Antibacterial Activity Test of Wet and Dried Extracts of Calabash Tree (Crescentia cujete L.) against Aeromonas hydrophilla

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ABSTRACT

Study to determine the antibacterial activity of wet and dry extract of the leaf, fruit, and bark of Calabash tree (Crescentia cujete L.) against the growth of Aeromonas hydrophila. The solvent extraction process was done by using 96% ethanol in the maceration method. Antibacterial test results using diffusion agar to decide clear zone and tube series of dilution test to provide MIC and MBC. Fresh leaf extract produces the highest clear zone diameter (20.06 mm), after which fresh bark extract (12.81 mm), and the last is fresh fruit extract (3.22 mm). In contrast to fresh extracts, the dried extracts are have not clear zone. MIC (Minimum Inhibitory Concentration) of Calabash Tree fresh leaf extract against Aeromonas hydrophila is 80%, and MBC (Minimum Bacterisidal Concentration) is 100%.

Introduction

The diseases commonly attack freshwater fish is Motil Aeromonas Septicemia (MAS) caused by the bacterium Aeromonas hydrophila (Kusumawardani, 2007). The bacterium has make the highest rate of fish mortality (80-100%) in a couple weeks (Purwaninigsih & Sudah, 2007). A. Hydrophilla is a bacterium that normally found in freshwater area. Infection of it make change of the environmental factor, like, the temperature of the water, resulted of secondary infection at a host (Kordi & Ghufran, 2004). Control of bacterium growth is hard to conduct because of wide spectrum strain, endogenous species in freshwater, and resistant on chemical pesticides.

Prevent of this epidemiology, either the fisherman or fish enterpreuner almost using the antibiotics or chemical pesticides. However, that solution can not persist for the long term period, the unwise use of chemical pesticides in longest time and did not consider for the specific dosage make the strain of bacterium becoming resistant, the chemical pollutant in the water field, and the less-safety food quality.

Calabash tree (Crescentia cujete L.), one of tropical plant, widespread distribution in South West Asia, America, and Africa has the main role as the source of herbal medicine by native people. The crude of seed, fruit, and leaf can use to decrease fever, infection of respiratory, prevent furthermore infection of hemorrhagic caused by bite snakes (Nielsen, et al., 2009). Vietnamese people have used calabash fruit as medicine, expectorant, antitussive, laxative, and stomachache.

(Lien, 2001) is mentioned that calabash tree has contained the chemical compounds, role as antimicrobial activities. Leaf, stem, and fruit of Calabash tree have many compounds such as polyphenol, saponin, and tannin.
Calabash tree either has tannin concentrate in fruit 9%, or in barks 20% (Anaf, 2009). Fresh fruit extract of Calabash tree is reported containing alkaloid (0.74%), flavanoid (0.52%), sapoin (0.7%), and phenol (0.46%) thus in dried fruit extract is containing alkaloid (0.46%), flavanoid (0.38%), sapoin (0.34%), and phenol (0.14%).

This study want to compare the antibacterial activities of crude extract of fresh and dried of leaf, fruit, and barks Calabash tree to the growth of *Aeromonas hydrophilla*.

### Materials and Methods

Sample of leaves, fruits, and barks Calabash tree collected from yard of Sepuluh Nopember Institute of Technology. The extraction solvent’s used ethanol 96% and aquades. The shaking process used a rotary shaker, centrifuge, and freeze drier. Antibactery assay is used Mueller-Hinton agar, Tryptic Soy Agar, and Tryptic Soy Broth, and the pathogenic agent bacterium *Aeromonas hydrophilla* collected from Fish Quarantine and Inspection Agency of Juanda, Surabaya, East-Java.

**Extraction of fresh and dried Calabash tree**

Maceration process was conducted for seven days. The preparing sample process divided into two types, there are fresh and dried samples. Dried extract resulted from 250 gr of leaves or barks which had cleaned by water and air-dried naturally, however the fruit was dried by oven at 65°C, until each of sample was reached the dried weight. On the other hand, fresh extract resulted from 250 gr of leaves; fruits; or barks which had cleaned by water and raised off until dried.

**Extraction of the polar compound**

Each of fresh or dried samples was sliced into the small pieces then blending smoothly by a blender. The smooth sample was soaked into ethanol 96% then shaked by rotary shaker for seven days. The filtrate was sentrifuged by 7,000 rpm. The supernatant begun to freeze at -30°C until 40°C to evaporate the polar solvent. Each of crude extracts was prepared to make the concentration treatments consist of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100% (without solvent). The tetracyclin antibiotic serves as positive control and the negative control used aquades.

