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Potential of Patikan Kerbau (*Euphorbia hirta*) as Antibacterial on *Aeromonas hydropilla* and *Vibrio alginolitycus* in Fish Culture

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Abstract

The aims of this study were to find out the potential of Patikan kerbau *(Euphorbia hirta*) as antibacterial on *Aeromonas hydropilla* and *Vibrio alginolyticus*. Phytochemical study on *E. hirta* leaves was carried out to determine the chemical compounds group and resulted in the presence of phenolic, terpenoid and tannin group in the leaves. The antibacterial assay by diffusion agar using paper disc showed that methanol and aqueou extract either of using dried powder leaves boiling method or fresh leaves boiling method were able to inhibit the growth of *Aeromonas. hydropilla* and *Vibrio alginolyticus*. The highest antibacterial activity against those bacteria was shown by methanol extract. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of this methanol extract was found to be 0.156 and 0.625 % by using agar dilution method. In addition, the toxicity study exhibited that treatment of cat fish (*Clarias batrachus*) larvae and humpback grouper (*Cromileptes altivelis*) larvae with *E. hirta* methanol extract at the concentration of 1 % for 5 minutes did not give any toxic effect.

Keywords: *Euphorbia hirta*, Antibacterial, *Aeromonas hydropilla*, *Vibrio alginolyticus*, *Clarias batrachus*, *Cromileptes altivelis*

1. Introduction

Bacterial disease has been a serious problem in fishery culture. *Aeromonas hydropilla* is one of pathogen bacterium which can attack all fresh water fishes. It is the causative agent of *Motile Aeromonas Septicemia* (MAS) resulting in mass mortality in fishery culture (Haniffa & Shanthi, 2012; Maduri, 2012; Erawati & Marsoedi, 2004). Another pathogen bacterium, *Vibrio alginolyticus*, has also attacked fresh and branckish water fishes and has been the causative agent of vibriosis. According to Yuasa *et al.*. (2000) and Dewi *et al.,* (2002), *Vibrio alginolyticus* has been the main problem in fishery culture since it has given mass mortality more than 50 %.

Efforts to overcome these bacterial diseases have used chemicals such as formalin and malachite green as well as antibiotics, such as chloramfenicol, oxytetracyclinn and prefuran (Raffi & Suresh, 2011). Since the bactericides have given a side effect on environment such as can accumulate in water and decrease the aquatic environmental quality and human as a consumer such as transfer pathogen to human, these treatments have become less effective (Nithikulworawong, 2012; Bhuvaneswari, 2012; Haniffa & Shanthi, 2012). Based on the problems, some researchers have attempted other alternative methods to control the diseases.

The rapid growth of knowledge on natural products with various biological activities has provided an alternative to overcome these problems. In recent years, the natural product research has focused the work on isolation and identification of compounds for application mostly in pharmaceutical area. *Euphorbia hirta* is one of plants which is abundant in the tropical area. In Indonesia, *E. hirta* is grown among the grass on the sidewalk, gardens or the grounds of the ungroomed house and found in scattered each other (Adedapo *et al.*, 2005; Arisandi & Andriani, 2006). Patikan kerbau was used as traditional drug in several tropical countres such as Indonesia and Malaysia (Kader *et al.*, 2013; Loh *et al.*, 2009).

Mostly people used *E. hirta* as cure sore throat, bronchitis, asthma, stomach inflammation, dysentery, diarrhoea, blood, urinary inflammations of mammary glands, swollen breasts and eczema (Kader *et al.*, 2013; Arisandi & Andriani, 2006). The ability of patikan kerbau in disease prevention is due to the existence of its bioactive compounds such as Flavanoid and tannin (kader *et al.*, 2013; upadhyay *et al*., 2010; Poornima & Prabakaran, 2012; Shih & Cherng, 2012), saponin and steroid (Kader *et al.*, 2013; Poornima & Prabakaran, 2012), alkaloid and terpenoid (Kader *et al.*, 2013; Upadhyay *et al*., 2010; Shih & Cherng, 2012). *E. hirta* was also reported to have antibacterial (Kader *et al*, 2013; Upadhyay *et al.,* 2010; Jyothirmayi & Prasad, 2011; Titilope *et al*., 2012; Poornima & Prabakaran, 2012; Mamun-or-Rashid *et al.*, 2013; Shih & Cherng, 2012), antifungal (Jyothirmayi & Prasad, 2011), anti-inflammation, antimalaria, antioxidant, anti-tumor, larvacidal and molluscicidal activity (Mamun-Or-Rashid *et al.*, 2013; Shih & Cherng, 2012).

