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Effects of the apple mangrove (*Sonneratia caseolaris*) on antimicrobial, immunostimulatory and histological responses in black tiger shrimp postlarvae fed at varying feeding frequency

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Abstract. The study aimed to evaluate extracts of the apple mangrove (*Sonneratia caseolaris*) as agent of prophylactic/therapeutant for the culture of *Penaeus monodon*. General types of compounds detected in the leaf and twig extracts were glycosides, steroids, triterpenes, sterols and flavonoids as determined by Thin Layer Chromatography (TLC). Based on the mean clear zone of inhibition, twig extract exhibited higher antibacterial activity than did the leaf extract. Furthermore, the methanolic extract of the twig gave the highest antibacterial activity and was thus employed for further tests. It 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 the immune responses against a control treatment (pure basal diet) fed three times daily. There were no significant differences in respiratory burst activities between treatments but shrimps fed medicated diets exhibited considerably higher values than did the control group of shrimps. Phagocytic and phenoloxidase activities were significantly higher in shrimps fed medicated diets at all feeding frequencies than did those fed the control diet; activities were not significantly different between those fed medicated diets. Bacterial survival index was significantly higher in shrimps fed the control diet than did those fed medicated diets; indices of shrimps fed medicated diets were not significantly different. There were no signs of toxicity or any adverse histological changes in shrimps fed medicated diets at 1000 µg mL^{-1} apple mangrove extract concentration.
Key Words: *Penaeus monodon*, *Sonneratia caseolaris, Vibrio harveyi*, immune responses.

Introduction. Antibiotic treatment of bacterial diseases in aquaculture has been applied for many years. Also, prophylactic chemo-therapeutants has commonly been used and practiced.

However, the abuse of broad-spectrum chemo-therapeutants has resulted in an increased number of antibiotic-resistant bacteria, the transfer of these drug-resistant genes to bacteria or virus that infects terrestrial animals and humans. Alterations of the bacterial flora both in sediments and in water column and residual act as antibiotics in seafood products (Cabello 2006). Therefore, it is necessary to exploit a non-chemotherapeutic method instead of the chemotherapeutic ones, such as the use of vaccine, probiotics, immunostimulants and natural therapeutics from plants (Sakai 1999).

The prevention and treatment of these infectious diseases by applying products from terrestrial plants appears as a possible alternative. Mangrove trees are a source of bioactive compounds characterized by a broad spectrum of biological activities (Manilal et al 2009).

The global shrimp industry has consistently suffered great production losses from the outbreak of infectious diseases, particularly from viruses and bacteria (Lightner & Redman 1998; Supungul et al 2002). Therefore, disease prevention has been considered a priority for this industry (Roch 1999). Traditionally, the control of bacterial problems

has relied on the indiscriminate use of chemical compounds in the culture of penaeid shrimp (Vaseeharan et al 2004). Recently however, probiotic microorganisms, vaccination have also been employed (Gomez et al 2000) as well as applications of natural extracts. The potential use of extracts from mangroves has been reported for use in medicine and other applications (Bandarayanake 1998).

Sonneratia spp. is one of the four most common general types of mangroves in the Philippines along with *Rhizophora, Avicennia,* and *Bruguiera* (Calumpong & Menez 1996). *Sonneratia caseolaris* (L.) Engler (family Sonneratiaceae; Visayan name: Pagatpat) is a small tree with oblong or obovate-elliptic coriaceous leaves and large, red flowers; fruit is eatable, sap is used as a skin cosmetic, leaves are used for goat food. *Sonneratia caseolaris* is a typical non-viviparous mangrove species and a key component of mangrove community in the Indo-West Pacific region (Chen et al 2008). Extracts of this plant are traditionally used as an astringent and antiseptic, in sprains and swellings, and in arresting hemorrhage (Sadhu et al 2006).

