



EFFECTS OF HYDROLYSABLE TANNINS ON THE GROWTH PERFORMANCE, TOTAL HAEMOCYTE COUNTS AND LYSOZYME ACTIVITY OF PACIFIC WHITE LEG SHRIMP *Litopenaeus vannamei*

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ABSTRACT

The present study was conducted to evaluate the efficacy of the dietary inclusion of commercial hydrolysable tannins (HT) on the growth performance and haemato-immunological parameters of Pacific white shrimp (*Litopenaeus vannamei*). Four isonitrogenous and isolipidic practical diets were formulated to contain 0; 0,1; 0,2 and 0,3% of HT obtained from the sweet chestnut (*Castanea sativa*). The 0,3% HT was designed to be included in a diet with a low level of fish meal (FM) (7,5%), while others included 10% FM. A total of 240 post larvae shrimp with an average initial body weight of $4.24 \pm 0,03$ g were randomly stocked in four groups, with four replicates per treatment and 15 shrimp per aquaria tank. Results showed that there were no significant differences in terms of final biomass, final mean weight, survival, weight gain (WG), feed conversion ratio (FCR) and thermal growth coefficient (TGC). However, biologically, a better performance was observed when the diets had higher inclusion levels of HT. Shrimp fed diets with 0,2 and 0,3% HT had a significantly higher total haemocytes count, and those fed the diet with 0,2% HT also had a significantly higher lysozyme activity, compared to shrimp from the control group. These results indicated that 0,2 and 0,3% HT could remarkably improve the growth performance and the haemato-immunological parameters of Pacific white shrimp, and have potential functional properties when included in commercial diets for shrimp.

1. Introduction

Pacific white shrimp (*Litopenaeus vannamei*) has become an important aquaculture commodity in several countries, including Indonesia (Wijayanto et al., 2017; Astiyani et al., 2020; Novriadi et al., 2021), Vietnam (Quyên et al., 2020), Ecuador (Rivera et al., 2018) and Thailand (Tookwinas et al., 2005), primarily due to the excellent market demand, acceptance of pelleted feeds, competitive market value and tolerance for a wide range of salinities to grow and survive (Roy et al., 2009; Qiu, 2017). Currently, the global shrimp production continues to grow significantly compared to other aquaculture species and the production increased from 0.15 million metric tons in 2000 to 5 million metric tons, with a market value up to USD 30,2 million (FAO, 2018).

In intensive culture systems, where shrimp are being cultured in

relatively high stocking densities, problems related to diseases and degradation of water quality within the rearing environments often occur and cause serious economic losses (Shang et al., 1998). Conventional treatments through the use of veterinary medicines and antibiotics are having limited value and only stimulate the development of bacterial resistance within the culture environment (Subasinghe, 1997; Defoirdt et al., 2007; Cabello, 2006). Another problem created by the application of these methods is the presence of chemicals and antibiotic residues in aquaculture products (Goldburg et al., 2001; Grave et al., 1999, 1996; Saitanu et al., 1994), that lead to allergy and toxicity in humans (Cabello, 2006; Aldeman and Hastings, 1998). Consequently, novel preventive approaches in aquaculture are needed to replace the use of antibiotics, especially in shrimp, that lack the complexity of the adaptive immune system and rely solely on innate immunity as their

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primary defence mechanism and response (Kurtz, 2005). Under these circumstances, natural products derived from plants, such as the sweet chestnut (*Castanea sativa*), have received considerable attention with respect to their properties and potential to strengthen the gut health and prevent pathogenic infections if applied properly in animal diets formulation (Miele et al., 2019; Bilić-Šobot et al., 2016; Lee et al., 2016; Maisak et al., 2013; Tosi et al., 2013).

