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Effects of the apple mangrove (*Sonneratia caseolaris)* on growth, nutrient utilization and digestive enzyme activities of the black tiger shrimp *Penaeus monodon* postlarvae

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ABSTRACT

A growth trial was conducted to evaluate the twig extracts of the apple mangrove (Sonneratia caseolaris) as growth-promoting agent of Penaeus monodon since it was shown previously to promote immune responses in this species. The twig methanolic extract was incorporated in the basal diet (commercially available shrimp starter feed) and was tested at three feeding frequencies (twice, three and four times daily) for its effects on growth, conversion efficiency and survival against a control treatment (pure basal diet) fed three times daily. Final average body weight (ABW) and length, specific growth rate (SGR) and food conversion ratio (FCR) of shrimps fed the medicated diets were all statistically similar with those fed the control diet with except those fed the medicated diet twice daily which exhibited poorest in all three parameters. Survival of shrimps fed medicated diets were all significantly higher than in those fed the control diet. Activities of amylase, total protease were measured following a 60-day feeding trial of the control diet fed 3 times daily and medicated diets at 2 times, 3 times and 4 times daily. Amylase activity was significantly the highest in shrimps fed medicated diet at 4 times daily while those fed the control and medicated diets at 2 and 3 times daily were statistically similar. Shrimps fed medicated diets at 3 and 4 times daily exhibited significantly the highest and statistically similar protease activities; those fed the control and medicated diets at 2 times daily were inferior and statistically similar.

INTRODUCTION

In the last decade, the production of shrimp, *Penaeus monodon* (Fabricius) has significantly declined in the Philippines and it has been assumed that it this is due to environment-related factors. The deteriorated environment have resulted in compromised health conditions of the cultured shrimp exacerbated by the proliferation of opportunistic shrimp pathogens and other disease-carrying organisms in ponds [1]. The use of antibiotics has been resorted to by some shrimp hatchery operators to combat this declining production despite its environmental hazards.

Research in the use of plant extracts for aquatic animals is increasing with the demand for eco-friendly and sustainable aquaculture, particularly for organic farming. Plant extracts decrease the selective pressure for developing antibiotics resistance **[2]**. One of these which was studied in this laboratory is the twig of the apple mangrove *Sonneratia caseolaris* [3]. We showed that shrimps fed apple mangrove-medicated diets exhibited significantly higher phagocytic and phenoloxidase activities at feeding frequencies of one up to 4 times daily than those fed diet without the supplement. Bacterial survival index was significantly higher in shrimps fed the control diet than did those fed medicated diets.

Apple mangrove tree has been reported as medicinal plant and food for human in many countries of Asia [4]. Squeezed flower juice of the tree was used as ingredient in antidiuretic drug formulation. Moreover, its flower and leaf could be grinded and used as poultice for healing bruised wound and smallpox .[5] Phenolic compound, such as gallic acid, and two flavonoids, e.g. luteolin and luteolin-7-O-glucoside, are the bioactive substances in the apple mangrove tree which have antioxidant activity [6]. Twenty-four compounds including eight steroids, nine triterpenoids, three flavonoids, and four benzene carboxylic derivatives were isolated and identified from stems and twigs of medicinal mangrove plant *S. caseolaris* [7]. In the leaves of the tree, ethanolic and acetone extracts gave positive results for alkaloids, carbohydrates, flavonoids and cardiac glycosides. In addition to that, it was found that saponins and phenolic compounds were present in ethanol and sterols are found in acetone extracts respectively. [8] Natural substances when fed in moderate to high concentrations could sometimes have positive or negative effects in the organisms that consume them. The positive effects of the apple mangrove twig extract in enhancing the shrimps' immune responses have already been mentioned [3]. Possible negative effects should also be documented which can include fall in food consumption, reduction of weight gain and inhibition of digestibility and assimilation of nutrients in the intestine (Johnson et al., 1986; Cheeke, 1996; Bureau et al., 1998). Thus, this study aimed to determine whether or not the diet treated with the extract affects survival, growth, feed conversion efficiency and the activities of the digestive enzyme α-amylase and total protease.

MATERIALS AND METHODS

Experimental animals

Post larvae of *P. monodon* were purchased from a commercial prawn hatchery in Oton, Iloilo, Philippines, transported in a Styrofoam boxes and immediately stocked in a continuously aerated fiberglass tank upon arrival at the university hatchery. Shrimps were acclimated for 15 d and were fed commercial diet. The conditioning tank was aerated throughout the conditioning period.