It is interesting to know whether or not there is an additional benefit brought by a prospective feed additive such as enhanced capability for immune defense. Given that vertebrates have no adaptive immunity, they rely on very strong and efficient nonspecific immune responses to combat external invasion of their system (Lemaitre et al 1997). There are times when natural extracts such as that of the apple mangrove may render the whole formulated feed unpalatable to the shrimp. It is also our hypothesis that to counteract such negative effect, we varied the feeding frequency and determine how the administration of such extract resulted in increased or decreased efficacy of the additive. The present study aims to determine the effects of the addition of the apple mangrove extract to the feed on the immune response of the shrimp fed at varying feeding frequency.

Material and Method. Post larvae of *P. monodon* were purchased from a commercial prawn hatchery in Oton, Iloilo. The postlarvae were transported in styroform boxes and were immediately stocked in a fiberglass tank upon arrival at the institute hatchery. The tank was covered and shrimps were acclimated for 15 days before experimentation. During this conditioning process, the shrimps were fed commercial diet. The conditioning tank was aerated throughout the conditioning period.

Extraction. Twigs and leaves samples of *S. caseolaris* were shade dried for a period of about two weeks. The dried samples were cut into small pieces and pulverized using hammer mill grinder. Pulverized samples were packed separately in small plastic bags which were stored in a tightly-covered glass container placed in a cool dark place at ambient room temperature.

The method of extraction used was that of Guevarra & Recio (1985). Pulverized twigs and leaves (about 200 g) were soaked in equal part of methanol (1:1) for 48 h. The slurry was filtered, washed, and the filtrate centrifuged (20,000 x g for 30 min). The whole process was repeated three times or until the filtrate becomes colorless or clear. The combined filtrates were concentrated using a rotary evaporator under reduced pressure at 40 – 50^{o}C. Three portions of condensate (10, 100 and 1000 µg L^{-1}) were impregnated in sterile paper discs, screened against *Vibrio harveyi* through replicates of disc diffusion test. Zone of inhibition of each mangrove extract was measured.

Antibacterial assay against *V. harveyi* was done and when detected, further extraction using organic solvents of increasing polarity was carried out.

Antibacterial Assay. Paper disc diffusion method was employed to evaluate the antibacterial activity of each fraction of *S. caseolaris* twig and leaf extracts. Sterile paper discs were impregnated with the extracts (10µg mL^{-1}, 100µg mL^{-1} and 1000µg mL^{-1}) and were dried partially and placed onto the nutrient agar based-layer plates seeded with *V. harveyi*. Diffusion was done in triplicates and Amoxicillin was used as positive control.

Pure culture of *V. harveyi* was streaked in nutrient agar and incubated at 37^{o}C for 24 h. Luminous colonies were detected in the dark, marked with pen, inoculated into 5 mL nutrient broth and incubated at 37^{o}C for 18 h. These served as the inoculum and its

concentration was adjusted by the addition of nutrient broth and compared to MacFarland standard. Antibacterial activity was evaluated by computing the antibacterial index, which is the clear zone of inhibition (cm) minus the diameter of the disc (cm), divided by the diameter of the disc (cm).

Extraction with Organic Solvents. Based on the result of the antibacterial assay of the crude extracts, the concentration which showed the highest mean value of the clear zone of inhibition was used for the succeeding assay using liquid-liquid extraction with organic solvents of increasing polarity, namely, *n*-hexane, dichloromethane, ethyl acetate and chloroform at three concentrations and in three replicates. Each fraction obtained in the process was concentrated using a rotary evaporator at 40 – 50^{o}C.

Thin Layer Chromatography. The types of compounds that may be present in the organic solvent fractions obtained were separated and analyzed using Thin Layer Chromatography (TLC). Briefly, concentrates were spotted on silica gel precoated plates and placed in chambers saturated with different solvent systems. The developed chromatograms were sprayed with reagents that give color reaction specific to certain group of bioactive compounds. The retardation factor (Rf) was estimated for each spot obtained. The two spray reagents used were antimony (III) chloride for detection of flavonoids, vitamins A and D, carotenoids, steroids, sapogenins, steroid glycosides, terpenes; and vanillin-sulfuric acid for higher alcohols, steroids, essential oils and terpenes giving blue, violet, green, brown or red zones.