The active ingredients in the sweet chestnut, classified as hydrolysable tannins (HT), are molecules that consist of a central core of a polyol, such as glucose, esterified with phenolic groups, such as gallic acid or hexahydroxydiphenic acid (Macáková et al., 2014; Okuda & Ito, 2011; Mueller-Harvey & McAllan, 1992; McLeod, 1974). Studies showed that the use of HT can help regulating the intestinal function and stimulating the production of digestive enzymes in grass carp (*Ctenopharyngodon idellus*) (Yao et al., 2019), as well as stimulating immunological parameters of rohu (*Labeo rohita*) fingerlings (Prusty et al., 2007) and act as an antibacterial activity against *Aeromonas* and *Streptococcal* pathogens of tilapia *Oreochromis niloticus* (Maisak et al., 2013). Recently, Zhu et al. (2021) demonstrated that the inclusion of 0,15% HT in a diet with 22% fish meal (FM) and 5% shrimp shell meal (SSM), could improve the growth performance, antioxidant capacity, and resistance against *Vibrio parahaemolyticus* in Pacific white shrimp. However, little is known about the optimum inclusion level of HT in low fish meal diets and their effects on growth and haemato-immunological parameters in Pacific white shrimp. Therefore, the objective of this study was to explore the biological response of Pacific white shrimp fed with various inclusion levels of commercial hydrolysable tannins. Besides, the inclusion of hydrolysable tannins in a diet with a lower content of fish meal than the others was evaluated. For this purpose, the growth performance and the haemato-immunological parameters were assessed.

2. Materials and Methods

2.1. Ethics Statement

All procedure were approved by the Committee on Animal Health, Ministry of Marine Affairs and Fisheries, Republic of Indonesia

2.2. Feed preparation

The basal diets were designed with 10% fish meal (FM), 44,9% soybean meal (SBM), 8% corn gluten meal (CGM) and 17% wheat products (WP). The ingredients and the proximate analysis of the basal and experimental diets are shown in Table 1. Hydrolysable tannins (Tanin Sevnica, Slovenia; purity >65%) were added to the basal diet at two concentrations (0,1 and 0,2%). The third experimental diet was considered as an additional treatment and designed by decreasing the amount of FM from 10 to 7,5% and increasing the inclusion levels of HT up to 0,3% to evaluate the efficacy of HT in lower inclusion level of FM. All experimental diets were produced at the Main Center of Mariculture Development of Lampung (Indonesia) using standard procedures for making shrimp feed. Dry pellets were crumbled, packed in sealed bags, and stored in a freezer until further use.

2.3. Growth trial

The growth trial was conducted at the PT. Batam Dae Hae Seng research station (Batam, Indonesia). Pacific white shrimp post larvae (PL) were obtained from PT. Maju Tambak Sumur (Kalianda, Lampung, Indonesia) and were acclimatized to the culture system. Post larvae were fed with a commercial feed (Evergreen Feed, Lampung, Indonesia) for three weeks until they reached the suitable size. Shrimp ($4,24 \pm 0,03$ g initial mean weight) were randomly distributed into 16 tanks ($70 \times 35 \times 40$ cm; 98 L) per aquaria tank and assigned the experimental diets: control, 0,1% HT, 0,2% HT and 0,3% HT. Four replicate groups of shrimp were fed with the experimental diets using a nutrition research

Table 1

Composition and proximate analysis (% as is) of diets containing hydrolysable tannins and fed to Pacific white shrimp for 60 days.

