Antibacterial activity of betel leaf (*Piper betle* L.) leaves extract on *Vibrio harveyi*

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Abstract. The use of herbs as a natural medicine for disease control in aquaculture is safer than using antibiotics. Antibiotic resistance from microorganisms is a problem that can impact public health. The purpose of this study was to determine the antibacterial ability of betel leaf (*Piper betle* L.) toward the pathogenic bacteria, *Vibrio harveyi* by in vitro. The research design used a completely randomized design. The number of treatments was 12 concentrations of betel leaf extract with three replications for each treatment. The parameters observed were Minimum Inhibition Concentration (MIC) and Minimum Bactericidal Concentration (MBC) from the ethanol extract of betel leaf. The OD value data of the MIC test results were analyzed by Analysis of Variance (ANOVA) and Duncan’s Multiple Range Test. The results proved that betel leaf extract has the potential to be antibacterial with the ability to inhibit the growth of *V. harveyi* at a minimum concentration of 0.19% (OD: 2.057) while at a minimum concentration of 0.39% it can kill it.

1. Introduction

The *V. harveyi* bacteria that cause vibriosis has become a significant problem in shrimp hatcheries [1]. Vibriosis control using antibiotics can cause bacterial resistance problems [2] and can be a significant obstacle in treating diseases caused by bacteria [3]. Abraham *et al.* [4] reported that the *V. harveyi* bacterial strains isolated from sick shrimp were resistant to antibiotics: ampicillin, chlorotetracycline, cephalexin, erythromycin, furazolidone, gentamycin, nalidixic acid, neomycin, nitrofurantoin, nitrofurazone, novobiocin, ofloxacin, oxytetracycline, penicillin G, polymyxin B, rifampicin, streptomycin, sulphasomidine, sulphamethoxazole, and sulphafurazole.

The use of antibiotics that are not appropriate for the target organism will hurt the aquatic environment. Most of these antibiotics settle in the bottom of the pond sludge, contributing to microbial communities’ development in a cultivation environment that is resistant to antibiotics [5]. The use of antimicrobials in cultivation can also produce antimicrobial residues in cultivated products that can endanger public health if consumers are exposed to residues by consuming products containing residues or handling products containing residues [6].

With the development of antibiotic resistance, it is necessary to explore natural materials as an alternative to antimicrobials. According to Angeh [3], herbs have been used in human medicine since centuries ago as antibacterial, anti-inflammatory, cytostatic, antifungal, and antiviral properties. The content of alkaloids, phenolic compounds, diterpenoids, steroids, glycoalkaloids, and other compounds in herbs can inhibit bacterial growth [7]. The use of natural ingredients as traditional medicines has fewer side effects than drugs derived from chemicals; besides, the price is more affordable [8].
Betel (*P. batle* L.) is included in the Peperaceae family and is a type of plant widely used for medicinal purposes. Parts of the betel plant (*P. batle* L.) such as roots, seeds, and leaves have the potential for treatment, but the most frequently used are the leaves [9]. The leaves have an antibacterial effect and can prevent foodborne pathogens [10], because they contain flavonoids and polyphenols [11]. The aroma of betel leaf is caused by the presence of essential oils consisting of phenols and terpenes. The terpenoids include 1, 8-cineole, cadinene, camphene, caryophyllene, limonene, pinene, chavicol, ally pyrocatechol, carvacrol, safolro, eugenol and chavibetol are the main phenols found in betel leaf [10]. The essential oil content of betel leaf is 56.5% and has antibacterial properties [12]. Khan and Kumar [13] reported that methanol and ethanol extracts of betel leaf were effective against the pathogenic bacteria *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*. Ataguba et al. [14] also proved that betel leaf extract with 40% ethanol could inhibit pathogenic bacteria in shrimp and fish, namely: *Vibrio parahaemolyticus*, *Vibrio parahaemolyticus*, *Edwardsiella ictaluri*, *Edwardsiella tarda*, *Aeromonas veronii*, and *Streptococcus agalactiae*. The antimicrobial activity of betel leaf crude extract against *V. alginolyticus* was maximal at high concentrations of 100 mg/mL, while intermediate concentrations between 10 and 80 mg/mL produced activities similar to commercial antibiotics such as oxytetracycline [15]. This study aims to determine betel leaf extract's ability to inhibit and kill *V. harveyi* with minimal concentrations. Bacteria of *V. harveyi*, known as the cause of Vibriosis in shrimp, is used as a target model of antimicrobial activity of betel leaf extract. Hopefully, the results of this study can be developed in vivo on shrimps affected by Vibriosis.