Feeds and Feeding. *Peneus monodon* larvae were purchased from a commercial hatchery, acclimatized to the laboratory conditions and to the basal diet (San Miguel Corp.) for 15 days. After acclimatization, 3 experimental groups and a control group all in three replicates were stocked at 100 shrimp postlarvae cubic meter^{-1} at the initial ABW of 0.008 g individual^{-1} in 1 cubic m-capacity rectangular tanks.

Shrimps were fed at 8 % of their body weight day^{-1} for the duration of the experiment. The commercial basal diet (starter shrimp feed) 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 (Pascual 1993). Feed was sprayed with methanolic twig mangrove extracts (1000 µg mL^{-1}) and dried for 24 h prior to the feeding experiments. Feeding was done in feeding trays using three feeding frequencies, namely, two times daily (800 and 1700); three times daily (at 800, 1200 and 1700); and four times daily (800, 1100, 1400 and 1700). Feed ration was adjusted after every sampling period (10 days) based on the total body weight per tank for the whole duration of the feeding study (75 days). The experimental tanks sufficiently aerated 24 h daily at more than 80% saturation and about 50% of the total water volume was replaced every 15 days.

Immunological Assay. Hemocyte and hemolymph from the shrimp were collected following the method described by Weeks-Perkins et al (1995). Humoral assay (bacterial growth inhibitory activity and phenoloxidase) was done using cell-free hemolymph of the individual shrimp sample while cellular assay (respiratory burst activities and phagocytic) was carried out using hemocytes extracted from *P. monodon* samples.

Respiratory burst activity (RBA) was measured using the reduction of nitroblue tetrazoleum to formazan which is an indirect measure of the formation of superoxide anion as described by Weeks-Perkins et al (1995).

Phagocytic activity (PA) was measured using Weeks-Perkins et al (1995) method. Phagocytic cells were counted under a Nikon fluorescence and phase contrast microscope. Phagocytic rate (PR) was estimated as follows:

$$PR = [(\text{phagocytic hemocytes})*(\text{total hemocytes counted})]^{-1} \times 100$$

Phenoloxidase activity was estimated spectrophotometrically using $_L$-3, 4 hihydroxyphenylalanine ($_L$-DOPA) as substrate (Soderhall 1981) and trypsin as elicitor following the method described by Smith & Soderhall (1991). One unit of enzyme activity was defined as an increase in absorbance of 0.001 min^{-1} mg $protein^{-2}$. Protein content was measured by the Bradford method (Bradford 1976) using bovine serum albumin as a standard protein.

For bacterial growth inhibitory activity, *V. harveyi* was cultured to reach the log phase and 100 µL of approximately 1 x 10^3 bacterial cells mL^{-1} was incubated with equal volume of cell-free hemolymph in a 96-well microtiter plates. Control reaction was made by incubating the bacterial suspension with phosphate buffered saline instead of hemolymph. The assay was done following turbidity test at 25°C for 18 h where aliquot samples were serially diluted and bacterial counts were obtained spectrophotometrically. Bacterial growth activity was estimated as relative growth inhibition (RGI):

$$RGI = \frac{[(\text{final bacterial count} - \text{initial bacterial count})]}{[\text{Initial bacterial count}]} \times 100$$

Histological Examination. The gills, hepatopancreas and muscle samples from *P. monodon* were used to examine the histological effects of *S. caseolaris* extract on the experimental animal. Shrimps were sacrificed after the feeding trial and sample tissues were fixed in Davidson's fixative, processed, embedded, sectioned (thickness of 5-6µm), and stained with haematoxylin and eosin (Bell & Lightner 1988). The tissues were examined for histomorphological changes under a fluorescent microscope.