Ingredients (% as is)	Diet code			
	Control	HT 0,1%	HT 0,2%	HT 0,3%
Menhaden Fishmeal ¹	10,00	10,00	10,00	7,50
Soybean meal ²	44,90	44,90	44,90	44,90
Corn Gluten Meal ³	8,00	8,00	8,00	8,00
Menhaden fish oil ⁴	5,66	5,66	5,66	5,66
Corn Starch ⁴	10,34	10,24	10,14	12,54
hydrolysable tannins ⁵	-	0,10	0,20	0,30
Wheat products ⁴	17,00	17,00	17,00	17,00
Mineral premix ⁶	0,70	0,70	0,70	0,70
Vitamin premix ⁷	1,90	1,90	1,90	1,90
KP dibasic ⁴	1,50	1,50	1,50	1,50
Proximate analysis (% as is) ⁸				
Crude protein	35,72	35,68	35,83	35,19
Lysine	1,89	1,86	1,83	1,81
Methionine	0,85	0,79	0,82	0,74
Moisture	7,68	7,82	7,51	7,59
Crude Fat	7,44	7,11	7,09	7,04
Phosphorus	2,14	1,89	2,08	2,16
Ash	8,54	8,76	8,87	8,85

¹ High protein fish meal (Peru) supplied by Agri Permata Asia, Jakarta, Indonesia.

² De-hulled solvent extract soybean meal, Bogor Ingredients, Indonesia.

³ FKS Multi Agro, Tbk, Jakarta, Indonesia.

⁴ PT Bright International (Bogor, West Java, Indonesia).

⁵ Farmatan AquaTM (Tanin Sevnica, Slovenia).

⁶ Trace mineral premix (g/100 g premix): cobalt chloride, 0.004; cupric sulfate pentahydrate, 0.550; ferrous sulfate, 2.000; magnesium sulfate anhydrous, 13.862; manganese sulfate monohydrate, 0.650; potassium iodide, 0.067; sodium selenite, 0.010; zinc sulfate heptahydrate, 13.193; alpha-cellulose, 69.664.

⁷ Vitamin premix (g/kg premix): thiamin-HCL, 4.95; riboflavin, 3.83; pyridoxine-HCL, 4.00; Ca-pantothenate, 10.00; nicotinic acid, 10.00; biotin, 0.50; folic acid, 4.00; cyanocobalamin, 0.05; inositol, 25.00; vitamin A acetate (500,000 IU/g), 0.32; vitamin D3 (1,000,000 IU/g), 80.00; menadione, 0.50; alpha-cellulose, 856.81.

⁸ Analysis conducted by the SUA Integrated Fish Farm, Bogor Agricultural University, West Java, Indonesia.

standard protocol for 60 days. Shrimp were fed by hand four times daily (07:00, 11:00, 15:00 and 20:00). Based on our historic results, feed inputs were pre-programmed assuming the normal growth of shrimp and a feed conversion ratio (FCR) of 1,5. Daily allowances of feed were adjusted based on observed feed consumption, weekly counts of the shrimp and mortality. Uneaten feed, feces, and molts were removed by siphoning the aquaria tank prior to the first feeding.

2.4. Water quality and growth performance

Regarding the water quality analysis, the pH, dissolved oxygen (DO), water temperature and salinity (‰) were measured four times daily using Aqua TROLL 500 Multiparameter Sonde instrument connected to AquaEasy apps (Bosch, Singapore) for data monitoring and recording system. Total ammonia-nitrogen (TAN), nitrate and nitrite were measured once in a week by using absorption spectrophotometry (DR890, HACH, USA). At the end of the feeding period, all shrimp were group and individually weighed to calculate the final biomass, final weight, weight gain (WG), feed conversion ratio (FCR), survival and thermal unit growth coefficient (TGC), as follows:

$$WG = \frac{(\text{average individual final weight} - \text{average individual initial weight})}{(\text{average individual initial weight})} \times 100$$

$$FCR = \frac{\text{feed given (g)}}{\text{alive weight gain (g)}}$$

$$\text{Survival} = \frac{\text{final number of shrimp}}{\text{initial number of shrimp}} \times 100$$

$$\text{TGC} = \frac{\text{FBW}^{1/3} - \text{IBW}^{1/3}}{\sum \text{TD}} \times 100,$$

where FBW is final body weight, IBW is initial body weight, T is water temperature (°C) and D is number of trial days.