**The antibactery assay**

In vitro assay guideline by (Boyd, 1995) consist of tube dillation test and disk diffusion test. Disk diffusion test adapted from Kirby-Bauer (NCCLS), 1999, (Osata et al., 2001).

**Disk diffusion test**

Each of 10 mm paper disk soaked into each of concentaration treatments, aquades, and tetracyclin then put on the surface of Mueller-Hinton agar which had the spread of 0.1 ml *Aeromonas hydrophilla* (equivalents to McFarland 0.5). Afterthat, the agar treatments were incubated for 48 h at room temperature. The inhibition zone diameter was measured for each of 18h, 24h, and 48h then the diameters were classified based on (Greenwood, 1995) (Table 1).

**Table 1. Inhibition zone classification of natural plant extracts**

| Inhibition zone diameter | Response power category* |
|--------------------------|--------------------------|
| > 20 mm                  | strong                   |
| 16 – 20 mm               | moderate                 |
| 11 – 15 mm               | weak                     |
| ≤ 10 mm                  | no                       |

*) category based on (Greenwood, 1995)

**Tube dilution test**

Dillution methode provided the Minimum Inhibitory Concentrate (MIC) and Minimum Bactericidal Concentration (MBC). Series of tubes were contained 0.25 ml *Aeromonas hydophilla* (equivalents to McFarland 0.5); 0.5 ml of the concentration of treatments; and 4.5 ml TSB. The tubes test was incubated at temperature room for 24 h. The result observed by the clear-dilution of the tube test (Boyd, 1995).

The first tube test whose the dilution was begun cleared reffered as MIC, the lowest concentrate of the drug that inhibited the microorganisms’ growth after 24 h, then was sub-culturing on TSA by pour plate methode, incubated at temperature room for 24 h. The coloniests of the bacteria were counted by total plate count methode. The MBC defined as there were have no colonolist growth or 99.9% killed.
Results and Discussion

Antibacterial Activity of Calabash Tree (Crescentia cujete L.) against Aeromonas hydrophilla by Disk Diffusion Test

The activity antibacterial of calabash tree have shown the various strength/power considered by dry or wet of leaves; fruits; and barks (Table 2).

Table 2. Inhibition zone diameter of wet/dried extract leaves, fruits, and barks of Crescentia cujete L. by disk diffusion test Kirby-Bauer for 24h

| Crude extracts | Inhibition zone diameter (mm) | Inhibition responses* |
|----------------|-------------------------------|-----------------------|
| Aquades        | 0a                            | no                    |
| Dried fruits   | 0a                            | no                    |
| Fresh fruit    | 3.22b                         | no                    |
| Dried barks    | 0a                            | no                    |
| Fresh barks    | 12.81c                        | weak                  |
| Dried leaves   | 0a                            | no                    |
| Fresh leaves   | 20.06d                        | strong                |
| Tetracyclin    | 31.11e                        | strong                |

*) category based on (Greenwood, 1995)

The activities antibacterial of Calabash tree would be provided by the clear zone were formed at around of the disk paper (Wattimena et al., 1991). The bacteriostatic category referred as the clear zone would be fading after 24 h, however the bacteriocidal provides as the inhibition zone diameter was still cleared for 48 h.

Table 3. The category of activities antibactery of wet/dried extract leaves, fruits, and barks of Crescentia cujete L. for 48 h

| Crude extract | Inhibition zone diameter (mm) | Antibacteria’s category* |
|---------------|-------------------------------|--------------------------|
| Dried fruit   | 0 0 0                         | -                        |
| Fresh fruit   | 3.22 3.22 3.22                | Bacteriocidal            |

Performed antibacterial activities or inhibited the growth of bacteria indicated by the clear zone that form on the surface of Mueller-Hinton. Calabash tree extract has significantly inhibited the growth of Aeromonas hydrophilla (Table 2). These activities can be caused by the compounds that have been contained in the body cell of Calabash tree. The plant has riched of secondary metabolites that have several antimicrobial activities: saponin, polyphenol, tannin, alkaloid, and flavonoid (Hutapea, 1993; Poeloengan et al., 2006; Cushine & Lamb, 2005). According to (Pelczar & Chan, 2005) the antimicrobial substance is a compound that can harm or disturbed the growth of microorganism by inhibited mechanisms. Many factors influence those mechanism; therefore, do effect any variant of diameters of inhibition zone (Jewetz & Adelberg’s, 1996).

