Macrothink Institute™

**Journal of Biology and Life Science**
ISSN 2157-6076
2015, Vol. 6, No. 1

# Phytochemistry And Antibacterial Activity Test From Methanol Extract of *Alstonia Acuminata* Tree Bark Against *Vibrio Harveyi* Bacterium in In-Vitro Manner

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Received: July 28, 2014 Accepted: August 15, 2014

doi:10.5296/jbls.v6i1.6496 URL: http://dx.doi.org/10.5296/jbls.v6i1.6496

**Abstract**

Grouper belongs to one of cultivated commodities whose high selling price gives bright prospects. Grouper belongs to one of cultivated commodities whose high selling price gives bright prospects. However, the greatest faced obstacle in this aquaculture industry lies in disease strike brought by bacterium. Synthetic antibiotic use made from chemical compounds gives long-term negative effect and accumulates the chemical compounds into body tissues. Natural material use as an antibacterial is highly recommended to reduce side effect of medicinal effect. The use of antibacterial extract made from *A. acuminata* tree bark will give new discourse in handling mischievously bacterial strike problem. The study aims to find out chemical compounds made from *A. acuminata* and to find out solvent and the best dose of *A. acuminata* inhibition activity as antibacterial against *Vibrio harveyi* bacterium. Based on research findings, it is known that *A. acuminata* extract with methanol solvent has the highest inhibition activity compared to ethyl acetate and n-hexane solvent. Then, a concentration of 100% has the highest inhibition activity compared to concentration of 15%, 25%, 50% and 75%. Phytochemistry test result shows that *A. acuminata* tree bark contains compounds of alkaloid, phenol, flavonoid, steroid, and terpenoids. Based on the findings, it is concluded that *A. acuminata* contains phenol compound which is able to increase inhibition activity against V. *harveyi* bacterium.

**Keywords:** *Alstonia Acuminata*, *Vibrio Harveyi*, Bacterium In-Vitro Manner

## 1. Introduction

One of sea fishery products belonging to leading export commodity is grouper fish.Grouper fish species going through export commodity are rat grouper (*Cromileptes altivelis*), tiger grouper (*Epinephelus fuscoguttatus*), mud grouper (*Epinephelus coioides*), and Napoleon (*Heilinus undulates*) (Sukardi, 2007).

Market demand on the commodity is stable; even it tends to increase over the years. Business development on grouper fish farming has quite good prospects. Major obstacle for tiger grouper fish farming is high mortality rate from husbandry to harvest time. One of main causes causing high mortality rate in grouper fish farming is fish disease strike causing mass mortality (Darwisito, 2002).

One of disease types to be serious problem in grouper fish farming is disease caused by pathogen bacteria infection especially Vibrio, i.e. when at the peak epidemic condition, body of fishes drop, stress and infected, even death. Vibriosis was still main problem in sea fish farming since it is able to raise mortality rate up to 100% (Dewi, et al., 2002).

In general, *Vibrio* infection in fish is known as *haemorrhagic septicaemia* causing wide-ranging injury on its skin, focal necrosis to its liver, spleen, kidney, and other tissues (Yanuhar *et al.*, 2004). According to Moriaty (1997), disease brought by *Vibrio spp* bacterium becomes causal factor of mass mortality to breeding grouper. One of *Vibrio spp* bacterium species which is pathogen to grouper is *V. harveyi*. Strike level of *V. harveyi* bacterium at tiger grouper hatchery may be counted in couple of hours. This Vibrio bacteria strike may damage breeding fish organ and injure skin.

*V. harveyi* is dangerous; if density in water is more than $10^8$ CFU/ml, it may cause 90% of mortality rate. Larvae may live normally, safe and sound if bacteria density in husbandry media is under $10^4$ CFU/ml (Sugama, *et al*., 1993).

Handling effort to fish disease, such as tetracycline has been done. However, it does not run well since it leads to other problem, i.e. environmental pollution (Hameed, *et al*., 2003; Kerry, *et al*., 1997; Rairakhwada *et al*., 2007; Khachatryan, 2006). Accumulation of antibiotic residue in fish tissue will affect its growth and resistance to medicine as well as immunosuppression (Maqsood et al., 2009).

