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THE INFLUENCE OF *Acanthus ilicifolius* EXTRACTS TO HISTOPATHOLOGICAL ON HEPATOPANCREAS OF TIGER SHRIMP (*Penaeus monodon* F.)

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ABSTRACT

Assesing the influence of the leaf extract to histopathological on hepatopancreas of shrimp as protection from *Vibrio harveyi*. Crude, ethyil acetate, and n-butanol fraction was given by dipping method. The best survival was the fraction of n-butanol 300 ppm, followed by n-butanol 200 ppm and 700 ppm etyl acetate fraction. It reduced the prevalence of attacks and improved the survival of shrimp. The best pathophysiological and pathological anatomy of shrimp was n-buthanol fraction, followed etyl acetat fraction and then crude. Histopathological of hepatopancreas was normal on n-butanol fraction, normal and minor in damage on etyl acetat fraction and crude, and minor to moderate damage on antibiotic

Key words: *Acanthus ilicifolius*, *Vibrio harveyi*, histopathological, hepatopancreas

INTRODUCTION

Vibrio bacteria are pathogens that can attack at any stadia shrimp, which causes high mortality. *Vibrio* attack can lead to death on larvae to adult shrimp, when it is cultivated grow-out pond (Kumaravel *et al*., 2010). *Vibriosis* in shrimp showed clinically symptoms of reddish-black, and some outside organs looked red, especially on the gills and limbs (Saptiani *et al*., 2012 [b]). Shrimp will looked light up at night or in a dark environment, then the disease severe attack is followed by occurrence of mass mortality. Antibiotics and chemicals are often used to treat the disease, which can lead to resistance. These conditions must be very detrimental to the surrounding farms and sustain the production and product trade. Bioactive compounds from natural ingredients can be done as an alternative to tackle the disease on fish, shrimp, and aquatic biota (Saptiani *et al*., 2012[a]). Bioactive of mangrove *Acanthus ilicifolius* that grows in the surrounding of ponds can be used for immunostimulan on shrimp. This plant contains glukosid, alkaloids, flavonoids, fatty acids, steroids, lignans, phenols and terpenoids components (Kanchanapoom *et al*., 2001; Wostmann and Liebezeid, 2008). Bioactive of *A. ilicifolius* is used to improve the immunity of shrimp and also safe to be used around the grow-out ponds environment. The purpose of this study was to determine the influence of *A. ilicifolius* leaf extract to the pathophysiological, pathological anatomy and histopathological of shrimp which was infected by *V. harveyi*.

MATERIAL AND METHOD

Extraction and Fractionation of Acanthus ilicifolius

A. ilicifolius was taken from the area of aquaculture in the sub district of Muara Badak Kutai regency of East Kalimantan, Indonesia. *A. ilicifolius* leaves were cleaned, washed with fresh water, drained and then chopped. Furthermore, the leaves were dried in a room that was not exposed by direct sunlight with temperatures 30 ^{0}C. The leaves were extracted by storing them in jar and macerated in methanol. The macerated result was extracted with evaporation method according to Aknin *et al*. (1999) and Manilal *et al.* (2009). The extraction result was taken partly to do fractionation by using silica gel with column method. Solvents used were n-hexane, etyl acetate, and n-butanol.

Shrimp Preparation

The shrimps were used as experimental measuring about 3 grams and came from Basuki Hatchery in Muara Badak District, Kutai Kartanegara Regency of East Kalimantan. Before bred, the healthy shrimps were not given antibiotics, chemical substances and other medicine. The healthiest and motile larvae in PL 7 stadia from the hatchery were chosen and cultivated in a pond until they grew 3 grams in weight. Furthermore, the healthy shrimps were selected, and taken to the laboratory, inserted into the aquarium to be adapted.

Treatment and Observation

The treatment consists of 3 active materials: crude extract (200, 450, 700 ppm doses), ethyl acetate fraction (200, 450, 700 ppm doses), n-butanol fraction (100, 200, 300 ppm doses), negative control (PBS) and positive control (antibiotic), with three replications of each. The shrimps were put in 33 aquariums (10 shrimps in each) and acclimated for 3 days. Then the treatment of extracts and fractions of *A. ilicifolius* were given by dipping or immersion method, and on the 7th day challenge test with 10^5 cfu/ml dose of *V. harveyi* as much as 0,1 ml was conducted intra-muscularly on dorsal part.