### 2. Materials and methods

#### 2.1. Materials

The materials used were *V. harveyi* isolates obtained from the Center for Brackish Water Cultivation Fisheries (BBPBAP) Jepara, cultivated betel leaves, Thiosulfate Citrat Bilesalt Sucrose Agar (TCBSA), Nutrient Broth (NB), Mc Farland standard number 1, aquades, ethanol, dimethyl sulfoxide (DMSO) 10%, crystal violet, safranin, lugol, and alcohol acetone.

#### 2.2. Extraction of betel leaf powder

The betel leaf is dried at room temperature, then ground into a powder. A total of 500 grams of betel leaf powder were immersed in 800 ml ethanol solution for 3x24 hours at room temperature [16]. Furthermore, the solvent is evaporated using a rotary vacuum evaporator at 40°C. The results of the betel leaf ethanol extraction obtained 50 ml of a thick and green solution. Betel leaf extract solution can be stored for 1 month in the refrigerator [16].

#### 2.3. V. harveyi bacterial suspension

Isolates of *V. harveyi* were grown on TCBSA media as a selective medium. Furthermore, bacterial isolates were identified by Gram staining and biochemical tests to confirm *V. harveyi*. To make a bacterial density of $3 \times 10^8$ CFU/ml, as many as 4-5 colonies of *V. harveyi* bacteria were inoculated on NB media and incubated at 37°C, then the turbidity was equalized to Mc Farland 1 ($3 \times 10^8$ CFU/ml) [17]. According to Saulnier et al. [18], the density of *V. harveyi* bacteria $10^7-10^5$ CFU/ml can infect postlarvae and juvenile *Penaeus monodon*, while a density of $10^6-10^7$ CFU/ml can infect postlarvae and juvenile *Penaeus vannamei*.

#### 2.4. Methods

The research design used a completely randomized design consisting of 12 concentrations of betel leaf extract solution with three replications for each treatment. The concentration of extract solution of betel leaf used in this study was: 6.25%, 3.12%, 1.56%, 0.78%, 0.39%, 0.19%, 0.09%, 0.04%, 0.02%, and 0.01%. The treatment concentration was obtained from the extract of betel leaf dilution sequentially with 10% DMSO solvent. DMSO solvent is non-toxic and carcinogenic at a 5-10% [19].

The treatment tube containing betel leaf extract was added with 1 ml of bacterial suspension containing $3 \times 10^8$ CFU/ml of *V. harveyi*. The positive control tube contained 1 ml of betel leaf extract

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**Note:** The text is a summary of the research methodology and findings related to the antimicrobial activity of betel leaf extract against certain bacteria. The full paper contains detailed methodology, results, and discussion. The abbreviations used (CFU, Mc Farland, DMSO) are standard in microbial and bioactivity research. The references cited (10, 11, 12, 13, 14, 15, 16, 17, 18, 19) are likely to the scientific literature validating the methods and findings presented. The use of betel leaf in traditional and medicinal practices is highlighted, with research aimed at understanding its potential medicinal properties. The specific target organisms (*V. harveyi*) indicate a focus on shrimp health and disease management. The experimental design (completely randomized design) and replication protocols (three replications) suggest a robust approach to validating the findings. The concentration range of betel leaf extract solution (6.25% to 0.01%) and the use of DMSO solvent indicate a thorough investigation into the optimal concentration for antimicrobial activity.
and 1 ml of 10% DMSO solvent, while the negative control tube contained 1 ml of \( V. harveyi \) 3 \( \times 10^8 \) CFU/ml and 1 ml of 10% DMSO. Furthermore, each treatment tube and replicates were incubated at 37°C for 24 hours.

Positive and negative controls were used as the standard for visually observing turbidity in all treatment tubes of betel leaf extract concentration. The cloudy media in the treatment tube indicated that \( V. harveyi \) was still growing, meaning that the betel leaf extract solution in the concentration tube could not inhibit these bacteria's growth. On the other hand, a clear treatment tube indicates a positive result, meaning that the betel leaf extract solution in the concentration tube can inhibit the growth of \( V. harveyi \) bacteria. Furthermore, the MIC test results of each treatment tube were read the Optical Density value with a spectrophotometer with a wavelength of 520 nm.

All MIC treatment tubes were inoculated on TCBSA media and incubated at 37°C for 24 hours to obtain the minimum concentration that killed \( V. harveyi \) bacteria. If the media does not grow bacterial colonies, it shows that the extract solution of betel leaf with a certain concentration is bactericidal, and vice versa.

2.5. Analysis of Data

The OD value data of the MIC test results were analyzed by Analysis of Variance (ANOVA) and Duncan's Multiple Range Test. According to Kusringrum [20], Duncan's Multiple Range Test with a confidence level of 5% is to determine the best treatment.