E. hirta has been used for disease prevention on huma. Nevertheless, this plant has not been used as antibacterial on fishery culture. Therefore, to increase the economical value of *E. hirta* and to search a new source of drugd as antibiotics instead in fishery culture it need to study potential of Patikan kerbau as antibacterial on *A. hydropilla* and *Vibrio alginolyticus* through determination of its chemical compound group and toxicity on cat fish (*Clarias*

batrachus) larvae and humpback grouper (*Cromileptes altivelis*) larvae.

2. Materials and Methods

2.1 Collection and Phytochemical Study

E. hirta was collected from Kupang City, East Nusa Tenggara province, Indonesia. Patican *E. hirta* were separated, cleaned and dried under room temperature. Dried *E. hirta* leaves then were ground to powder form. A part of powder form was used for phytochemical study and the rest was used for extraction work. Alkaloid test used Culvenor-Fiztgerald method, saponin test used saponification test and phenolic test was carried oud by addition of $FeCl_3$. Moreover, terpenoid and steroid test was done by Lieberman-Burchard method (Wibowo *et al.*, 2008; Jagessar & Cox, 2010).

2.2 Extraction of E. hirta

Fifty gram of powder form leaves was soaked in 500 mL n-heksane (1 : 10) for overnight and filtered through Whatmann No.1 filter paper fitted in a Buchner funnel using suction. Filtrate obtained was evaporated under reduce pressure by a rotary evaporator (Buchi-type) to get n-hexane crude extract and residue was extracted with 500 mL ethyl acetate for overnight and filtered. Filtrate was then evaporated to yield ethyl acetate crude extract. Residue was again soaked with 500 mL methanol for overnight, filtered and evaporated to give 10 mL metanol crude extract by using vacuum evaporator (Salosso & Jasmanindar, 2009).

A part of dried powder form leaves (50 g) was added with 500 mL distilled water and boiled. Aqueous extract was then precipitated overnight, filltered and evaporated to get 10 mL aqueous crude extract (Salosso & Jasmanindar, 2009). Aqueous extract was also obtained from boiling of cutted fresh *E. hirta* leaves and boiled to be continued filtering through Whatmann No.1 filter paper fitted in a Buchner funnel using suction and collected for concentrated under reduce pressure by a rotary evaporator (Buchi-type) to yield aqueous extract crude extract (Salosso, 2012).

2.3 Antibacterial Assay

All extracts (n-hexane, ethyl acetate, methanol, and both aqueous extracts) of Patikan Kerbau leaves were tested their antibacterial against *A. hydropilla* and *V. alginolitycus* by agar diffussion using paper discs at the concentration of 30 mg/mL. The target bacteria were cultured by using TSA (Triptic Soy Agar) and incubated at 37 ^{0}C for 24 hours. The bacteria are prepared by suspending in 10 ml of sterile water. The concentration of bacteria is adjusted in sterile water to match the density of a 0.5 McFarland Standard. The filter paper discs (6 mm in diameter) were individually impregnated with 20 µL of the all crude extracts (30 mg/mL) and 20 µL of the solven controls (n-hexane, ethyl acetate, methanol, and distilled water) dried in a laminar air flow and then placed onto the TSA plates previously inoculated with the tested microorganisms. The plates were incubated at 37°C for 24 h. The presence of inhibition was indicated by the occurrence of clear inhibition zone around the disc.