Observation and inspection were covered the pathophisiological, mortality, pathological anatomy and histological of hepatopancreas tissue. Pathophysiology was

done by observing changes in behavior, activity, reflex, appetite, and completeness of the shrimps' body. Mortality was also observed and calculated based on the percentage of the number of dead shrimps every day. Pathological anatomy and histopathological hepatopancreas were examined on the 7th, 14th and 21st day. Pathological anatomy observations were done by observing a change of color, shape, organs consistency as well as abnormal changes. Histopathological hepatopancreas were observed by hepatopancreas preparation. After it was fixed in Davidson solution and then followed with the test procedure by Lightner (1996).

RESULT AND DISCUSSION

Pathophysiological, indicate the color was changed to be more blue on the shrimps' carapace, after 2-4 days of the extract treatment, especially in the n-butanol fraction. The color change of the shrimps was caused by enlargement of pigment or cuticle chromatophore of the shrimps. The color change indicated the reaction of shrimp immunity against the presence of foreign into the body. Discoloration is also a sign of a foreign into the body of the shrimp, because shrimp chromatophore is one of the body's defense patterns on shrimp (Saptiani *et al*., 2012 [b]). After 4 days of extract treatment, shrimps' body is back to normal. Pathophisiologicals of shrimp after challenge test showed that the shrimps on the extract treatment was better than the negative control. Shrimp mortality occurred on the 14th day in the *A.ilicifolius* leaf extract treatment, namely 7,33 to 12 %, 12% in the positive control (antibiotic) and 20,33% in the negative control. Furthermore, on the 21th day in the *A.ilicifolius* leaf extract treatment was 10 to 22.33%, 22.00% in the positive control and 42.33% in the negative control. *A.ilicifolius* leaf extract could reduce shrimp mortality against *V. harveyi* compared to the control treatment. *A.ilicifolius* leaf extract could inhibit the growth of *V. harveyi* and protect the shrimsp from *V. harveyi* attacks. Inhibition and protection from *A.ilicifolius* leaf extracts were better than the antibiotics. The bioactive of *A. ilicifolius* leaves extract can be used as antibacterial to against *V. harveyi* (Saptiani *et al*., 2012[a]; Saptiani *et al*., 2013). Mechanism of antimicrobials in killing or inhibiting the growth of microbes was inhibiting cell wall synthesis, protein synthesis and nucleic acid synthesis and inhibits the function of the cell membrane (Jawetz *et al*., 1989).

Vibrosis symptoms in the negative control began to appear on the 10th day or 3 days after the infectition. The symptoms included changes appearance of reddish black, red patches on the legs and tail, haemorhagi on the body, deformity and molting death, as could be seen in Table 2. Transmission of bacteria was carried by immersion, so the bacteria entered through the phase of adhesion and penetration into the gills, mouth and carapace to reach the target organ. It took time to get to the habitat and to multiply. In addition, the shrimp also have a defense system against pathogens. If the shrimp health was decreased and water quality changes, it will cause physiological disturbances by lowering the immune system and susceptibility to pathogen infection (Saptiani, 2001: Saptiani *et al*., 2008). *A.ilicifolius* leaf extract and fractions are safe and does not cause problems on the shrimp, this is evidenced by the absence of death and specific pathophisiologicals (Saptiani *et al*., 2012 [b]).

Observations of anatomical pathology on the 14th day showed that the shrimp hepatopancreas of negative control changed color to brown and mushy were smaller. The hepatopancreas and stomach became reddish and hardened. Acutely infected shrimp showed a very rapid decrease in feed consumption and became lethargic. It was suspected that pathogens already developed in the body and interfered the metabolism, thus affecting the immune system; decreased appetite and the shrimp became lethargic and over time will die. Immersion of shrimp in the *V. harveyi* solution would lead to that the bacteria transferred from the mouth to the posterior part of the alimentary canal. The presence of bacteria in the digestive system of shrimp hepatopancreas was interfered because the bacteria broke down various polysaccharides and carbohydrates, took the necessary nutrients shrimp, and caused the death of the shrimp. Austin and Zhang (2006), *V. harveyi* on fish cause vasculitis, gastro-enteritis and lesions of the eye, while the shrimp pathogens are often associated with vibrioluminous. Pathogenic bacteria associated with its extracellular products, such as protease, haemolysin and lipopolisaccharide. In the treatment of leaf extracts and fractions of *A.ilicifolius*, it changed pathological anatomy getting better, though initially there was some shrimp hepatopancreas brownish change. This indicated that the bioactive of *A.ilicifolius* leaves could improve the immune system of shrimp, so as to inhibit infection and protect shrimp from *V. harveyi* attack.