Preparation of twigs extract

Twigs of *S. caseolaris* were shade dried for a period of about two weeks. The dried twigs were pulverized using hammer mill, packed separately in plastic bags and stored in canisters in a cool dark place at ambient room temperature until extraction.

The method of extraction used was as described in Guevarra and Recio [9] and Vinod and Guruvayoorappan [10]. Pulverized twigs (about 200 g) were soaked in equal part of analytic-reagent grade methanol (1:1 w/v) for 48 h. The slurry was filtered, washed to remove non-soluble fractions, centrifuged (20,000 x g for 30 min) for clarification, and the supernatant stored at 4^oC. The whole process was repeated (about three times) until the solution became clear. The combined solutions were concentrated using a rotary evaporator under reduced pressure at 40 – 50^oC and the resulting concentrated solution was stored at 4^oC until use.

Feeds and Feeding

P. monodon larvae (ABW=0.008 g) were randomly divided into 12 concrete rectangular tanks (1 m^3-capacity) at 100 shrimp postlarvae cubic $meter^{-1}$. The tanks consisted of three replicates of 3 treatments (medicated diets at three feeding frequencies) and a control treatment (non-medicated diet at feeding frequency of three times d^{-1}). The treatments were as follows: control diet (not medicated), medicated diet fed twice daily (2X feeding scheme), medicated three times daily (3X feeding scheme) and medicated fed four times daily (4X feeding scheme). All medicated diets contained the same levels of mangrove twig extract at 20 mg kg^{-1} diet.

Shrimps were fed their respective diet at 8 % of their body weight day^{-1} for the duration of the experiment. The commercial basal diet was composed of 45.9% crude protein, 3.6% crude fat, 35.8% nitrogen-free extract, 1.43 % crude fiber, 13.3 % ash, and 4.24% moisture [11]. Methanolic twig mangrove extracts were sprayed on feed pellets at 20 ml kg^{-1} dry diet (containing 1000 µg ml^{-1} twig condensate) and dried for 24 prior to the feeding experiments. The control diet was sprayed with 20 ml of distilled water kg^{-1} diet. Feeding was done in feeding trays using the following feeding schemes: the control diet (unmedicated diet) was fed three times daily, and the three test diets containing the same levels of the twig condensate (20 mg kg^{-1}diet) fed twice times daily (800 and 1700, 2X); three times daily (at 800, 1200 and 1700, 3X); and four times daily (800, 1100, 1400 and 1700, 4X). Feed ration was adjusted every sampling period and the feeding trial lasted for 75 days. The experimental tanks were sufficiently aerated 24 h daily; about 50% of the total water volume of the recirculating system was replaced every 15 days.

At the end of the experiment, survival, growth rate and conversion efficiency were estimated by measuring length and weight of experimental shrimps from each group. Specific growth rate (SGR) was calculated using the following formula:

$$SGR = [(\ln w_2 - \ln w_1)/(t_2 - t_1)] \text{ X } 100$$

Enzyme assay and protein determination

All assays procedures were carried out at 0-4^{o}C unless otherwise stated. Assays were conducted at 25^{o}C (unless otherwise stated) using stopped-flow type of method; zero-time reactions were also carried out.

Live shrimps were sacrificed, hepatopancreas excised, washed with cold extraction solution (50mM citrate phosphate buffer pH 7.0), weighed and homogenized in the same solution at 1:20 ratio (wet tissue to volume) in an Ultraturrax homogenizer. The homogenates were centrifuged at 4000 rpm for 15 min and the supernatant used for enzyme assay. Total soluble protein was measured following the procedure of Lowry *et al.* [12] modified by Marichamy *et al.* [13] with bovine serum albumin as a standard. All enzyme assays were conducted within 4 h of homogenization and all samples for a single enzymatic assay were run in the same day. Blanks (i.e. absence of either enzyme or substrate) and controls (i.e. zero time reaction) were also run during the assay.

α-Amylase activity was assayed as described by Bernfield [14] modified by Mukesh *et al.* [15]. Briefly, the assay mixture consisted of 0.1 ml soluble starch solution, 0.5 ml of enzyme preparation and 0.5 ml homogenizing buffer. The reaction was stopped by adding DNS solution, the mixture heated for 5 min in boiling water, cooled in running water, diluted and optical density read at 546 nm. Amylase activity was expressed in terms of µg maltose liberated from starch min^{-1} mg protein^{-2}.