2.4 Determination of Minimum Inhibitory Concentration (MIC) and Minium Bactericidal Concentration (MBC)

Methanol extract of *E. hirta* leaves was obtained to give the highest antibacterial activity against *A. hydropilla* and *V. alginolitycus* and was continued to determine its minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) by using agar dilution method. Ten sterile tubes were prepared in which the first tube was added with 10 mL Nutriet Broth (NB), whilst the second until the tenth tube were added with 5 mL Nutrien Broth (NB). The first tube was added methanol extract until reach the concentration of 10 %. From this concentration, a serial two fold dilution was prepared to give the concentrations of 5%, 2.5%, 1.25%, 0.625%, 0.313%, 0.16%, and 0 %. One loop of *A. hydropilla* and *V. alginolyticus* was separately mixtured into NB in the all tube except the ninth and tenth tube. In the ninth tube, it only contained NB whilst, in the tenth tube, it contained NB plus aqueous extract without bacteria. All tube tested were vortexed and incubated at 37°C for 24 hours. After 24 hours, 20 µL filtrate of each tube was spread on TCBSA and incubated at 37°C for 24 hours. The presence of growth inhibition was indicated by the absence of bacterium colony growth on TCBSA. The lowest concentration which was able to inhibit the growth of bacteria until 24 hours and after 48 hours the bacteria regrew was determined as MIC. Meanwhile, the lowest concentration which was able to kill bacteria (no bacteria grew after 48 hours) was determined as MBC.

2.5 Toxicity Test of E. hirta Active Methanol Extract on Cat Fish (C. batrachus) Larvae and Humpback Grouper (C. altivelis)

8-10 cm of cat fish (*C. batrachus*) larvae and 7-10 cm of and humpback grouper (*C. altivelis*) larvae obtained from Balai Benih Ikan Noekele, Kupang and BBAP Takalar, South Sulawesi, Indonesia, respectively were used as tested animals. The culture density was 6 fish/20 liter of water. Acclimatization process was conducted for 3 to 4 days in the rearing water preceding the experiment. On the first day of toxicity test, Fish larvae were treated with the active Patikan kerbau leaves methanol extract at the concentrations of 10.0 %, 1.0 % and 0.1 % in steril sea water 30 ppt. Thereafter, the observation of soaking time was carried out untill fish larvae died. From the data of soaking time which caused died fish larvae was determined the appropriate fish larvae soaking time period for each concentration of the active methanol extract. Experiment was continued on fish larvae treated with the active methanol extract at those concentrations at the soaking time period obtained 5 minutes in which in this time, fish larva did not show any mortality. Fish larvae treated with the active aqueous extract at those concentrations by using bath method were reared in another aquarium with the density of 6 fish/10 L water for 7 days

3. Results and Discussions

3.1 Phytochemical Test

Phytochemical study on *E. hirta* leaves showed the presence of fenolic, flavanoid, tanin and triterpenoid group (Table 1), which have potential to be developed as antibacterial in fishery culture. *E. hirta* collected from different countries has the different bioactive compounds. Flavanoid, tannin and fenolic were obtained from *E. hirta* collected from Malaysia (Kader *et*

al., 2013), Jaipur india (Upadhyay *et al*., 2010) Tamiluede India (Poornima & Prabakaran, 2012) and Cina (Shih & Cherng, 2012). Meanwhile, saponin and steroid were only isolated from Patikan Kerbau collected from Malaisia (Kader *et al.*, 2013) and Tamiluede India (Poornima & Prabakaran, 2012) and alkaloid and terpenoid were found to be contained in *E. hirta* collected from Malaysia (Kader *et al.*, 2013), Jaipur India (Upadhyay *et al*., 2010) and Cina (Shih & Cherng, 2012).