The inhibition zone diameter of the dried extract is respectively differed from wet extract (Table 2). This trend may be caused of the drying process of the calabash tree had changed the quantitative of the total antibacterial compounds in its. Besides, there are might be the contaminant agent done harmful on its process. Some unknowns agents may be followed in while the fruit, leaf, and bark are been drying. It was decreasing the effectivity of antimicrobial compounds. This unknowns agent may effect the change of chemical structure of the antibactery compounds that in other way change the diffusion’s capability of its (Osata et al., 2001).
There is a significant difference among the clear zone diameter of fresh leaf, fruit, and bark. The fresh leaf has the greatest value of diameter clear-zone among the others (Fig. 1), that is 20.06 mm. Whereas, the fresh bark extract is considerably 50% smaller than fresh leaf extract (12.81 mm) and the fruit fresh extract produces the lowest number of clear-zone diameter (3.22 mm).

The diameter clear-zone of fresh leaf is more high than the fresh fruit (Figure 1) it caused by the lower quantitave of bio-active compound that solved in fresh fruit. (Intan, 2008) and has reported that leaf fruit extract calabash tree is more competitive to inhibited the growth of Staphylococcus aures than fruit extract.

The diameter clear-zone fresh bark extract is bigger than fresh fruit be suspected by the higher concentrad of tannin in fresh bark (20%) than in fruit (9%) (Anaf, 2009).

**Antibactery Activity of Calabash Tree (Crescentia cujete L.) Leaf againts Aeromonas hydrophilla by Tube Dillution Test**

Tube dillution test was applied on the types of extract that had the strongest responses. Fresh leaf extract was the best candidate to inhibited of the growth of Aeromonas hydrophilla (Table 2) that classified as the strong power bacteriociadic antibactery, the others were adressed as weak and no power antibacterial activities.

The Minimun Inhibitory Concentration (MIC) of wet leaf Calabash Tree extract can be provided by comparing the turbidity of the tube series to the aquades tube (0% concentrate). The turbidity of the series tube can calculate the density of the bacteria, whereas the murkier of the dilution; the more colonists have grown. On 80% concentrate after 24h, of fresh leaves, the tube getting more evider than the lower concentration of its (Table 4). It means the growth of Aeromonas hydrophilla starting to be inhibited. MIC, the lowest concentrations that prevent visible growth of bacteria, of fresh leaves extract of Calabash Tree goes to 80%.

**Table 4. MIC and MBC of wet leaf extract of Calabash Tree (Crescentia cujete L.) againts Aeromonas hydrophilla by Tube Dillution Test**

| Tube series (%)| MIC indicator | avg Σ colonist (CFU/ml) | % change of avg Σ colonist | Provided |
|----------------|---------------|------------------------|---------------------------|----------|
| 0              | turbid        | 3700000                | 24566.66667               | -        |
| 10             | turbid        | *                      | *                         | -        |
| 20             | turbid        | *                      | *                         | -        |
| 30             | turbid        | *                      | *                         | -        |
Saponin antibacterial mechanism is the hydrogen bind of saponin is bind to cytoplasm membrane of bacteria resulted in enzyme synthesis disrupted, ruined permeability system of membrane plasma, as of killing the cell in its process (Aulia, 2008) (Noer & Nurhayati, 2006).

This study was conducted that extract of calabash tree has considerable change to the growth of *Aeromonas hydrophilla*. The activity of fresh extract is more potential as drug than dried extract. The MIC of fresh leaf extract againsts *Aeromonas hydrophilla* is 80%, while, the MBC is 100%.

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Table 5. The phytochemical test of metabolite secondary of Calabash Tree Leaf Extract

| Phytochemical test | Percentage compound* |
|--------------------|----------------------|
| Polyphenols        | 0.43                 |
| Saponin            | 1.56                 |
| Flavonoid          | 1.48                 |
| Alkaloid           | 1.22                 |

*) 100 mg of leaves

Alkaloid have been reported causing the abruptly of DNA bacteria by its nitrogen of basa group (Cushnie et al., 2014).

(Pachanawan et al., 2008) reported that flavonoid is a bacteriostatic drug to treat fish that was infected by *A. hydrophilla*. Flavonoid is a secondary metabolite compound that inhibited the growth of bacteria whose mechanisms are interrupting protein synthesis of bacteria cell, inhibiting cytoplasm membrane system, and inhibited metabolism energy (Cushine & Lamb, 2005).
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