One of alternatives to handle this problem is by utilizing bioactive compound from land plant as antibacterial. *Alstonia acuminate* is traditional land plant species from Southeast Maluku which is traditionally utilized as medicine for liver, diarrhea, malaria taken from bark of the rod. Bitter taste as indicated from the rod bark contains chemical content as antibacterial material which can restrain the disease in fish.

Based on previous research on antibacterial test of *V. harveyi* bacteria against *A. scholaris* and *A.acuminata*, it turns out that *A.acuminata* has greater inhibitory capacity (15mm) compared to *A. scholaris* (12mm**)**.Ability of *A. acuminata* extract in inhibiting bacteria growth in in vitro manner shows that *A. acuminata* contains an antibacterial active compound. According to

Heyne (1988), bioactive compound with *A. acuminata* therein shows antibacterial activity and it contains ditamin and ekitamin phenol from the tree bark. It is presumed that mechanism bringing out inhibition of bacteria growth after being extracted by *A. acuminata* is caused by bioactive compound content where one of them called as phenol compound and the derivatives. Research on land plant use from *Alstonia acuminate* as antibacterial has not been conducted a lot, hence it needs scientific study on *A. acuminata*.

## 2. Methodology

### *2.1 Active Material Extraction*

Fresh A. acuminata tree bark is taken from Southeast Maluku Regency, then it is put out to dry in a windy place, cut into small pieces, and blended to make it finely powdered. A 50 gram of refined *A. acuminata* powder is soaked into 200 ml methanol liquid for 2x24 hours by maceration method at room temperature. When methanol extract and its waste have been separated, methanol extract output is steamed by using rotary evaporator device at temperature of ± 40 ℃, so this liquid turns to thick methanol extract. Then, the extract is partitioned by n-hexane and ethyl acetate solvent where the solvent ratio and material is 4:1 (Harbone, 1987).

### *2.2 Phytochemistry Test of A. Acuminate Tree Bark*

Phytochemistry content test is conducted by analyzing compound groups of alkaloid, flavanoid, saponin, terpenoids, steroid, and polyphenol.

**1.** Alkaloid Compound Analysis (Culvenor-Fiztgerald Method**)**

- Four gram of refined *A. acuminata* is ground down in dies and added by a few chloroform to make it pasta.
- Next, 10 ml of ammonia-chloroform 0.05N solvent is added and ground it down once again, 10 ml H2SO4 2N layer is firmly mixed, keep it from moving to form two layers.
- Use pipette with cotton buds on top to filter, take sulfuric acid layer and put it inside a small test tube (storage the chloroform layer for terpenoids test).
- Filtrate is tested by Meyer reagent; when white or muddy sediment is formed through this Meyer reagent, it indicates the presence of alkaloid.

2. Flavonoid Compound Analysis (Shinoda/Sianidin Test Method)

- A number of 0.5 mg refined sample is extracted by 5 ml methanol and heated up for 5 minutes in test tube.
- Extracted sample is added by some drops of thick HCl and a few magnesium powders.
- When the color turns to red or yellow, it indicates that the sample contains flavanoid.

3. Saponin Compound Analysis (Foam Test)

- In this saponin test, it is best to utilize dried sample since the applied test in this section is foam formation test.

- Refine the dried sample, put it into the test tube and add 10 ml of distilled water and boil it for 2-3 minutes. Cool it down, and then mix it firmly when it is cool already. The presence of stable foam for 5 minutes means that the sample contains saponin.

4. Polyphenol Compound Analysis

- One ml of extracted sample (ethanol, n-hexane-ethanol) is added by FeCl3 1%. Terpenoids compound is indicated by the appearance of blue to black or purple.