2.5. Total haemocyte count

At the end of the growth trial, hemolymph was sampled from two intermolt shrimp per tank, or eight shrimp per treatment, and the total hemocytes count was determined. Hemolymph (100 µL) of individual shrimp was withdrawn from the pleopod base of the second abdominal segment with a sterile 1 mL syringe (25 G ×13 mm needle). Before hemolymph extraction, the syringe was loaded with a precooled (4 °C) solution (10% EDTA, Na2) used as an anticoagulant. The haemolymph with anti-coagulant solution was diluted in 150 µL of formaldehyde (4%) and then 20 µL were placed on a hemocytometer (Neubauer) to determine the total haemocyte count (THC) using an optical microscope (Olympus, DP72).

2.6. Lysozyme activity analysis

Lysozyme activity was measured by using a lysozyme detection kit (Sigma-Aldrich, Cat. no. LY0100) according to the manufacturer's instruction. The results were defined by the lysis of the *Micrococcus lysodeikticus* cells. The reactions were conducted at a temperature of 25 °C and the absorbance was measured at a wavelength of 450 nm in the ultraviolet/visible spectrophotometer (Perkin Elmer, Lambda XLS, USA):

$$\text{Lysozyme activity} \left(\frac{\text{Units}}{\text{mL}} \right) = \frac{(\Delta A_{450}/\text{minTest} - \Delta A_{450}/\text{minBlank}) (df)}{(0,001)(0,03)}$$

df = dilution factor

0,001= ΔA₄₅₀ as per the unit definition

0,03= Volume (in milliliters) of enzyme solution

2.7. Statistical analysis

All data were analyzed using a one-way analysis of variance to determine the significant difference ($P < 0.05$) among the treatment means, followed by the Tukey's multiple comparison test to determine the differences between the treatments means in each trial. The pooled standard errors were used across all the growth parameters, as the variance of each treatment was the same. Statistical analyses were conducted using SAS system (V9.4, SAS Institute, Cary, NC, USA).

3. Results

3.1. Water quality and growth performance

The water quality data during the morning and afternoon daily measurements are displayed in Table 2. Based on the mean and standard deviation of the data, all parameters including pH, salinity (‰), water temperature (°C) and dissolved oxygen (DO, mg L⁻¹), together with ammonia (mg TAN L⁻¹) and nitrate (mg NO₂-N L⁻¹), were within the acceptable range for Pacific white shrimp. Regarding the growth parameters of shrimp, shown in Table 3, there were no significant differences among the treatments in final biomass, final mean weight, survival, WG, FCR and TGC. However, biologically, the inclusion of HT induced better growth compared to the control diet. The optimum growth performance was obtained from the use of 0,2% inclusion level of HT in the diet. The inclusion of 0,3% HT compensated the decreasing

Table 2

Overall water quality measurements during the grow-out phase of the experiment. Data were presented as mean ± standard deviation (range).

Time	Parameter					
	Temperature (°C)	D.O (mg L ⁻¹)	pH	Salinity (‰)	Ammonia (mg TAN L ⁻¹)	Nitrate (mg NO ₂ -N L ⁻¹)
AM	27,56 ± 0,54	5,64	7,72	24,28 ± 2,24	0,09 ± 0,08	27,25 ± 3,39
		±	±			
		0,42	0,17			
PM	29,41 ± 0,72	5,91	7,82	24,45 ± 1,62	0,09 ± 0,08	27,25 ± 3,39
		±	±			
		0,43	0,34			

Table 3

Growth performance of Pacific white shrimp (mean initial weight 4.25 ± 0,03 g) fed the experimental diets for 60 d.