Histopathological of hepatopancreas on the negative control was indicating a change at the bacteria-infected cells, with signs of cytopathic effect (CPE) formation, hyperplasia and necrotic. The CPE could be cell size increasing (hypertrophy), cell shrinking (atrophy), and cell fusion. Including cell necrosis, nuclei were enlarged and moved to side, granulated of nucleus and lysis. Hyperplasia was cell structure that became irregular. Besides, bacterial granulomas that formed bacterial colonies were found on the tissue. Haemopoiesis also occurred. It is the expenditure of haemocyte cells into hepatopancreas tissues. The occurrence of cell fusion in the treatment of *A.ilicifolius* leaf extract was including minor to normal criteria. Cell fusion occurred due to the loss of cell boundaries, so that the cells underwent cell fusion where two cells fused to form a cell and made its appearance larger than the other cells. Besides that, the nucleus of the cell swelled and retained cells fluid because it exceeded the tolerance emphasis of cell wall elasticity, and then the cell would be broken. The nucleus cell would out of the cells fluid together with the other cells that were next to them and to be cell debris. *A.ilicifolius* extracts could overcome the attack of bacteria that did not cause damage to cells and hepatopancreas tissues.

Necrosis began with the enlargement of the cell nucleus, and then pressed to the side until the karyolysis. Swollen nucleus would suppress the liquid cell and caused the cell to burst. The mixed rupture nucleus cell with the nucleus adjacent to another rupture formed debris cell. It caused damage to cells, tissues and organs function so that ultimately caused the death of the shrimp. Bell (1988) stated that the nuclei of normal cells that have been infected by visible light reddish, because the nucleus of eosinophilik was so absorbing dye eosin. Where as in the cells that have been in serious infected became visible dark blue because it was so absorbing dyes basophilik haematoxylin. Necrose is a level of the mayor damage cells, but if the number of cells undergoing necrose of the hepatopancreas tissue is still below 25%, then the extent of tissue damage is minor. Because this is the cell and tissue property that constantly regenerate, so it does not disturb the work of an organ. In accordance with the opinion of Sudarto (1988) and Lightner (1996), that the criteria for an organ or tissue damage were based on the level of cell damage and the extent of tissue damage.

A. ilicifolius leaves extract caused a few hemocytes infiltrating into some parts of the hepatopancreas or midgut. In case of an extract treatment, by the 7th day, *V. harveyi* induced an increase in hemocyte numbers in the intertubular tissue of the hepatopancreas compared to the control tissue. Extract of *A. ilicifolius* leaves could inhibit the growth of *V. harveyi* which could overcome the attacks of bacteria and did not change the hepatopancreas tissue. According of Manilal *et al.* (2009), *A. ilicifolius* is vibriosidal with the inhibition of three vibrio species, namely *V. Alcaligenes* (8 mm), *V. vulnificus* (9 mm), and *V. alginolyticus* (10 mm). *A. ilicifolius* mangrove contains bioactive compound that is potential to use as antibacterial agent (Manilal *et al*., 2009; Khajure and Rathod 2010; Thirunavukkarasu *et al*., 2011; Saptiani *et al*., 2012 [a]). Saptiani *et al*. (2012 [a]) stated that the leaf extract of *A. ilicifolius* is the best compared to the stem, fruit and flowers, with a 12 mm zone of inhibition. Soonthornchai *et al.* (2010), generally, stated that *V. harveyi* is highly pathogenic to post larvae of shrimp and causes damage to the front of the hepatopancreas and intestine. Hepatopancreas damaged at epithelial cells, hyperplasia and necrotic. Extract of *A. ilicifolius* leaves can be used as an antibacterial and immunostimulant for shrimp culture in the hatchery or in cultivation ponds. According to Bray *et al.* (2006), excessive use of antibiotics oxytetracycline will cause damage on the hepatopancreas tubules and necrotic. Herbs are alternative that can be used in the culture system because they have growth promoting ability and a tonic to improve the immunity system and an appetite stimulant, increase consumption, stimulate maturation and have the capacity as antibacterial and antistress when they are used in shrimp or fish farming without causing trouble and disturbing environment (Citarasu, 2009).