Total protease activity was measured using casein as substrate according to the methods of Walter [16] modified by Abirami *et al.* [17]. Reaction mixture consisted 0.75 ml of 1% (w/v) aqueous solution of casein, 0.1 ml of enzyme extract and 0.75 ml buffer in a final volume of 1.6 ml. After 1 h of the reaction, 2.25 ml ice-cold trichloroacetic acid (5%) was added, the mixture left at 2^{o}C for 30 min and the absorbance of the supernatant solution was read at 280 nm. One unit of total protease activity was expressed as µg of tyrosine produced min^{-1} mg^{-2} protein.

Statistical Analysis

Statistical analysis of the data was performed using a graph- statistical software package (Statistica, Stat Soft., Inc., USA). Homogeneity of variances and normality were tested (Levene's test and Shapiro–Wilk's test, respectively) prior to ANOVA. Differences between final length and body weight, arcsine-transformed survival, FCR, SGR, α-amylase and total protease activities were tested using one-way ANOVA. Post hoc analysis among groups after finding significant differences were performed by Tukey tests, with the level of significance preset at $P<0.05$. Data were reported as mean ± standard error.

RESULTS

Table 1 shows that **t**he final length of shrimp fed the control diet was not significantly different from those fed medicated diets using 3X or 4X feeding scheme but was significantly higher than that of shrimps fed medicated diet using 2X scheme; final length between shrimps fed the medicated diets were not significantly different.

Similarly, the final ABW of shrimps fed the control diet was not significantly different from those fed medicated diets using 3X or 4X schemes but was significantly higher than those fed medicated diets using 2X scheme (Table 1).

Survival of shrimps fed the medicated diets at all feeding frequencies were significantly higher than those fed the control diet. The trend in SGR was similar to those of the final length and final ABW. Growth rate of shrimps fed the control diet was significantly higher than those of shrimps fed medicated diet at 2X scheme but were not significantly different from those fed medicated diets at 3X or 4X schemes.

FCR was significantly different between shrimps fed medicated and control diets, only when fed either at 3X or 4X feeding schemes. Shrimps fed medicated diet at 2X feeding scheme recorded significantly the poorest FCR.

Feeding shrimps with medicated diet at 4X feeding scheme resulted in significantly the highest amylase activity while no significant differences were recorded in amylase activities of those fed the control, medicated diet at 2X and 3X schemes (Fig. 1).

Feeding the shrimp diets at 3X and 4X schemes resulted in significantly higher total protease activities than feeding them with the control diet; however activities were not significantly different between those fed the control diet and the medicated diet at 2X scheme (Fig. 2).

Table 1. Specific growth rate, feed conversion ratio and survival of *P. monodon* fed the control diet (commercial shrimp starter feed fed three times daily) and *S. caseolaris*-medicated diets fed at twice daily (2X), three times daily (3X) and four times daily (4X). Values in the same row not sharing the same superscript are significantly different (*P*<0.05) according to Tukey's Honestly Significant Difference Test.

	Control	2X	3X	4X
Initial length (cm)	2.05	2.05	2.05	2.05
Final length (cm)	6.83^{a}	5.24^{b}	6.32^{ab}	6.36^{ab}
Initial ABW (g shrimp^{-1})	0.008	0.008	0.008	0.008
Final ABW (g shrimp^{-1})	1.965^{a}	0.815^{b}	1.557^{ab}	1.669^{ab}
Survival (%)	69.2^{b}	75.3^{a}	76.2^{a}	78.5^{a}
SGR	6.49^{a}	5.9^{b}	6.35^{a}	6.26^{a}
FCR (g intake g gain^{-1})	2.43^{b}	2.70^{a}	2.23^{b}	2.10^{b}

