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%.

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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 & Suwidah, 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 planth, 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 decrese 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 calasbash 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

(Hutapea, 1993). Calabash tree either has tannin consentrate 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%), saponin (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 colleted 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 65^0C, untill 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 rised 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 freez at -30^0C until 40^0C 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 dillution 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 concenctaration treatments, aquades, and tetracyclin then put on the surface of Mueller-Hinton agar which had the spread of 0.1 ml *Aeromonas hydrophila* (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 dillution 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 dillution 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 cololonist growth or 99.9% killed

by the extracts (Gillespie, 1994; Krishnan et al., 2010; Scorzoni et al., 2007).

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 streght/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 diffusin test Kirby-Bauer for 24h

Crude extracts	Inhibition zone diameter (mm)	Inhibition responses*
Aquades	0[a]	no
Dried fruits	0[a]	no
Fresh fruit	3.22[b]	no
Dried barks	0[a]	no
Fresh barks	12.81[c]	weak
Dried leaves	0[a]	no
Fresh leaves	20.06[d]	strong
Tetracyclin	31.11[e]	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 inbition 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)			Antibacteries' category*
	18	24	48	
Dried fruit	0	0	0	-
Fresh fruit	3.22	3.22	3.22	Bacteriocidal
Dried bark	1.67	0	0	Bacteriostatic
Fresh bark	12.25	12.81	12.89	Bacteriocidal
Dried leaves	3.56	0	0	Bacteriostatic
Fresh leaves	20.06	20.06	20.06	Bactericidal
Tetracyclin	31.11	31.11	26.11	Bacteriostatic

*) category based on (Wattimena et al., 1991)

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 coumpounds that in other way changed 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).

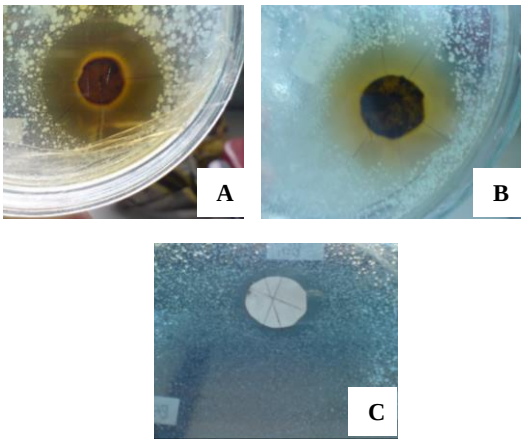

Figure 1. Clear-zone diameter fresh extract of *Crescentia cujete* L. : (A) leaf, (B) bark, and (C) fruit

Relatively there are any varians of responses to perform clear-zone diameter in single treatment. It may be caused of the intensity of antibacterial-coumpounds that contain in every single treatment. The lowest quantitative of active-compound can not do any activities to inhibited or killed the cell of bacteria. The more concentrated of bio-active coumpund that contained in drugs solvent, the more ability to control the growth of bacteria (Pelczar & Chan, 2005).

Clear-zone diameter of fresh fruit extract calabash tree is remarkable differ from dried fruit extract, whereas, the dried fruit extract has no clear zone diameter. This may caused of the drying process has been eliminated the bioactive compound that dissolved in it. According to (Oghubuagu, 2008) bio-active compound in fresh fruit calabash extract is higher than in its dried fruit.

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 bacteriocidal 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	*	*	-

40	turbid	*	*	-
50	turbid	*	*	-
60	turbid	*	*	-
70	turbid	*	*	-
80	clear	1133.33	-99.2	MIC
90	clear	6156.67	-95.9	-
100	clear	3.33	-99.9	MBC

Note:

- First colony calculated is $15x10^3$ CFU/ml
- (*) series of tube consentrate are not calculated

This study is complementing with the MBC's value, the lowest concentrations required to kill bacteria, by calculating the total number of bacteria colonists that growth on TSA subculture plate. The MBC of fresh leaves extract of calabash tree is 100% caused by the colonists of *A. hydrophilla* was inhibited for 99.9% (from the first colony count at 0%) (Table 4).

The metabolite compounds contained in leaf of calabash tree: alkaloid, flavonoid, polyphenol, and saponin (Hutapea, 1993) consider the strength of inhibited mechanism of bacteria. According to phytochemical test whose calabash tree has (Table 5), the leaves have polyphenol (0.43%), saponin (1.56%), flavonoid (1.48%), and alkaloid (1.22%).

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

Phytochemical test	**Percentage compund***
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).

Saponin antibacterial mechanism is the hydrogen bind of saponin is bind to cytoplasm membrane of bacteria resulted in enzyme synthesis distrupted, 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 againts *Aeromonas hydrophilla* is 80%, while, the MBC is 100%.

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