Diet code	Final Biomass (g)	Final Mean Weight (g)	Survival (%)	WG (%)	FCR ²	TGC ³
Control diet	189,14	14,83	85,00	248,49	2,78	0,0485
HT 0,1%	216,79	15,48	93,33	263,30	2,62	0,0506
HT 0,2%	211,54	15,67	90,00	271,00	2,61	0,0515
HT 0,3 %	196,74	15,46	85,00	262,03	2,62	0,0505
<i>p-value</i>	0,1811	0,4924	0,3379	0,4489	0,4032	0,4318
PSE ⁴	10,6985	0,4603	4,2310	11,1107	0,0955	0,0015

Values represent the mean of four replicates.

Note: ¹ WG = Weight gain; ² FCR = Feed conversion ratio; ³ TGC = Thermal growth coefficient; ⁴ PSE = Pooled standard error.

levels of FM in the diet and still maintained an optimum growth, similar to that obtained with the control diet.

3.2. Total haemocyte count and total lysozyme activity

The THC and lysozyme activity are shown in Figs. 1 and 2. The THC in shrimp fed diets including 0,2 and 0,3% of HT was significantly higher ($P < 0.05$) compared to that of shrimp from the control group. Regarding the lysozyme activity, the inclusion of 0,2 % HT in the diet was able to significantly enhance it ($P < 0.05$), when compared to the control group.

4. Discussion

Initially, tannins were considered as an anti-nutritive agent and also reported to create palatability problems due to their astringent taste

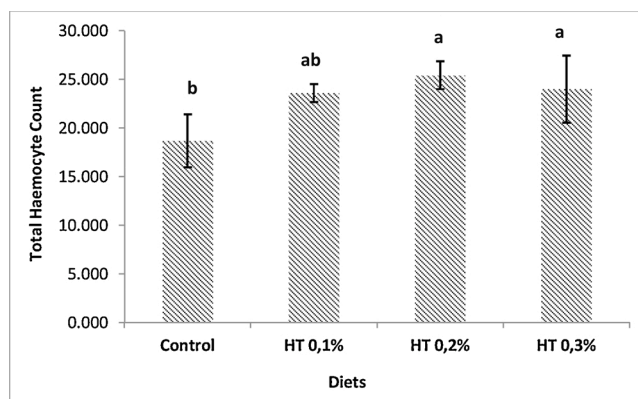


Fig. 1. Total haemocyte count of Pacific white shrimp (10⁶ cell mL⁻¹) fed the experimental diets at the end of the growth trial. Values represent the mean of four replicates (P -value: 0,0029).

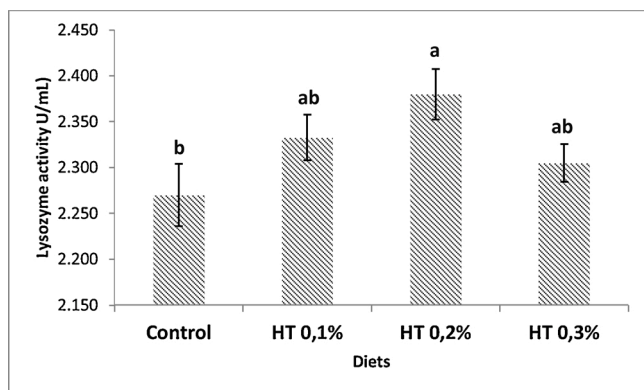


Fig. 2. Lysozyme activity of Pacific white shrimp (U mL^{-1}) fed the experimental diets at the end of the growth trial. Values represent the mean of four replicates (P-value: 0,0023).

(Joslyn & Goldstein, 1964; Chung et al., 1998). However, as it is well known now, the effect of tannins can vary according to the type of tannin and their structural diversity, the inclusion levels used in the diet, the physiological state of the animal, and the species involved (Candek-Potokar et al., 2015; Makkar et al., 2007). In aquaculture production systems, two different types of tannins, HT or condensed tannins (CT), have been used to evaluate the growth of fish fed with diets containing different levels of HT and CT (Buyukcapar et al., 2011). In our study, the inclusion of 0,1 and 0,2% of HT in diets for Pacific white shrimp maintained the growth of the animals in a similar way than the control diet. Interestingly, the inclusion of 0,3% HT in a diet containing a low level of FM, compensated the reduction in the content of FM. Despite there were no statistically significant differences in the growth parameters, our results showed an increasing trend in terms of growth as the inclusion level of HT increased in the diet. This is in line with a study from Zhu et al. (2021), who reported that the growth performance of shrimp was optimized as the inclusion of HT in diets with a high inclusion level of FM increased. These authors also reported an increase in the trypsin activity, which could enhance the nutrient availability and absorption.