Table 1. Compounds group from *E. hirta* leaves

Compound group	Result
Phenolic	+
Flavonoid	+
Alkaloid	-
Steroid	-
Triterpenoid	+
Saponin	-
Tannin	+

3.2 Antibacterial Activity of E. hirta Extracts

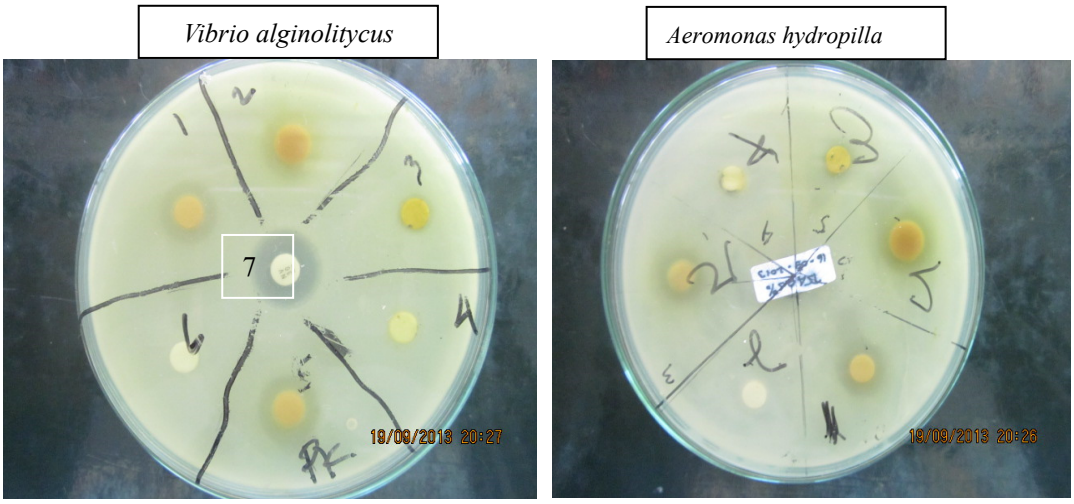
The antibacterial activity test of all extracts from *E. hirta* leaves exhibited that methanol and aqueous extract of this plant were able to inhibit the growth of *A. hydropilla* and *V. harveyi* indicated by the presence of clear inhibition zone around paper disc (Table 2 and Figure 1). The formed inhibition zone is the ability measure of antimicrobe compounds against target bacteria. Inhibition around paper disc is depended on diffusion of antibacterial compounds used. If the antimicrobe compounds has a function for inhibiting, the bacteria growth will stop and give a clear inhibition zone around paper disc after incubation for 18-24 hours (Fadjar *et al*., 2005).

The strong antibacterial activity of plant is commonly effect of solvent polarity used in the extraction process. In this study, methanol extract of *E. hirta* leaves showed the higher inhibition zone than that of aqueous extract (Table 2). It indicated that the antibacterial compounds of *E. hirta* leaves were concentrated in methanol extract. The antibacterial activity of bioactive compounds is caused by several factors such as fuctional group activity, resistence of bacteria on bioactive compounds, concentration of bioactive compounds, and the density of bacteria (Mallawa & Halid, 2006).

Table 2. The antibacterial activity of all extracts of *E. hirta* leaves against *A. hydropilla* and *V.alginolitycus*

No	Extract	*A. hydropilla*	*V.alginolitycus*
1	Aqueous extract of fresh leaves	+	+
2	Methanol extract	+	+
3	n-Heksane extract	-	-
4	Ethyl acetate extract	-	-
5	Aqueous extract of dried powder form leaves	+	+
6	Solvent (-) control	-	-
7	Streptomycine ((+) control)	+	+

Note : +: Inhibition zone; -: No inhibition.



1. Aqueous extract of fresh leaves
2. Methanol extract
3. n-Heksane extract
4. Ethyl acetate extract
5. Aqueous extract
6. Solvent (without extract)
7. Streptomycine (positive control)

Figure 1. Inhibition zone of *E. hirta* extracts on *V. alginolyticus* and *A. hydropilla*

3.3 Determination of Methanol Extract Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Minimum Inhibitory concentration (MIC) and Minimum Bactericidal Concentration (MBC) of *E. hirta* leaves methanol extract by using agar dilution method was shown in Table 3.

