Application of piper betel leaf (piper betle linn) extract to control fish pathogenic bacteria in-vitro

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Abstract. Betel leaf extracts have some natural antimicrobial. This extracts has been used as antimicrobial agent in some aqua farmer in Indonesia for an alternative method to control fish diseases. This study aims to determine the effects of betel leaf extract on fish pathogenic bacteria in-vitro. Three kinds of solvents were used in betel leaf extracts. There were ethanol, n-hexane and ethyl-acetate. About 100 g of betel leaf simplcia were diluted by 1000 ml of solvents in stainless container cap for 24 hours maceration. This extracts were filtered with 0.42 µm paper filter and then evaporated at 50°C during 30 minutes in 50 rpm with vacuum rotary evaporator. This concentrated extracts were used to antimicrobial testing. The pathogenic bacteria for in-vitro testing were from Gram-negative bacteria such as Aeromonas hydrophila, Vibrio alginolyticus and Edwardsiella tarda. The in-vitro test of herbal extract from betel leaf against Gram-negative pathogenic bacteria indicated that the betel leaf extract could inhibited the growth of pathogenic bacteria with diameter mean of inhibition zone were more than 14 mm. The solvents variations were not significantly different (p > 0.05) to diameter inhibition zone of V. alginolyticus and A. hydrophila but significantly different (p < 0.05) to E. tarda.

Keywords : Betel leaf, pathogenic bacteria, A. hydrophila, V. alginolyticus, E. tarda

1. Introduction
In fish culture, many fish farmers complain of a decrease in productivity due to fish disease attacks by pathogenic bacteria and it was very detrimental. Vibriosis and Motile Aeromonas Septicemia (MAS) occurs in many cases of fish disease. Edwardsiellosis/Emphysematous Putrefactive Disease of Catfish (EPDC) or Edwardsiella Septicaemia (ES) was caused by Edwarsiella tarda [1]. These Gram-negative bacteria are widely spread into the water in any condition. Aeromonas hydrophila can affect fish farming and often lead to outbreaks of disease with mortalities occasionally approaching 80-100 percent in a short period of 1-2 weeks [2]. Outbreaks of E. tarda have occurred only in fish larger than 450 g during the period of July through October. Disease outbreaks are most numerous when water temperatures are above 30°C and when high levels of organic fertility are present [3]. Vibriosis is caused by bacteria belonging to the genus Vibrio. Some species are pathogenic and can generate serious epizootic disease, but some other species are only opportunistic pathogens which impact disease if the fish was physically injured, parasitic injuries and stress [4].

Handling of fish diseases caused by pathogenic bacteria using antibiotics such as oxytetracycline, erythromycin, and kanamycin has been widely studied, but the results were still unsatisfactory. The use of antibiotics or medicine to control bacterial diseases can cause new problems. It could affect or
kill non-target organisms, the emergence of pathogens that are resistant to antibiotics, residues in fish meat, affect growth and breeding and cause environmental pollution [5]. It might be safer to use natural antibiotics or antimicrobial to prevent fish disease in aquatic culture.

Indonesia as a tropical country has a wealth of plants that have the potential to become medicinal plants. Many types of plants compounds are antimicrobial. A number of plants compounds are bactericidal (bacteria killers), and bacteriostatic (inhibitors of bacterial growth). Betel leaves are known to be antioxidant, antiseptic, bactericidal and fungicidal. This extract has been used as an antimicrobial agent in some aqua farmer in Indonesia for an alternative method to control fish diseases. The use of betel leaves to control fish diseases include for African catfish [6-7], carp [8], tetra ornamental fish [9], tiger grouper fish [10], disinfecting giant prawn larvae [11], catfish [5,12] and tilapia GIFT [13].

The ability of betel leaf as a medicinal plant is due to the many active compounds contained in it, such as essential oils, fatty acids and fat esters [14], while the identified betel extract contains alkaloids flavonoids, tannins, steroids, and triterpenoid which are antibacterial [15-16]. Based on the function and application of betel leaf extract, a technical study is needed to determine the effect of betel leaf extract on fish pathogenic bacteria in vitro.

