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A comparative study on the efficacy of mixed tannins, hydrolysable tannins, and condensed tannins of *Avicennia marina* as anti-ectoparasite against *Trichodina* sp.

[1]Musri Musman, [1]Aulia Rahmad, [1]Irma Dewiyanti, [2]Chairin Sofia, [2]Hendro Sulistiono

[1] Faculty of Marine and Fishery, Syiah Kuala University, Darussalam-Banda Aceh, Indonesia; [2] Brackish Water Aquaculture Centre, Ujung Batee-Aceh Besar, Indonesia.
Corresponding author: M. Musman, musrimusman@gmail.com

Abstract. The research objective was to compare the efficacy of mixed tannins, hydrolysable tannins and condensed tannins from *Avicennia marina* leaves against protozoan ectoparasite *Trichodina* sp. Mixed tannins obtained from the leaves of *A. marina* was further separated into hydrolysable tannins and condensed tannins. The condensed tannins were to be highly toxic (LC_{50} = 19.82 ppm) to *Trichodina* sp. compared to both hydrolysable tannins (LC_{50} = 61.76 ppm) and mixed tannins (LC_{50} = 64.81 ppm) displayed also toxic to the ectoparasite. The results addressed that condensed tannins have a good prospect to be used as an agent for controlling the ectoparasitic diseases in fish.
Key Words: Grey mangrove, white mangrove, LC_{50}, ciliate protists, protozoa, ectoparasite control.

Introduction. Tilapia (*Oreochromis niloticus*) has been cultivated in several countries, including Indonesia. Tilapia can be cultured in a variety of habitats (in fresh, brackish, and marine water) due to its euryhaline nature. Hence, it can be a subsistence level to meet local protein needs and move the mainstream seafood markets (FAO 2014). An inhibiting factor in tilapia fish farming is a trichodina disease that cause many deaths addressed by parasitic protozoa groups, especially the type of *Trichodina* sp. Symptoms of infected fish among others are a white spots on their skin and gills, gasp for air at the surface, flashing, scraping, and weight loss. The fish are showing signs of damage to the skin are often accompanied by secondary infections (Hendrick 1998).

The ectoparasite *Trichodina* sp. has shape likes a plate or a hat that covered his body cilia tip section. Usually *Trichodina* sp. attack to the outer part of the body organs such as the skin, fins, and gills through denticle organs that functions as suction on the host infestation. *Trichodina* sp. uses cilia to migrate from host to host where the parasite lives and foraging (Lom & Dykova 1992). Protozoan parasite control can be done by breaking the cycle of life. To break the cycle of life, the fish after treatment was quarantined for 3 days at a temperature of about 25-27°C, then transferred it to the place of cultivation.

The use of synthetic chemical-based antiparasitics against ectoparasites infection it is already clear that is not recommended, because the synthetic antibiotics can cause resistance to the pathogens. Therefore, a natural antibiotic substance to control this disease should be sought. A number of experts are eager to study natural substances derived from the mangrove to lead to the discovery of bioactive compounds which can be used for pharmaceuticals, antibiotic substances, and feed. The studies shown constituents of *Avicennia marina* leaf, which contain alkaloids, terpenoids, saponins,

glycosides, flavonoids, and tannins (Poompozhil & Kumarasamy 2014; Mouafi et al 2014).

Plant tannins based on the building block of their chemical moieties are grouped into two main classes, i. e. hydrolysable tannins, and condensed tannins (Ribereau-Gayon 1972; Sarkar & Howarth 1976; Hahn et al 1984; Khanbabee & Ree 2001). Hydrolysable tannins are hexahydroxydiphenic acid esters of glucose or other polyols whereas condensed tannins are flavonoid polymers (Haslam 1979). The most common modes of tannins action are interference with the cell membrane and cell wall, interference with nucleic acids and enzyme interactions (Mukhopadhyay & Peterson 2006; Hugo & Russell 1982; Tenover 2006). Tannins have ability to bind with protein (Bate-Smith & Swain 1962; Hahn et al 1984) and preserve animal hides (White 1957; Maxson & Rooney 1972). Tannins have shown antibacterial (Banso & Adeyemo 2007), anticarcinogenic, antimutagenic, antimicrobial (Cos et al 2004; Awika et al 2006), antioxidant and antiradical (Amarowicz et al 2004; Alasalvar et al 2006) properties.