Results. Tables 1 and 2 show the bioactive compounds screened from the leaves and twig extracts of *S. caseolaris.* The leaf solvent fractions of dichloromethane, chloroform, ethyl acetate and *n*-hexane were positive to the spray reagents for the presence of steroids, glycosides, triterpenes and sterols. In the twig solvent fractions, the four types of compounds were also present with the addition of flavonoid for the *n*-hexane extract, which was not used on the leaf extract.

Table 1

Bioactive compounds found in the leaves of *S. caseolaris*

	Visualizing agents	Rf values	Remarks
SDCM extract	Antimony (III) chloride	0.08, 0.17, 0.2, 0.5, 0.63	Steroids
		0.42	Glycosides
Chloroform extract	Antimony (III) chloride	0.03, 0.2	Steroids
		0.67	Glycosides
Ethyl acetate extract	Antimony (III) chloride	0.08	Steroids
		0.25, 0.42, 0.55	Glycosides
	Vanillin-sulfuric acid	0.08, 0.17, 0.25	Triterpenes and Sterols

Table 2

Bioactive compounds in the twigs of *S. caseolaris*

Twig Sample	Visualizing agents	Rf values	Remarks
SDCM extract	Antimony (III) chloride	0.03, 0.05, 0.08	Steroids
		0.17, 0.5	Glycosides
	Vanillin-sulfuric acid	0.22, 0.25, 0.3, 0.33	Triterpenes and sterols
Chloroform extract	UV light	0.13	Flavonoids
	Antimony (III) chloride	0.05, 0.06	Steroids
		0.58	Glycosides
	Vanillin-sulfuric acid	0.2, 0.33	Triterpenes and sterols
Ethyl acetate extract	Antimony (III) chloride	0.75	Glycosides
n-Hexane extract	UV light	0.25	Flavonoids
	Antimony (III) chloride	0.13	Steroids
		0.47	Glycosides
	Vanillin-sulfuric acid	0.17, 0.25, 0.33, 0.58, 0.75	Triterpenes and sterols

Table 3 shows the antibacterial activity of the different organic solvents used for *S. caseolaris* assay.

Extracts at 10 µg mL^{-1} for both the leaf and twig did not show any significant difference in antibacterial activity between extracts using various solvents; the 100 µg mL^{-1} exhibited slight differences while the highest concentration of 1000 µg mL^{-1} gave the greatest differences.

For both *S. caseolaris* leaf and twig extracts (1000, 100 and 10 µg mL^{-1}), the methanolic extracts exhibited the highest mean values of clear zone of inhibition while the aqueous extracts exhibited the lowest. Furthermore, leaf extracts exhibited lower values than did twig extracts. Thus, in the subsequent tests, only the methanolic twig extracts were employed for evaluation.

Feeding diets with the methanolic extract of the apple mangrove twigs did not enhance the respiratory burst activity of *P. monodon* juveniles (Fig. 1).

P. monodon juveniles fed the control diet exhibited significantly the lowest phagocytic rate among treatments, lower than did those fed the medicated diets at all feeding frequencies (Fig. 2).

Feeding shrimps the control diet resulted in the lowest hemolymph phenoloxidase (PO) activity while those fed the medicated diets exhibited significantly higher PO activities at all feeding frequencies (Fig. 3).

Table 3

Mean clear zone of inhibition (mm) using paper discs antibacterial assay (n= 3; ± = Standard deviation) against *Vibrio harveyi* of *S. caseolaris* extracts using various organic solvents at three concentrations (10, 100 and 1000 µg mL^{-1}). Values in the same row not sharing the same superscript are significantly different ($P<0.05$) according to Tukey's Honestly Significant Difference Test