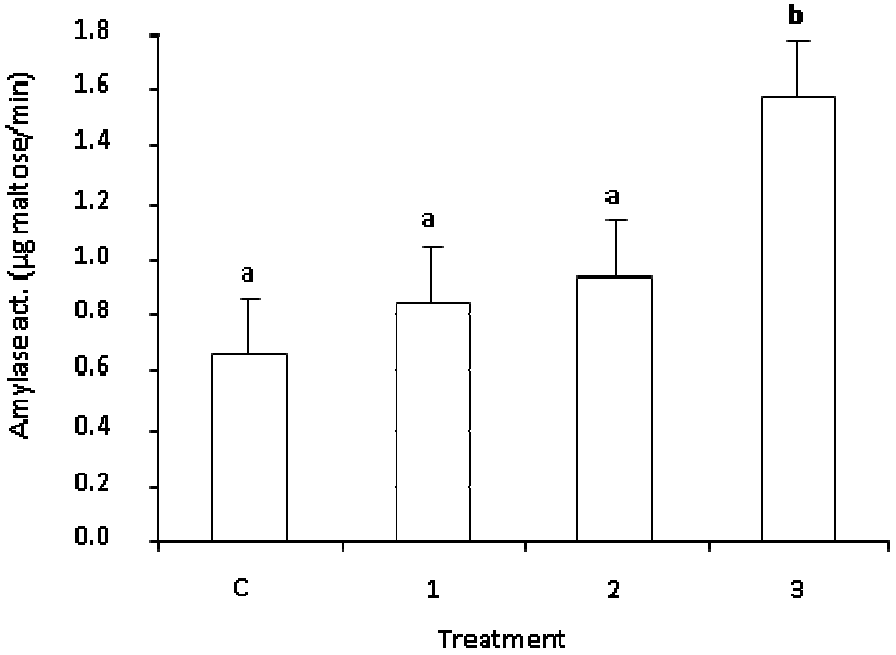

Fig. 1. Amylase activities of *P. monodon* juveniles fed the control diet (C) (commercial starter diet fed three times daily) and diets with methanolic twig extracts of *S. caseolaris* fed twice daily (1), three times daily (2), and fed four times daily (3) for 60 days. Error bars indicate standard error of the mean (SEM). Values not sharing the same superscript are significantly different (*P*<0.05) according to Tukey's Honestly Significant Difference Test.

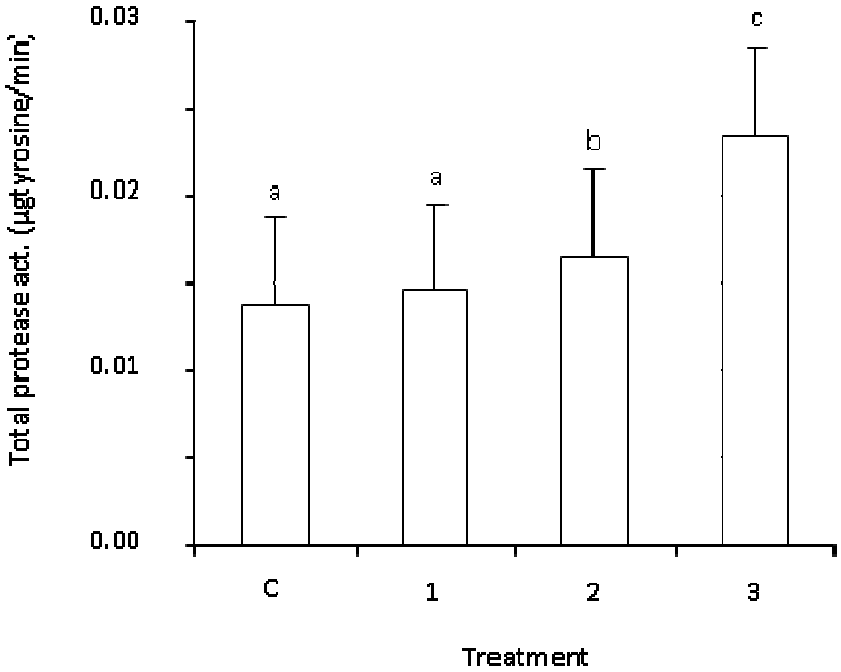

Fig. 2. Total protease activities of *P. monodon* juveniles fed the control diet (C) (commercial starter diet fed three times daily) and diets with methanolic twig extracts of *S. caseolaris* fed twice daily (1), three times daily (2), and fed four times daily (3) for 60 days. Error bars indicate standard error of the mean (SEM). Values not sharing the same superscript are significantly different (P<0.05) according to Tukey's Honestly Significant Difference Test.

DISCUSSION

Shrimp growth, survival and conversion efficiency

Final ABW and length, SGR and FCR of shrimps fed medicated or control diets were not significantly different when shrimps were fed medicated diet 3 or 4 times daily. Only survival was significantly higher in shrimps fed the medicated than in those fed the control diet. This finding is significant since it demonstrated that there was no growth inhibiting or promoting effect by the active principle of the apple mangrove twig extract. It was also an important observation that survival was increased and in the hatchery, even a small promotion of survival means a considerable increase in profitability.