Research on an efficacy of mixed, hydrolysable, and condensed tannins of *A. marina* against ectoparasites *Trichodina* sp., as far as authors knows based on the literatures searching has not been done. Therefore, study to control ectoparasite using the tannins extracts of *A. marina* against *Trichodina* sp. needed.

Material and Method

Identification of organism. The host of ectoparasite *Trichodina* sp. was tilapia (*O. niloticus*) captured using nets at the Kuta village (coordinate 5° 57' 41" N 95° 33' 08" E), Lamprit Subdistrict, Banda Aceh. The fishes taken for this study were the size of 3-7 cm, amounting to 240 fishes was attacked by the ectoparasites. Ectoparasite *Trichodina* sp. found on the outside of the body organs of fish was identified (Noga 2010; Purwanti et al 2012). Scraping mucus from skin surface of infected fish on both sides of the fish with a cover glass, and then placed it on an object glass and spilled some water, and observed using a Nikon Eclipse E200 microscope. After soaking 48 hours, the fish was put in a container with clean water so that the fish fresh back. *Trichodina* sp. found in fish body was observed to approve its death due to exposure the treated extracts.

Plant material. 1.4 kg of *A. marina* fresh leaves collected at Gano village (coordinate 5° 58' 67" N 95°32' 94" E) of Syiah Kuala Subdistrict, Banda Aceh were dried under the sunbeam for 5 days to give 750 g dried material. The amount of moisture removed was calculated and the sample was then stored in a herb room at 10°C with a relative humidity of less than 50% until used.

Isolation of the A. marina-leaf extract. Isolation of the dried material was run at the chemical laboratory of Teacher Training and Education Faculty of Syiah Kuala University, Banda Aceh. 750 g of *A. marina* dried leaves were ground in a blender to get the powder in 40 meshes. 250 g of the powder was transferred to dark-colored flasks, poured 1500 mL of 96% (v/v) methanol on the powder and then let to rest for 24 hours. Afterwards, the slurry was filtered through Whatman #1 filter paper. The filtrate was evaporated to dryness under vacuum at 65°C. 13.4 g of the crude extract was obtained as the mixed tannins (MTs) and stored at 4°C until further analyses.

Separation of a mixed tannins using Sephadex LH-20. Method to separate the mixed tannins was based on Hagerman (2002) method. 4 g of the mixed tannins were suspended in 5 mL of 95% (v/v) ethanol and then applied to a chromatographic column (45×180 mm) packed with Sephadex LH-20 that had been equilibrated with 95% (v/v) ethanol. The column was rinsed with 2500 mL of ethanol 95% (v/v) to get hydrolysable tannins, and condensed tannins were eluted from the column using 1500 mL of 65% (v/v) acetone.

Test for tannins and phenolic compounds. About 2 mg of each of the extract was boiled with 10 mL of water for 5 minutes, then cooled and filtered.

Lead acetate test. To each of the 1 mL aliquot extracts, 5 drops of 1% lead acetate

solution was added. The formation of white precipitate indicated the presence of tannins (Kokate 1997).

Ferric chloride test. To 1 mL aliquot of each of the extracts, 5 drops of 5% ferric chloride solution was added. Hydrolysable tannins forms bluish-black precipitate whereas condensed tannins forms greenish-brown precipitate (Jain et al 2013).

Gelatin test. To 1 mL aliquot of each of the extracts, 5 drops of 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicated the presence of tannins (Tiwari et al 2011).

Toxicity tests. Bioassays were conducted in the laboratory of Brackish Water Aquaculture Center (BBAP) Ujong Batee, Aceh Besar District in four replications for each experimental unit in a completely randomized design using tannins extracts of *A. marina* against ectoparasite *Trichodina* sp. The tannins were dissolved in water to make concentration 60-100 ppm (mixed tannins experimental unit), 50-90 ppm (hydrolysable tannins experimental unit), 10-50 ppm (condensed tannins experimental unit) on the basis of preliminary testing. Ten individuals *Trichodina* sp. were introduced into a 0.5 L vessel containing 400 mL of treated extract. Mortalities were recorded 48 hours later. To verify that *Trichodina* sp. lives normally, a control was prepared and used in the same condition. Mortality was recorded 48 h after treatment and the mortality was defined as the body structure of *Trichodina* sp. stunted (Ajizah 2004).

