacteria with the same concentration treatments of 5%, 10%, 15%, 20%, and 25% resulted in inhibition zones of 7.280, 7.640, 7.713, 8.027, and 8.070 mm; and 6.67, 6.87, 7.43, 7.50, and 7.93 mm, respectively. In the inhibition zone test against *S. aureus* as a Gram-positive bacterium with the same concentration

treatments of 5%, 10%, 15%, 20%, and 25%, the inhibition zones were 6.70, 6.87, 8.33, 9.21, and 10.36 mm, respectively. Based on the three tests, the highest concentration treatment produced the largest inhibition zone. Additionally, the extract of limberry leaves demonstrated a more effective inhibitory effect against Gram-positive bacteria compared to Gram-negative bacteria.

Herdiana et al., was also found that the extract of limberry fruit has significant antibacterial potential against *S. aureus* [27]. The results indicate that the increase in extract concentration is directly proportional to the resulting inhibition zone. The lowest concentration (5%) showed an inhibition zone of 6.52 mm, while the highest concentration (25%) reached 11.94 mm. These findings indicate that the active compounds contained in limberry can play an important role in controlling pathogenic bacteria.

4. CONCLUSIONS

Based on the results of phytochemical screening, it is known that the ethanol extract of limberry leaves (*T. trifolia*) contains antibacterial compounds such as steroids, tannins, alkaloids, flavonoids, and phenolics. The ethanol extract of limberry leaves shows natural antibacterial activity in inhibiting the growth of *Vibrio sp.*, with the diameters of the inhibition zones formed at concentrations D1 (5%), D2 (10%), D3 (15%), D4 (20%), and D5 (25%) being 7.280, 7.640, 7.713, 8.027, and 8.070 mm, respectively. The results of this study indicate that increasing the concentration of the ethanol extract of limberry leaves correlates positively with the size of the inhibition zone, with the largest zone observed at concentration D5 (25%) measuring 8.070 mm. The implications of these findings indicate the potential of limberry leaf extract as a source of natural antibacterial agents that can be used in the treatment of bacterial infections, particularly those caused by *Vibrio sp.*, which often poses health problems in the community. Given the increasing antibiotic resistance experienced by many pathogenic bacteria today, the use of limberry leaf extract may serve as a promising alternative. For future research, it is recommended to conduct effectiveness tests of this extract under *in vivo* conditions to assess its safety and efficacy in living organisms. Additionally, further exploration of the effects of limberry leaf extract on various other bacterial strains is also necessary to understand the broader antibacterial potential of this plant.

AUTHOR INFORMATION

Corresponding Author

Novita Herdiana — Department of Agricultural Product Technology, University of Lampung, Bandar Lampung-35145 (Indonesia);
orcid.org/0000-0003-1876-6525
Email: novitaherdiana1976@gmail.com

Authors

Yosua Musada Sagala — Department of Agricultural Product Technology, University of Lampung, Bandar Lampung-35145 (Indonesia);
orcid.org/0009-0000-8521-5809

Samsul Rizal — Department of Agricultural Product Technology, University of Lampung, Bandar Lampung-35145 (Indonesia);
orcid.org/0000-0002-6898-4013

Maria Erna Kustyawati — Department of Agricultural Product Technology, University of Lampung, Bandar Lampung-35145 (Indonesia);
orcid.org/0000-0001-7980-1844

Author Contributions

Conceptualization, N. H., Y. M. S., S. R. and M. E. K.; Methodology, N. H., Y. M. S., S. R. and M. E. K.; Software, N. H., Y. M. S., and S. R.; Validation, N. H., Y. M. S., S. R. and M. E. K.; Formal Analysis, N. H., Y. M. S., and S. R.; Investigation, N. H., Y. M. S., and S. R.; Resources, N. H., Y. M. S., and S. R.; Data Curation, N. H., Y. M. S., and S. R.; Writing – Original Draft Preparation, N. H. and Y. M. S.; Writing – Review and Editing, N. H., Y. M. S., S. R. and M. E. K.; Visualization, N. H. and Y. M. S.; Supervision, N. H., Y. M. S., S. R. and M. E. K.; Project Administration, N. H. and Y. M. S.; Funding Acquisition, N. H.

Conflicts of Interest

The authors declare no conflict of interest.

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