A.ilicifolius leaf extract could be used as immunostimulant on shrimp to *V. harveyi* attacks and did not cause any vibrosis symptom. It could reduce mortality of shrimp against *V. harveyi*. *A.ilicifolius* leaf extract could inhibit the growth of *V. harveyi* and protect shrimp from *V. harveyi* attacks. Anatomic pathology of hepatopancreas and any other organs were normally. Histopatological of hepatopancreas were normal, except for 200 ppm crude there was minor damage.

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ATTACHMENT

Figure 1

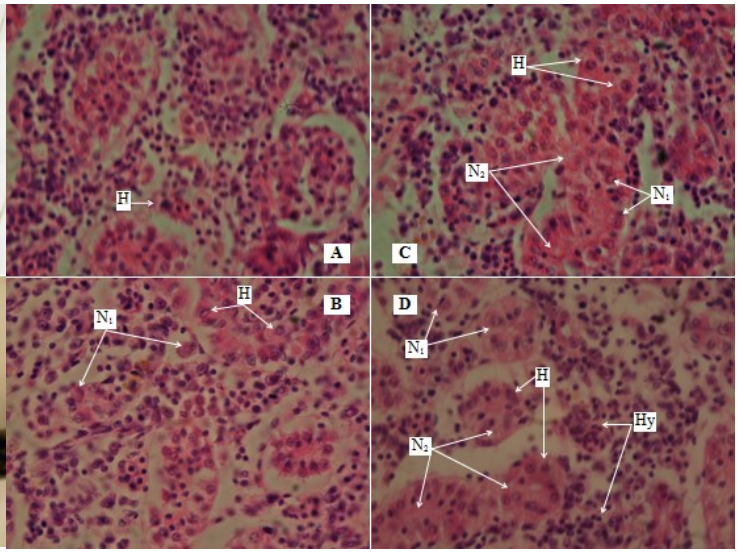

Figure 2

Figure 1. Pathophysiological of the shrimp on day-14 (1) and day 21 (3), head of the shrimp (2), Hepatopancreas of shrimp infected with *Vibrio harveyi*, smaller, brown and mushy consistency (4). Description: B= n-Butanol, etyl Acetate (E), Crude (C), positive control (K^+), and negative control (K^-).

Figure 2. Hepatopancreas on the n-Butanol (A), etyl Acetate (B), Crude (C) and negative control (D). Description: H = hypertrophy; N_1 = enlarged cell nuclei and to the side; N_2 = cel lysis Hy = hyperplasia. Magnification 10 x 100, enlarged 50%. Smear: HE, fixatives: Davidson, Thickness: 8µ.

Table 1. The average mortality of shrimp

Treatment	Mortality (%)	
	14th	21th
Crude 200 ppm	12,00	22,33
Crude 450 ppm	10,67	17,67
Crude 700 ppm	9,33	15,67
Etyl acetate 200 ppm	9,33	16,00

Etyl acetate 450 ppm	9,00	15,33
Etyl acetate 700 pp m	9,00	11,33
n- Buthanol 100 ppm	9,33	13,33
n- Buthanol 200 ppm	9,00	10,67
n- Buthanol 300 ppm	7,33	10,00
Negative control	20,33	42,33
Positive control	12,00	22,00

Table 2. The average of pathophisiologicals shrimp

Treatment	Day	Pathophisiological			
		Completeness body	Appetite	Activity/reflex	Body condition
Crude 200 ppm	7	Normal	Good	Active	Good
	14	Normal	Decline	Lethargic	Blackish
	21	Normal	Decline	Lethargic	Good
Crude 450 ppm	7	Normal	Good	Active	Good
	14	Normal	Decline	Lethargic	Good
	21	Normal	Good	Active	Good
Crude 700 ppm	7	Normal	Good	Active	Good
	14	Normal	Good	Active	Good
	21	Normal	Good	Active	Good
Etil asetat 200 ppm	7	Normal	Good	Active	Good
	14	Normal	Good	Active	Good
	21	Normal	Good	Active	Good
Etil asetat 450 ppm	7	Normal	Good	Active	Good
	14	Normal	Good	Active	Good
	21	Normal	Good	Active	Good
Etil asetat 700 ppm	7	Normal	Good	Active	Good
	14	Normal	Good	Active	Good
	21	Normal	Good	Active	Good
n-butanol 200 ppm	7	Normal	Good	Active	Good
	14	Normal	Good	Active	Good
	21	Normal	Good	Active	Good
n-butanol 450 ppm	7	Normal	Good	Active	Good
	14	Normal	Good	Active	Good
	21	Normal	Good	Active	Good
n-butanol 700 ppm	7	Normal	Good	Active	Good
	14	Normal	Good	Active	Good
	21	Normal	Good	Active	Good
Negative control	7	Normal	Good	Active	Good
	14	Failed moulting	Decline	Lethargic	Redness
	21	Deformity	Decline	Lethargic	Haemoraghi
Positive control	7	Normal	Good	Active	Good
	14	Normal	Decline	Active	Reddish black
	21	Normal	Good	Active	Good