The main parameters observed in this study were *Trichodina* sp. attacked to the body of the fish by looking under a microscope and the extracts. The physico-chemical water parameters, i. e. pH, temperature, dissolved oxygen were also monitored.

Statistical analysis. Data analyses were performed with SPSS version 18.0. Prior to analysis all data were transformed using arcsin square root transformation in order to reach the assumptions of the analysis of variance (ANOVA). The results of the acute toxicity experiments were analyzed for each experimental unit separately, using a one-way ANOVA followed by Duncan's test at 5% of significance. For each experimental unit, the four replicates used for each extracts concentration yielded a mortality percentage. The data obtained in the form of dead parasites due to exposure the tannins of *A. marina* then analyzed using Trimmed Spearman-Karber (TSK) program version 1.5 to calculate LC_{50} with confidence intervals at 5% level. Means are given ± SE.

Results and Discussion. The results shown in Table 1 revealed that the tannins of *A. marina* have significant effect on mortality of protozoan ectoparasite *Trichodina* sp. attacking tilapia (*Oreochromis niloticus*) where p = 0.05. Mortality of ectoparasites *Trichodina* sp. increased with increasing concentration of the given extract on each experimental unit. Mortality data of *Trichodina* sp. after exposure the tannins of *A. marina* was incorporated into the program TSK version 1.5 to obtain results LC_{50}.

A one-way between treatments ANOVA was conducted to compare the effect of tannins of *A. marina* on mortality of ectoparasite *Trichodina* sp. There was a significant effect of concentration of mixed tannins [F(5, 18) = 126.9, p = 0.0], hydrolysable tannins [F(5, 18), 119.1, p =0.0], and condensed tannins [F(5, 18) = 322.8, p = 0.0] on mortality of ectoparasite *Trichodina* sp. at the p = 0.05 level for the six conditions of each experimental unit. Post hoc comparisons using the Duncan's test indicated that the mean for the control condition was significantly different than the other concentrations for each experimental unit.

Mortality data of *Trichodina* sp. were analyzed using the TSK program version 1.5 in order to get LC_{50} (median lethal concentration) values of tannins of *A. marina* against ectoparasite *Trichodina* sp. on tilapia. The LC_{50} values of mixed, hydrolysable, and condensed tannins of *A. marina* to *Trichodina* sp. on tilapia were 64.81, 61.76, and 19.82 ppm, respectively. The values indicated that the condensed tannins were highly toxic to *Trichodina* sp. than hydrolysable tannins and mixed tannins. The highly toxic condensed tannins to *Trichodina* sp. were due to condensed tannins most ready precipitate proteins at pH values near the isoelectric point of the proline-rich proteins which have a very high affinity for tannins (Hagerman 1989; Hagerman & Butler 1980). However, the loss of

conformational mobility of intramolecular biphenyl linkages in mixed tannins and hydrolysable tannins reduce capacity to bind to protein (Haslam & Lilley 1985). The death of *Trichodina* sp. in the treatment was due to the presence of tannin-toxic substances of *A. marina*. Therefore, the tannins of *A. marina* can act as an anti-ectopasite against *Trichodina* sp.

Table 1

Percent mortality and LC_{50} of *Trichodina* sp. due to introduce mixed, hydrolysable, and condensed tannins of *Avicennia marina* in 48 hours exposure time

Extract concentration	*% Mortality (mean±SE*, n = 40)*	*A one-way ANOVA result*	*Median lethal concentration value*
MTs (ppm)			
Control	0^a	There was a significant effect of MTs on mortality of *Trichodina* sp. at the p = 0.05 for the six treatments [F(5,18) =126.9, p_{sig} = 0.0]	LC_{50} [confidence interval, p=0.05](ppm)/48h = 64.81 [54.74-76.73]
60	4.3 ± 0.3^b		
70	5.8 ± 0.6^c		
80	8.0 ± 0.4^d		
90	9.8 ± 0.3^e		
100	10^e		
HTs (ppm)			
Control	0^a	There was a significant effect of HTs on mortality of *Trichodina* sp. at the p = 0.05 for the six treatments [F(5,18) =119.1, p_{sig} = 0.0]	LC_{50} (confidence interval, p=0.05](ppm)/48h = 61.76 [50.98-74.82]
50	2.5 ± 0.3^b		
60	4.5 ± 0.3^c		
70	5.5 ± 0.3^c		
80	6.8 ± 0.3^d		
90	7.5 ± 0.3^d		
CTs (ppm)			
Control	0a	There was a significant effect of CTs on mortality of *Trichodina* sp. at the p = 0.05 for the six treatments [F(5,18) =322.8, p_{sig} = 0.0]	LC_{50} (confidence interval, p=0.05](ppm)/48h = 19.82 [11.46-34.26]
10	3.3 ± 0.3^b		
20	4.8 ± 0.3^c		
30	6.3 ± 0.3^d		
40	8.8 ± 0.3^e		
50	10^f		