2. Material and methods
2.1. Sterilization of Tools
Equipment such as needles of use before used first was sprayed alcohol 70% then conducted burning directly. Petri dish was sterilized by autoclave at a temperature of 121 °C with pressure 1 atm for 10-15 minutes.

2.2. Preparation of Gram-negative bacteria isolation
The Gram-negative bacterial isolates were from the Laboratory of Microbiology of SIFHE Serang. The types of bacteria isolates were Aeromonas hydrophila, Edwardsiella tarda, and Vibrio alginolyticus. Bacterial isolates that have been frozen in a deep freezer (-80°C) are thawed at room temperature inside the Biosafety Cabinet (BSC). When the temperature of bacterial isolates was the same as the temperature of the isolation room (25°C), the bacterial isolates were cultured into Tryptic Soy Agar (TSA) and saline TSA media. Bacterial isolates that have been replanted in TSA media were incubated in an incubator at 37°C for 18-24 hours. The Gram-negative bacterial isolates were examined for bacterial biochemical tests to ensure their purity.

2.3. Preparation of betel leaf extract
Betel leaf extract was made by dissolving 100 g of betel leaf simplicia, respectively, into 1000 ml of several solvents types (ethanol, n-hexane, and ethyl-acetate). The extraction was conducted by maceration method for 24 hours in a closed container. It was filtered with 0.42 μm filter paper and then concentrated using vacuum rotary evaporator at 50°C and 50 rpm for 30 minutes. The concentrated extract was stored in a 3 mL microtube at room temperature. Pieces of filter paper with a diameter of 5-6 mm were used to be paper disc put into a solution of betel leaf extract during 2 x 24 hours.

2.4. Bacterial sensitivity testing
Gram-negative bacteria isolates from TSA media were re-isolated into Mueller Hinton media with a scratch method on all parts of the petri dish evenly. Then, the isolates were tested respectively with paper discs filled with betel leaf extract and incubated at 37°C for 18-24 hours. The experimental design of this sensitivity testing in three replications for each type of extract treatment. After 24 hours, the inhibition diameter zones were measured if the inhibition zone around the disc has been formed for each treatment.
2.5. Data analysis
All data from diameter inhibition zone were analyzed with one-way ANOVA. The hypothesis of this study was to find out the differentiation of betel leaf extract with various types of solvents to the size of the diameter of the inhibition zone formed.

3. Result and Discussion
The in-vitro test results of betel leaf extracts to the diameter of the inhibition zone in Gram-negative bacteria isolates were shown as follows:

**Table 1. Diameter of the inhibition zone (mm) of V. alginolyticus**

| Solvents | Repetition | Mean       |
|----------|------------|------------|
|          | 1          | 2          | 3          |
| A        | 19.00      | 19.00      | 21.00      | 19.67 ± 1.15<sup>a</sup> |
| B        | 16.25      | 13.25      | 22.00      | 17.17 ± 4.45<sup>a</sup> |
| C        | 14.00      | 18.75      | 14.25      | 15.67 ± 2.67<sup>a</sup> |

(p > 0.05), the same letter on superscript is not significant

**Table 2. Diameter of the inhibition zone (mm) of E. tarda**

| Solvents | Repetition | Mean       |
|----------|------------|------------|
|          | 1          | 2          | 3          |
| A        | 34.00      | 32.75      | 34.25      | 33.67 ± 0.80<sup>a</sup> |
| B        | 18.25      | 16.50      | 25.75      | 20.17 ± 4.91<sup>c</sup> |
| C        | 33.25      | 24.25      | 27.50      | 28.33 ± 4.56<sup>b</sup> |

(p < 0.05), the different letter on superscript is significant

**Table 3. Diameter of the inhibition zone (mm) of A. hydrophila**

| Solvents | Repetition | Mean       |
|----------|------------|------------|
|          | 1          | 2          | 3          |
| A        | 23.00      | 27.00      | 23.00      | 24.33 ± 2.31<sup>a</sup> |
| B        | 12.50      | 15.75      | 26.00      | 18.08 ± 7.04<sup>a</sup> |
| C        | 15.25      | 23.75      | 16.75      | 18.58 ± 4.54<sup>a</sup> |

(p > 0.05), the same letter on superscript is not significant

Solvent A gave the largest diameter of the inhibition zone than solvent B and C for each type of Gram-negative bacteria tested (Table 1, Table 2, Table 3). Solvent A was an herbal extract made from extracting betel leaves using ethanol solvents. While B and C were using n-hexane and ethyl-acetate. This could have happened that the ethanol is a polar solvent which can dissolve the antibacterial compounds contained in betel leaves higher than other organic solvents.