5. Terpenoids and Steroid Analysis (Lieberman-Burchard Method)

- Some drops of chloroform to alkaloid test is put on drop plate, added by 5 drops of anhydride acetate and let it runs dry. Then, add 3 drops of thick H2SO4.
- The presence of terpenoids compound is indicated by the appearance of orange or purple, while steroid presence is indicated by blue color.

*2.3 Antibacterial Activity Test*

This step covers preparation and making of bacteria growth media, bacterial test culture, and antibacterial activity test.

*2.4 V harveyi Bacterial Culture*

Pure culture of V. harveyi bacteria is acquired from Microbiology Laboratory, Faculty of Medicines, Brawijaya University. Before executing antibacterial test, the bacteria are formerly rejuvenated since the utilized bacteria for the test is 24 hours old. Procedure of bacteria culture rejuvenation are: Prepare 5 ml of NB solution in the test tube, take one dose of *V. harveyi* bacteria pure culture aseptically, put it into prepared NB media. Then, incubate NB solution with bacteria therein for 24 hours at 37 ℃. When 24 hours goes through, NB media with bacteria herein is cultured into thick media, i.e. TCBSA/TSA, and then incubate the sample once again for ±24 hours. After 24 hours pass, bacteria are ready to be utilized for antibacterial test.

*2.5 Antibacterial Test of A. acuminata Extract by Disk Method*

Each of obtained thick extract gets antibacterial test to find which one of them may inhibit *V. harveyi* bacteria. In this test, disk test is applied where 6 mm in diameter of sterile disk paper is sinked into each of extract at concentration of 15%, 25%, 50%, 75%, and 100%. After 15-30 minutes, disk paper is stuck onto TCBSA media and TSA media which were inoculated by *V. harveyi* bacteria. Observation is conducted after incubation period for 24 hours at temperature of 37 ℃ by observing the presence and absence of formed transparent zone.

## 3. Discussion

### Active Materials Extraction in *Alstonia acuminate* tree bark and Antibacterial Activity Test of *Vibrio harveyi*

*3.1 Active Materials Extraction in A. acuminata Tree Bark*

Extraction result of *A.acuminata* can be seen in Table 1. Extracts resulted from extraction

process of *A. acuminata* are different based on used solvent type consisting of methanol extract, ethyl acetate extract, and n-hexane extract by ratio of 1:4. Yield of every extract may be seen in Table 1.

Table 1. Raw extract yield of *A. acuminate* tree bark containing methanol, ethyl acetate, and n-hexane.

| Type of Solvent | Yield percentage (w/w) |
|---|---|
| Methanol | 11.12 |
| Ethyl Acetate | 0.78 |
| N-Hexane | 0.68 |

In Table 1, yields of extraction process of *A. acuminata* tree bark continually are: methanol extract gets 11.12% (w/w), ethyl acetate extract gets 0.78% (w/w), and n-hexane extract gets 0.68% (w/w) where initial weight of *A. acuminata* is 273.86 g and each of utilized solvent is 800 ml. Table 1 shows that the highest extraction result is produced by methanol extract since in maceration process, there occurs mixing process with the extracted materials, so it may increase the probability of inter-particle sediment. Sediment formation causes cell fragmentation under expectation that expected component may leave material tissue and fuse into its solvent. The process also aims to increase bonding and reaction of inter-active component with the utilized solvent (Gritter, 1991). Based on extraction yield, *A. acuminata* contains a lot of polar substances.

The greatest resulted yield is the yield by using methanol solvent. It is in accordance with a statement that methanol is able to extract organic compound, small number of fat and tannin which produce methanol extraction yield quite high (Heath and Reineccius, 1987). Extraction yield is affected by some factors, i.e. natural characteristic of natural materials, extraction method, particle size of the sample, and condition and time of sample storage.

*3.2 Phytochemistry Test to the Raw Extract of A. acuminata Tree Bark*

Qualitative test result given by chemical compound content to raw extract of *A. acuminata* tree bark may be seen in Table 2.