The haemato-immunological response is a primary physiological mechanism to protect the animals against diseases and environmental stressors (Chen et al., 2012). In shrimp, in order to stimulate and initiate an immune response, there has to be some compound present in the body of animals which can recognize foreign invaders that have gained entrance to the body (Holmblad & Söderhäll, 1999). Using a highly selective recognition process, signalling cascades are thus activated. These cascades will stimulate the production of defence substances in crustaceans (Vargas-Albores et al., 1996). Tannins possess various biological activities, such as antimicrobial, antioxidant, and immunomodulatory (Mueller-Harvey, 2006), thus may have potential to be used as part of the immune activation system.

In this study, the THC of shrimp that received 0,2 and 0,3% inclusion of HT into the diet increased compared to the control treatment. Haemocytes are the primary mediator of cellular response in crustaceans, and their role includes the non-self-recognition mechanisms, phagocytosis, reactive oxygen intermediates production, wound healing and melanization with encapsulation of foreign materials (Burge et al., 2007; Martin et al., 1993; Söderhäll and Cerenius, 1992). An increase of THC provides enhanced immune capability during the culture periods, and this could also enhance disease resistance in crustaceans against pathogenic infection (Truscott & White, 1990; Le Moullac et al., 1998). Little is known about the antimicrobial properties in HT that can induce the increasing number of THC. However, we speculate that by consistently feeding the shrimp with HT, we could be inducing the non-specific immune system of shrimp.

Other than THC, we also quantified the total lysozyme activity of

shrimp after the experimental feeding period. Lysozyme has been described in invertebrates as one of the primary tools to evaluate the innate immune system, and functions as an antimicrobial protein (Sotelo-Mundo et al., 2003). Our results showed that the inclusion of HT enhanced the lysozyme activity, following a similar trend as in the case of THC, and indicating that HT were beneficial in terms of enhancing the non-specific immune defense in shrimp. In line with this, Zhu et al. (2021) reported that the supplementation of 0,15% HT in a challenge test significantly enhanced the resistance of Pacific white shrimp against *Vibrio parahaemolyticus* and also increased the growth, antioxidant capacity and beneficial intestinal microflora of the animals. Taking the results from Zhu et al. (2021) and from the present study into consideration, the HT extracted from the sweet chestnut, can be used as feed supplement in practical diets for Pacific white shrimp.

5. Conclusion

Under the conditions of the present study, HT act as potential functional feed additives and can be used at levels of 0,2 % or 0,3% in lower inclusion levels of FM to promote the growth and the haemato-immunological parameters in Pacific white shrimp. Further studies should be conducted to determine the potential efficacy of HT in the long-term culture period using commercial culture ponds until shrimp reach the consumption size.

Data Availability Statement

Research data are not shared.

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Tanin Sevnica provided the studied product, as well as the funding for this study.

Statement of the authors

Romi Novriadi: Research conceptualization, Methodology, Validation, formal analysis, investigation, Draft creation, supervision and Writing the original draft.

Aldy Eka Wahyudi: Research work, Investigations, formal analysis.

Rifqi Fadhilah: Research work, Investigations, formal analysis.

Clara Trullàs: Research conceptualization, Methodology, Validation, formal analysis, investigation, and funding acquisition.

Declaration of Competing Interest

Clara Trullàs is employed by Tanin Sevnica. The rest of the authors state no conflict of interest.

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