However, the p-value showed that the differentiation of solvent extraction for betel leaf was not significant to the diameter of the inhibition zone for V. alginolyticus and A. hydrophila. Therefore the diameter of the inhibition zone of E. tarda bacteria could be influenced by differences solvents extracted from herbal medicines given.

Diameter of the inhibition zone formed in the colonies of Gram-negative bacteria tested with herbal extracts showed that antibacterial active compounds in herbal medicine extracts. The constituent components of betel leaf essential oil consisted of 82.8% phenol compounds and 18.2% compounds not phenols [17]. Phenol compounds which are the main components of essential oils are thought to act as anti-microbes of betel leaves [18]. The slow growth of Gram-negative bacterial diameter in the
treatment of betel leaf extract was suspected that there has been a reaction between antibacterial compounds from betel leaf extract to the three types of bacteria.

The phenol content will be higher as the concentration of betel leaf extract increases so that the reaction will be stronger. Antioxidant activity will levitate as more as phenol compounds increased [19]. The presence of phenol as anti-microbial agent found in betel leaf extract has damage bacterial cell walls, causing slow growth of bacteria so that the inhibition zone of *A. hydrophila*, *V. alginolyticus* and *E. tarda* were formed. Phenol compounds are able to break the cross linkage of peptidoglycan in an attempt to break through the bacterial cell wall [20].

Betel leaf can be used as a general antimicrobial such as bacteria, fungi and protozoa [21]. Antibacterial activity can be effective in Gram positive and negative bacteria [21]. The diameter of the inhibition zone of bacterial mix by 20% betel leaf extract was better than 3% hydrogen peroxide [22]. About 25% of betel leaf extract could inhibited *A. hydrophila* growth [5]. Arifin reported that betel leaf extract had antibacterial effect on *Haemophilus influenzas*, *Staphylococcus aureus* and *Streptococcus haemoliticus* [23]. On the antimicrobial activity of 10 medicinal plants used in traditional medicine in Colombia showed that betel and water extracts respond to antimicrobial activity against *Staphylococcus aureus*, but were not effective for hemolitic β *Streptococcus* and *Pseudomonas aeruginosa* [24].

Betel leaf can be used as a medicine that functions as antibacterial, anti protozoa and antifungal [25]. In addition, it can be used to cure several diseases such as abscesses, rheumatism, abrasion, hysteria, mastoiditis and so on [25]. This antibacterial activity occurs because betel leaves contain essential oils in which there are phenolic compounds that are bactericidal. Phenol compounds when interacting with cell walls of microorganisms will cause protein denaturation and increase the permeability of microorganisms [25].

Interaction between microorganisms results in changes in the balance of charge in protein molecules and causes coagulation. Proteins that are denatured and coagulated will lose physiological activity so they cannot function properly. Changes in the structure of proteins in the bacterial cell wall will increase cell permeability so that cell growth will be inhibited and then cells become damaged.

In addition, the kavikol compound (giving a distinctive odor to betel leaf) has a bacterial killing power five times greater than phenol. Charyophilic compounds are antiseptic and local anesthetics, while eugenol compounds are antiseptic and topical analgesics [25].
5. Conclusion
Betel leaf extract is effective in inhibiting the growth of pathogenic bacteria *Aeromonas hydrophila*, *Vibrio alginolyticus* and *Edwardsiella tarda* in vitro. The average diameter of the inhibition zone of betel extract formed more than 14 mm for each type of test pathogenic bacteria. The type of solvent has significantly difference to the diameter of the inhibitory zone of the bacteria *E. tarda* bacteria in vitro, except to *A. hydrophila* and *V. alginolyticus*.

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**Figure 3.** *A. hydrophila* inhibition zone
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