Based on qualitative test result to *A. acuminata* compound content, as seen in Table 2, it is known that *A. acuminata* contains compounds of alkaloid, phenolic, flavonoid, and steroid. These compounds allow *A. acuminata* to be developed as natural antibacterial and immunostimulant.

According to Satria (2005), flavonoid compound has special virtue as antioxidant by inhibiting various oxidation reactions and it is able to act as reducing hydroxyl radical, superoxide, and peroxyl radical. Antioxidant compound has role as free radical captor, metal chelation, and damper of singlet oxygen formation.

Table 2. Phytochemistry test product from raw extract of *A. acuminata* tree bark

| Active material type | Methanol extract | H-hexane extract | Ethyl acetate extract | Water extract | Information |
|---|---|---|---|---|---|
| alkaloid | + | - | - | + | if red sediment exists |
| Phenolic | + | - | + | + | if the solution turns to |

| | | | | | |
|---|---|---|---|---|---|
| | | | | | black |
| Flavonoid | + | - | + | - | if the solution turns to orange |
| Steroid | + | + | + | - | if the sediment is brown |

Steroid mechanisms in inhibiting microbe are by destructing plasma membrane, so it will leak the cytoplasm, even cell death (Putra, 2007). Others, according to Cowan (1999), phenolic compound is one of antibacterials working by infringing function of cytoplasm membrane.

Phenol compound and its derivative (flavonoid) is one of antibacterials working by infringing function of cytoplasm membrane. The presence of this phenol compound causes destruction to cytoplasm membrane. $H^+$ ion from phenol compound and its derivative (flavonoid) will attack polar group (phosphat group) that make phospholipid molecule on bacteria cell wall loose into glycerol, carboxylic acid, and phosphoric acid (Rowe, 1989).In this state, phospholipid is unable to maintain the form of cytoplasm membrane, and as the consequence, cytoplasm membrane will leak and bacteria will suffer growth retardation even mortality (Rowe, 1989).

*3.3 Antibacterial Compound Activity Test*

**Antibacterial Activity from Raw Extract of *A. acuminata* Tree Bark by Various Solvents**

Antibacterial test by raw extract of *A. acuminata* tree bark against *V. harveyi* by using methanol thick extract, ethyl acetate extract, and n-hexane extract.

Table 3. Antibacterial test result from raw extract of *A. acuminata* tree bark by various solvents

| Solvent | Average diameter of transparent zone (mm) |
|---|---|
| Control | - |
| Methanol Solvent | - |
| Ethyl acetate Solvent | - |
| N-hexane Solvent | - |
| Methanol | 14.24 ± 0.10 |
| Ethyl Acetate | 10.00 ± 0.05 |
| N-hexane | 9.11 ± 0.10 |

Methanol thick extract has a greater inhibitory capacity compared to ethyl acetate and n-hexane extract as seen in Table 3. Methanol extract has greater inhibitory capacity. Methanol belongs to polar solvent and it is frequently used for extraction process of a simplicia. It has been known that methanol is able to extract organic compound, small number of fat and tanin that makes methanol extraction result quite great (Heath and Reineccius, 1987). Presumably, the existing of active compound in methanol extract is greater than ethyl acetate or n-hexane extract, besides the possibility effect which occurs due to diffusion rapidity difference of antibacterial compound in agar media.

Mallawa and Halid (2006) state that the size of growth drag zone becomes bioactivity benchmark which were affected by multiple factors such as function group activity, bacterial resistance against bioactive substance, grade of active substance, and amount of bacterial test density. Active material mechanism in diminishing bacteria is conducted by denaturing protein and destructing cell membrane, i.e. by dissolving fat that exist on the cell wall. This

compound is able to migrate from liquid phase to fat phase. The occurrence of destruction in cell membrane causes the inhibition of activity and specific enzymes biosyntheses since they are required in metabolism reaction and finally, this condition causes bacterial death (Astuti, 1997).

**Antibacterial Activity in Raw Extract of *A. acuminata* Tree Bark on the Basis of Concentration**

Calculation on antibacterial test and drag zone measurement to the effect of methanol extract of *A. acuminate* tree bark against *V. harveyi* based on concentration of 15%, 25%, 50%, 75%, and 100% are presented in Table 3.