From the Table 3 it can be seen that the minimum inhibitory concentration (MIC) of *E. hirta* Leaves methanol extract was 0.156 % on *V. alginolitycus* and *A. hydropilla*. It was indicated by the presence of the target bacteria growth after 48 hours incubation meaning that at this concentration, methanol extract of this plant was bacteriostatic. Meanwhile, minimum

bactericidal concentration (MBC) of this plant methanol extract was 0.625 % indicated by the absence of the target bacteria growth after 48 hours incubation meaning at this concentration, methanol extract of this plat was bactericide. The low MIC value exhibited by this methanol extract showed that *E. hirta* leaves strongly inhibited the growth of both bacteria. The antibacterial activity of *E. hirta* was earlier reported by Hamdiyati *et al.,* (2008). They found that *E. hirta* leaves extract was able to inhibit the growth of *Staphylococcus epidermis* at the concentration of 20 mg/mL. Ogbulie *et al*., (2007) also reported that this plant leaves extract gave an antibacterial against *Staphylococliercus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Bacillus subtilis* at the concentrations of 50, 100, 150, 200, and 250 mg/mL. Furthermore, *E. hirta* leaves extract was also obtained to give an antibacterial activity against *S. aureus* dan *P. aeruginosa* dengan MIC (Minimum Inhibitory Concentration) value of 2 mg/mL (Ngemenya *et al*., 2006). In addition, Assidqi *et al.,* (2012) proved that this plant leaves extract showed a strong antibacterial activity towards *A. hydrophila* in fish culture with the MIC value of 0,156 % and MBC value of 0,312 %.

Table 3. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of *E. hirta* leaves methanol extract

Concentration	***Vibrio alginolitycus***		***Aeromonas hydropilla***	
(%)	MIC	MBC	MIC	MBC
10.000	-	-	-	-
5.000	-	-	-	-
2.500	-	-	-	-
1.250	-	-	-	-
0.625	-	-	-	-
0.313	-	+	-	+
0.156	-	+	-	+
0.078	+	+	-	+
Bacteria control	+	+	+	+
Extract control	-	-	-	-

Note: - Inhibition zone.

3.4 Toxicity Test of E. hirta Leaves Methanol Extract on Cat Fish (C. batrachus) Larvae and Humpback Grouper (C. altivelis) Larvae

Toxicity test of *E. hirta* leaves methanol extract on cat fish (*C. batrachus*) larvae and humpback grouper (*C. altivelis*) larvae was done by using bath method and the survival rate of cat fish (*C. batrachus*) larvae and humpback grouper (*C. altivelis*) larvae is shown in Table 4.

Table 4. Survival rate of cat fish (*C. batrachus*) and humpback grouper (*C. altivelis*) larvae soaked with *E. hirta* methanol extract at different concentrations for 5 minutes

No	Concentration (%)	Survival rate (%)	
		Cat fish larvae	Humpback grouper larvae
1	10.0	0	0
2	1.0	100	100
3	0.1	100	100
4	Control (untreated sample)	100	100

Table 4 displayed that the survival rate of cat fish and humpback grouper larvae treated with *E. hirta* methanol extract at the concentration of 0.1-1.0 % was 100 %. It showed that treating cat fish and humpback grouper larvae with methanol extract at these concentrations for 5 minutes did not give any toxic effect. It indicated that *E. hirta* leaves methanol extract may be an environment friendly antibacterial compounds source in fishery culture. As reported by Pelczar and Chan (2005) that one of ideal antibacterial characteristics is no toxic effect on test animal but it may kill pathogen microorganisms.

4. Conclussion

E. hirta leaves has the potential as an antibacterial compounds source in fish culture as its methanol extract showed a strong antibacterial activity against *A. hydropilla* and *V. alginolitycus in vitro*. The phytochemical study exhibited the presence of phenolic, tannin, and triterpenoid in *E. hirta* leaves. The antibacterial activity assay showed that methanol extract of this plant give the highest antibacterial activity against *A. hydropilla* and *V. alginolitycus* with the MIC and MBC value of 0.156 and 0.625 %, respectively. This methanol extract has no any toxic effect on cat fish and humpback grouper larvae at the concentrations of 0.1 and 1% for 5 minutes of bath method.

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