Conc.(µg mL^{-1})	Inhibition zone (mm) against *V. harveyi*					
	n-Hex	Chloro	DCM	E. Acet	MeOH	Water
Leaves						
1000	4.33 ± 1.15^{b}	2.67 ± 0.58^{c}	3.33 ± 1.53^{bc}	3.33 ± 0.58^{bc}	6.00 ± 1.73^{a}	2.67 ± 1.15^{c}
100	2.33 ± 0.58^{b}	1.67 ± 1.15^{c}	1.67 ± 0.58^{c}	1.33 ± 0.58	4.33 ± 0.58^{a}	1.67 ± 0.58^{c}
10	1.33 ± 0.58	0.67 ± 1.15	1.00 ± 0.00	1.00 ± 1.00	1.67 ± 0.58	1.00 ± 1.00
Twigs						
1000	7.33 ± 1.53^{b}	4.67 ± 1.53^{de}	6.00 ± 1.73^{c}	5.33 ± 0.58^{cd}	10.33 ± 1.53^{a}	3.67 ± 0.58^{e}
100	4.67 ± 1.15^{a}	2.67 ± 0.58^{b}	4.33 ± 0.58^{a}	4.00 ± 1.00^{a}	4.67 ± 1.53^{a}	2.33 ± 1.53^{b}
10	2.67 ± 0.58	2.00 ± 0.00	3.00 ± 1.00	2.67 ± 1.15	3.00 ± 1.00	1.33 ± 0.58
- Control	0.00	0.00	0.00	0.00	0.33 ± 0.58	0.00
+ Control	14.67 ± 3.06	14.00 ± 2.00	16.00 ± 1.00	15.00 ± 3.61	19.33 ± 3.06	17.33 ± 1.53

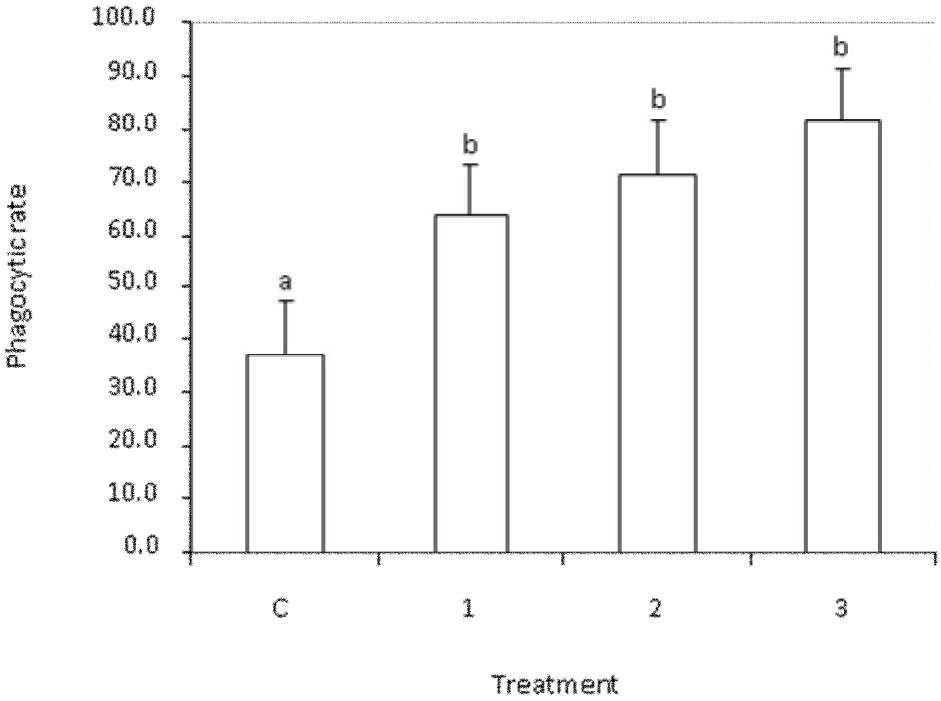

Figure 1. Respiratory burst activities of *P. monodon* juveniles fed the control diet (C) (commercial starter diet fed three times daily) and diets with methanolic twig extracts of *S. caseolaris* fed twice daily (1), three times daily (2), and fed four times daily (3) for 60 days. Error bars indicate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