*Means followed by same letter are not significantly different at 5% level (Duncan's test following ANOVA).
MTs - mixed tannins, Hts - hydrolysable tannins, CTs - condensed tannins.

Observation of the behavior of infected tilapia *Trichodina* sp. showed that at 15 minutes after contact to tannins tilapia look more active, where the movement was faster and openings the cover of the operculum was also faster than usual. This is caused by the reaction caused by exposure to toxic substances such as tannins in the test container. Compared with controls, tilapia was seen moving calm with normal operculum openings. The value of physico-chemical parameters of the water in this study, i.e. temperature was in range 28°-30°C, dissolved oxygen 5.1-7.8 mg/L, and pH 6.8-8.9. The value range is not much different from the control, i.e. temperature was in range of 29°-30°C, dissolved oxygen 6.2-8.5 mg/L, and pH was in range of 7.5-9.0 in which the *Trichodina* sp. found as much as 100% live. These clues can be stated that the death of *Trichodina* sp. due solely to tannins of *A. marina*.

Observation under the microscope showed the differences of the body structure of *Trichodina* sp. in the living and the dead form as shown in Figure 1. The body structure of dead *Trichodina* sp. showed the organs were not intact and the body was more faded (B) than the alive (A). Tannins have the ability to interact with and precipitate proteins via

the formation of cross links between collagen fibers in animal skins (Gupta & Haslam 1980). The deformations due to tannins action to disorder and to damage of cell membrane (Akiyama et al 2001) disrupted the permeability of the cell itself.

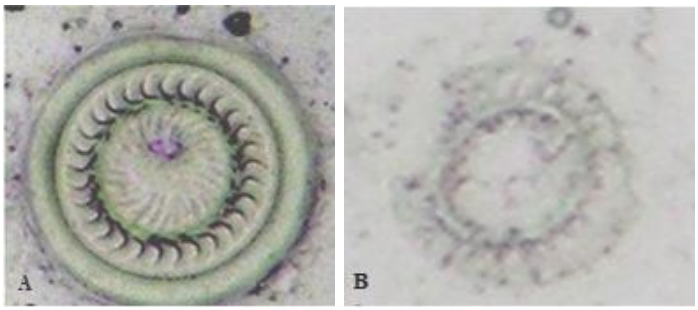

Figure 1. *Trichodina* sp. in alive form (A) and in a dead form (B) after exposure of the tannins extracts.

Conclusions. The condensed tannins been proved to be highly toxic against ectoparasite *Trichodina* sp. than the hydrolysable and mixed tannins.

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Authors:
Musri Musman, Syiah Kuala University, Faculty of Marine and Fishery, Indonesia, Darussalam-Banda Aceh 23111, e-mail: musrimusman@gmail.com
Aulia Rahmad, Syiah Kuala University, Faculty of Marine and Fishery, Indonesia, Darussalam-Banda Aceh 23111, e-mail: aulia.rm34@gmail.com
Irma Dewiyanti, Syiah Kuala University, Faculty of Marine and Fishery, Indonesia, Darussalam-Banda Aceh 23111 e-mail: irma_alfian@yahoo.com
Chairin Sofia, Brackish Water Aquaculture Centre, Indonesia, Ujung Batee-Aceh Besar 23381, e-mail: chairin_sofia@yahoo.com
Hendro Sulistiono, Brackish Water Aquaculture Centre, Indonesia, Ujung Batee-Aceh Besar 23381, e-mail: hendrosulistiono@gmail.com

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