3. Results and discussion

3.1 Results

| Parameter                        | Result of Observation |
|----------------------------------|-----------------------|
| Colony color (TCBSA)             | yellow                |
| Swarming                         | -                     |
| Colony forms                     | circular              |
| Gram stain                       | negative              |
| Cell shape                       | Rod                   |
| Motility                         | +                     |
| Oxidase                          | +                     |
| H2S production                   | -                     |
| Nitrate reduction                | +                     |
| Indol Production                 | +                     |
| Acid production from:            |                       |
| - L-arabinose                    | -                     |
| - Salcin                         | +                     |
| - Sucrose                        | +                     |
| - Xylose                         | -                     |
| Gas production from glucose      | -                     |
| Urease                           | +                     |
| Decarboxylase:                   |                       |
| - Arginine                       | -                     |
| - Lysine                         | +                     |
| - Ornithine                      | +                     |

Based on observations of bacterial colony morphology, Gram staining and biochemical tests of pure \( V. harveyi \) cultures listed in Table 1, these isolates are by the characteristics of \( V. harveyi \) described by [21,
The results of MIC test observations showed the inhibitory activity of *V. harveyi* by betel leaf extract with a minimum concentration of 0.09% (Table 2). The growth of *V. harveyi* bacteria colonies was not found in TCBSA media inoculated with betel leaf extract with a concentration of 6.25% to 0.39% (Table 2).

### Table 2. The results of the observation of the antimicrobial ability of betel leaf extract in inhibiting and killing *V. harveyi* in various treatment concentrations

| The concentration of extract of betel leaf (%) | Visual observation of the treatment tube | Value of optical density from visual observation | Observation of bacterial colony growth on TCBSA media |
|-----------------------------------------------|-----------------------------------------|-----------------------------------------------|---------------------------------------------------|
| 6.25                                          | Clear                                   | 2.818 ± 0.054a                               | -                                                 |
| 3.12                                          | Clear                                   | 2.449 ± 0.034e                               | -                                                 |
| 1.56                                          | Clear                                   | 2.318 ± 0.068cd                             | -                                                 |
| 0.78                                          | Clear                                   | 2.209 ± 0.074de                             | -                                                 |
| 0.39                                          | Clear                                   | 2.108 ± 0.060df                             | -                                                 |
| 0.19                                          | Clear                                   | 2.057 ± 0.047g                             | +                                                 |
| 0.09                                          | Clear                                   | 2.013 ± 0.024h                             | +                                                 |
| 0.04                                          | Cloudy                                  | 1.747 ± 0.166d                             | +                                                 |
| 0.02                                          | Cloudy                                  | 1.655 ± 0.151l                             | +                                                 |
| 0.01                                          | Cloudy                                  | 1.541 ± 0.185k                             | +                                                 |
| Control (+)                                   | Clear                                   | 2.804 ± 0.04b                              | -                                                 |
| Control (-)                                   | Cloudy                                  | 1.879 ± 0.04i                              | +                                                 |

**Note:**
- Different superscripts in the same column show a significant difference (p < 0.05).
- Control (+) = 1 ml betel leaf extract + 1 ml DMSO 10%
- Control (-) = 1 ml of 10% DMSO + 1 ml of *V. harveyi* bacterial suspension
- (+) = There is a growth of *V. harveyi* colony because betel leaf extract is unable to kill bacteria.
- (-) = There is no growth of *V. harveyi* colony because betel leaf extract can kill bacteria.

#### 3.2 Discussion

The results of visual MIC test observations showed betel leaf extract concentrations ranging from 6.25% to 0.09% were still clear, but the clarity of the 0.09% concentration was almost close to the positive control tube but not cloudy like the negative control tube. Based on observations with a spectrophotometer, the OD value of 0.09% concentration (2.013) was not significantly different from the negative control, which had an OD value of 1.879. The negative control was a cloudy tube due to the growth of *V. harveyi* bacteria. The OD value of 0.09% concentration (2.057) was not significantly different from the 0.19% concentration (2.057), but the OD value of 0.19% concentration was significantly different from the OD value of negative control. According to Michel and Blanc [24], visually observing the MIC test results has a weakness; namely, it is difficult to distinguish the turbidity level with certainty, so it is necessary to make observations with a spectrophotometer.

The OD value of positive control was 2.804, not significantly different from the OD value of betel leaf extract, with a 6.25% (2.818). This indicates that *V. harveyi* bacteria's growth is inhibited by betel leaf extract with a concentration of 6.25%. Meanwhile, 0.19% concentration is the minimum concentration that is still able to inhibit *V. harveyi* based on visual observation. This is also evidenced by the results of the Duncan Multiple Range test on the OD value, which shows that the OD value of 0.19% concentration (2.057) is not significantly different from the concentration of 0.09% (2.013) but is significantly different from the negative control (1.879) (Table 2).