Table 4. Antibacterial test result by methanol raw extract-based from A. acuminata tree bark

| Concentration (%) | Average diameter of transparent zone (mm) |
|---|---|
| Control of methanol solvent | - |
| 15 | 9.5 ± 0.15 |
| 25 | 10 ± 2.00 |
| 50 | 12.5 ± 1.00 |
| 75 | 12.75 ± 0.05 |
| 100 | 14.75 ± 0.33 |

Table 4 points out that all concentration of methanol raw extract from *A. acuminata* tree bark is able to produce transparent zone which is differently produced based on extract concentration. Calculation on drag zone of methanol raw extract from *A. acuminate* tree bark against *V. harveyi* based on concentration of 15%, 25%, 50%, 75%, and 100%. Calculation on drag zone diameter indicates that methanol extract from *A. acuminata* tree bark has moderate and strong inhibitory capacity against *Vibrio* gram-negative bacteria. This finding is in line with the report of Fardiaz (1988) stating that provision of antibacterial strength depends on its inhibition zone, when inhibition zone is 20 mm or more, it is categorized as very strong, strong category for 10-20 mm in width, moderate for 5-10 mm, and if inhibition zone ranges from 5 mm or less, it is categorized as weak.

Methanol raw extract from *A. acuminate* tree bark in all concentration shows growth inhibition process of *V. harveyi* bacteria meaning that all extracts are able to produce transparent zone. Transparent zone delivered by methanol extract may occurs since methanol solvent belongs to universal solvent having capability of dissolving almost all compound components contained in the extract. According to Jawetz et al. (1996), formed drag zone is strength size of antimicrobial substance against the observed bacteria. Inhibition surrounding the disk depends on absorbing power of utilized active material. If antimicrobial substance has inhibiting characteristic, the bacterial growth will stop while surrounding condition of the disk will emerge transparent circle where bacteria do not grow after being incubated for 18-24 hours.

Schlagel (1994) suggests that capability of antibacterial in destroying organism viability depends on concentration of antibacterial materials. Amount of antibacterial material in bacterial environment highly affects the exposed bacterial life. It is proved that extract concentration of 100% has a higher drag zone, while 25% less concentration only has 9 mm drag zone. Other than concentration percentage, antibacterial material types also affect

inhibitory capacity of bacterial growth.

*3.4 Minimum Inhibitory Concentration (MIC) Test*

Calculation on Minimum Inhibitory Concentration (MIC) from methanol raw extract of *A. acuminata* tree bark indicates that *V. harveyi* is bacterium having great resistance against extract of *A. acuminata* tree bark, i.e. 4.96%. It means that minimum concentration having capability in inhibiting bacteria is 4.96%. Bacterial inhibition indicates the capability presence of bioactive compound from *A. acuminata*, while capability of *A. acuminata* bioactive compound may kill bacteria in MBC value of 19.84%. This MIC calculation is in line with bacterial test by means of disk method which is calculated statistically.

Table 5. *Minimum Inhibitory Concentration* (MIC) and *Minimum Bacterial Concentration* (MBC)

| Value of MIC | Value of MBC |
|---|---|
| 4.96 % | 19.84 % |

Mechanism of bacterial inhibition or mortality affected by chemical compound may vary, such as cell wall damage, cytoplasm membrane leak, inhibition of enzyme activity and the presence of metabolite and also inhibition of protein syntheses, inhibition against spore formation and growth (Pelczar and Chan, 2005).

## 4. Conclusion

Based on qualitative test result, *A. acuminata* compound contains alkaloid, phenolic, flavonoid, and steroid compounds from methanol extract, and these compounds allow *A. acuminata* to be developed as natural antibacterial.

Methanol raw extract of A. acuminate in all concentrations may inhibit the growth of *V. harveyi* bacteria, yet when the concentration is 100%, its inhibitory capacity activity is higher than other concentrations.

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