Table 3. The average of pathological anatomy of shrimp hepatopancreas

Treatment	Day	Organ damage Criteria			
		Shape	Color	Consistency	Other changes of organ
Crude 200 ppm	14	Minor	Minor	Normal	Gills reddish
	21	Normal	Minor	Normal	-
Crude 450 ppm	14	Normal	Minor	Normal	-
	21	Normal	Normal	Normal	-
Crude 700 ppm	14	Normal	Normal	Normal	-
	21	Normal	Normal	Normal	-
Etil asetat 200 ppm	14	Normal	Minor	Normal	-
	21	Normal	Normal	Normal	-
Etil asetat 450 ppm	14	Normal	Normal	Normal	-
	21	Normal	Normal	Normal	-
Etil asetat	14	Normal	Normal	Normal	-

700 ppm	21	Normal	Normal	Normal	-
n-butanol	14	Normal	Normal	Normal	-
200 ppm	21	Normal	Normal	Normal	-
n-butanol	14	Normal	Normal	Normal	-
450 ppm	21	Normal	Normal	Normal	-
n-butanol	14	Normal	Normal	Normal	-
700 ppm	21	Normal	Normal	Normal	-
Negative	7	Moderate	Mayor	Mayor	Gills reds
control	14	Mayor	Mayor	Mayor	Gills and stomach reds
Positive	14	Minor	Minor	Normal	Gills reddish
control	21	Minor	Normal	Normal	-

Table 4. The average of damage cells and hepatopancreas tissues of shrimp

Treatment	Day	The level of cell damage		The level of tissue damage		
		Cell damage	criteria	Score (%)	extent of damage	Criteria
Crude 200 ppm	14	- CPE - hyperplasia - necrosis	Mayor	46,67 33,33 13,33	Minor Minor Normal	Minor
	21	- CPE - hyperplasia - necrosis	Mayor	43,33 26,67 6,67	Minor Minor Normal	Minor
Crude 450 ppm	14	- CPE - hyperplasia	Moderate	26,67 10	Minor Normal	Minor
	21	- CPE	Minor	16,67	Normal	Normal
Crude 700 ppm	14	- CPE	Minor	10	Normal	Normal
	21	- CPE	Minor	8,33	Normal	Normal
Etil asetat 200 ppm	14	- CPE - hyperplasia	Moderate	33,33 13,33	Minor Normal	Minor
	21	- CPE	Minor	16,67	Normal	Normal
Etil asetat 450 ppm	14	- CPE	Minor	13,33	Normal	Normal
	21	- CPE	Minor	13,33	Normal	Normal
Etil asetat 700 ppm	14	- CPE	Minor	10	Normal	Normal
	21	- CPE	Minor	6,67	Normal	Normal
n-butanol 200 ppm	14	- CPE	Minor	13,33	Normal	Normal
	21	- CPE	Minor	13,33	Normal	Normal
n-butanol 450 ppm	14	- CPE	Minor	10	Normal	Normal
	21	- CPE	Minor	6,67	Normal	Normal
n-butanol 700 ppm	14	- CPE	Minor	6,67	Normal	Normal
	21	- CPE	Minor	6,67	Normal	Normal
Negative control	14	- CPE - hyperplasia - necrosis	Mayor	76,66 33,33 53,33	Mayor Minor Moderate	Mayor
	21	- CPE - hyperplasia - necrosis - granuloma	Mayor	75 50 63,33	Mayor Moderate Moderate	Mayor
Positive Control	14	- CPE - hyperplasia	Moderate	46,67 13,33	Moderate Normal	Moderate
	21	- CPE - hyperplasia - necrosis	Minor	33,33 13,33 6,67	Moderate Normal Normal	Minor