Since restricted feeding was used during the growth trial, there was no basis to infer on the difference between the palatability of the medicated and control diets. But basing on the poor performance of shrimps fed 2X diet, the medicated diets appeared to be less palatable. Jiny Varghese *et al.* [8] has reported the morphological characteristics of the apple mangrove leaves as bitter in taste but without any specific characteristic odor; we assumed that it was also the case with the twig extract. This characteristics could have resulted in decreased palatability for the shrimps. Given that the control and medicated diets were provided at the same feeding rate (8% of BW d^{-1}), feeding medicated diet twice daily resulted in poorer FCR. It could be a result of either feeds taken in but not efficiently converted to flesh or it could be a result of feeds not taken in and just went to waste. If the latter was so, then it could mean that the medicated extract was less palatable. Further experiment should be done on this aspect. In fish, such as tilapia, feed intake is significantly lower in fish consuming raw moringa leaves or large amounts of moringa methanol extracts [18]. They attributed it to the relative concentration of various antimetabolic substances present in moringa leaf products. We are tempted to infer that the palatability-reducing principles were soluble in methanol as Afuang *et al.* [18] also concluded in their report.

The case of uneaten feed in the present study could be the cause of the poorer growth and conversion efficiency of shrimps fed 2X diet as compared to those fed the control diet. Despite this possibility, shrimps could otherwise acclimatize to the experimental diet only in a matter of days as was observed in common carp fed diets containing crystal amino acids [18] and feeding frequency can be increased to improve acceptability [19]. Feeding frequency as a feeding strategy can greatly influence the efficacy of the feed in crustaceans by improving acceptability by the animal over time. This factor is important for slow feeding benthic crustaceans such as shrimp and lobsters [20]. With shrimp, these concerns have been addressed by offering the pelleted feed at frequencies of up to six times daily [21]. Smith *et al.* [22] have improved the acceptance of lobster *P. ornatus* by incorporating krill hydrolysate and krill meal and also by increasing feeding frequency from 2 to 4 times daily.

The overall nonsignificant effect of the apple mangrove extract on growth compared to those fed the control diet differed from the results of other investigators using other extracts on shrimp. For example, Immanuel *et al.* [23] bioencapsulated *Artemia* with diets in which extracts of six herbs were separately incorporated and the brine shrimps fed to *P. indicus*. Feeding herbs to the shrimps results in higher SGR and better survival than did those fed the control diet. In the present study, growth rates were similar and only survival was improved by the apple mangrove extract-medicated diet.

Amylase and total protease activity

α-Amylase and total protease activities were enhanced by incorporating the mangrove extract to the feed. However, this finding does not account for the statistically similar SGR and FCR exhibited by shrimps fed either the control or medicated diets. Shrimps fed the medicated diets were expected to have better FCR in view of the observation that they all exhibited significantly higher digestive enzyme activities. At any rate, the overall significant finding with regard to the digestive enzyme activities was that there was no adverse effect on the chemical digestive capability of shrimps fed the twig methanolic extract of the apple mangrove.

There is always the possibility that even digestive enzyme activity levels could have daily cycles and to capture this, higher feeding frequencies increase the possibility of feeding the shrimps at natural peaks of enzyme production or activation. In fish, for example, it has been documented that they demonstrate distinct preferences for feeding in the morning or afternoon [24]. Feeding time may influence the phase or amplitude of some of the endocrine cycles involved in the physiological regulation of feeding, perhaps coinciding with natural rhythms of secretion, activation or synthesis of digestive or metabolic enzymes [25]. In the present study, we have measured activities of digestive enzymes at various feeding frequencies and variations in these enzyme levels were observed. These variations in enzyme activity levels could be one of the main reasons why growth rates and conversion efficiency of shrimps fed mangrove extract medicated diets ultimately approached those of shrimps fed the control diet when feeding frequencies were increased.

CONCLUSION

The apple mangrove extract did not affect growth rate and feed conversion efficiency but increased survival. It promoted digestion of carbohydrate and protein by increasing the activities of amylase when fed at 4 times daily and total protease when fed at 3 and 4 times daily. The apple mangrove extract could be employed in shrimp culture as a prophylactic/therapeutant as well as an immunostimulant without negative effects on growth, nutrient utilization and carbohydrate and protein digestion.

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