The MBC test results show that the lowest concentration of betel leaf extract that can kill *V. harveyi* is 0.39%. This can be seen from the results of MBC observations, which showed no colony growth on TCBSA media ranging from tubes with a concentration of 6.25% to 0.39% betel leaf extract. Compared
to ketapang leaf extract, the minimum concentration of betel leaf extract needed to inhibit and kill V. harveyi bacteria is lower. Kharisma et al. [25] reported that ketapang leaf extract can inhibit and kill V. harveyi at a minimum concentration of 1.56% and 3.12%.

The antibacterial activity of betel leaf extract is played by the sterols contained in the extract. The interaction of the surface of the sterol molecule with the bacterial cell wall and membrane causes changes in the cell wall and membrane's main structure, which in turn leads to pore formation and degradation of bacterial components [26]. Sterols act through the disruption of microbial membrane structures [27].

Piper betel plant leaves are rich in various secondary metabolites such as phenolic compounds (chavicol, hydroxyl chavicol), essential oils (safole, eugenol, isoeugenol, eugenol methyl ester), fatty acids (stearic and palmitic) and hydroxyl fatty acids (stearate, palmitic, myristic) which is antibacterial [28]. Palmitic acid, stearic acid, and fatty acid hydroxyl esters showed potent antimicrobial activity against various pathogenic microorganisms [29]. Likewise, hydroxychavicol, the active compound in betel leaf, has a strong antimicrobial effect [29, 30].

4. Conclusion
The minimum concentration of betel leaf extract to inhibit bacterial growth is 0.19%, while the 0.39% concentration is the minimum concentration to kill V. harveyi bacteria. The results of this study confirm that piper betel leaf extract has the potential to be antibacterial.

5. References

[1] Karunasagar I, Pai R, Malathi G, and Karunasagar I 1994 Aquacul. 128 203-209.
[2] Chythanya R, Karunasagar I, and Karunasagar I 2002 Aquacul. 208 1-10.
[3] Angeh J E 2006 Isolation and characterization of antibacterial compound present in members of Combretum section, Hypocrateropsis Thesis (Petoria: University of Pretoria).
[4] Abraham T J, Manley R, Palaniappan R, and Dhevendaran K 1997 J Aquat Trop. 12 1-8.
[5] Bhakta J N and Munekage Y 2010 eJBio. 6 1-5.
[6] Aly S M and Albutti A 2014 J Aquac Res Develop. 5 1-6.
[7] Najiah M, Nadirah M, and Zahrol MAS 2011 Int J of Curr Res. 3 84-86.
[8] Carolia N and Noventi W 2016 Majority 5 140-145 [in Indonesian].
[9] Damayanti R and Mulyono 2003 Khiasat dan manfaat daun sirih: obat mujarab dari masa ke masa (Jakarta: Agromedia Pustaka) [in Indonesian].
[10] Mazumder S, Roychowdhury A, and Banerjee S 2016 Annals of Food Sci. Tech. 17(2) 367-376.
[11] Durgaprasad M, Amarendra C H, Anusha G, Knodeti B, Durga Prasad A, Durga Rao A and Kuchi R 2011 LISID 1 109-114.
[12] Duke J A 1987 Handboo of medicinal herbs (Florida: Boca Raton).
[13] Khan JA and Kumar N 2011 JPBMS. 11 1-3.
[14] Ataguba G A, Dong H T, Rattanarojpong T, Senapin S and Salin K R 2018 Turk J Fish Aquat Sc. 18 671-680.
[15] Othman AB, Zamri-Saad M, Nik-Haiha NY and Siti-Zahrah A 2018 J App Biol Biotech. 6 46-48.
[16] Depkes (Departemen Kesehatan Republik Indonesia) 2000 Parameter Standar Umum Ekstrak Tumbuhan Obat. Edisi 1 (Jakarta: Direktorat Pengawasan Obat Tradisional, Direktorat Jenderal Pengawasan Obat dan Makanan) [in Indonesian].
[17] Baron E J, Petersen L R and Finegold S M 1994 Bailey and Scott’s diagnostic microbiology 9th Ed. (St. Louis: Mosby).
[18] Saulnier D, Phillipe H, Cyrille G, Peva L and Dominique A 2000 Aquacul. 191 133-144.
[19] Horváth A, Wayman W R, Urbányi B, Ware K M, Dean J C and Tiersch T R 2005 Aquacul. 247 243-251.
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