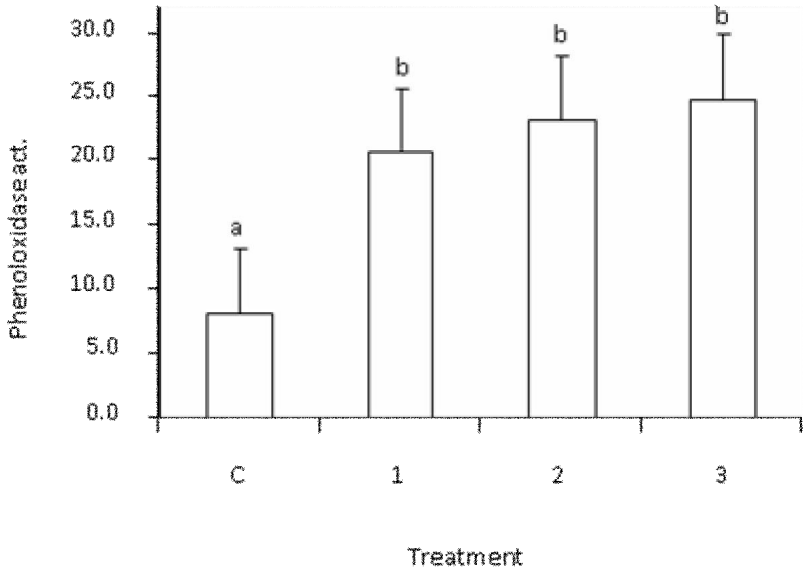

Figure 2. Phagocytic activities of *P. monodon* juveniles fed the control diet (C) (commercial starter diet fed three times daily) and diets with methanolic twig extracts of *S. caseolaris* fed twice daily (1), three times daily (2), and fed four times daily (3) for 60 days. Error bars indicate ±1 standard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

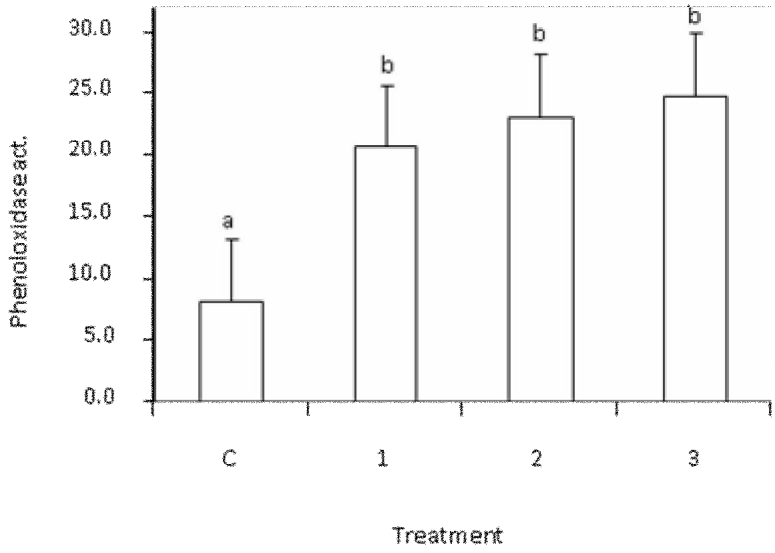

Figure 3. Phenoloxidase activity *P. monodon* juveniles fed the control diet (C) (commercial starter diet fed three times daily) and diets with methanolic twig extracts of *S. caseolaris* fed twice daily (1), three times daily (2), and fed four times daily (3) for 60 days. Error bars indicate ±1 standard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

Feeding shrimps the control diet resulted in significantly the highest bacterial survival. RGI of shrimps fed the medicated diet, at all feeding frequencies, exhibited lower values than did those fed the control diet (Fig. 4).

Histological examination on gill, hepatopancreas and muscles tissues of the experimental *P. monodon* showed that the methanolic extract from the twigs of *S. caseolaris* enhanced the health of the experimental shrimp postlarvae. Hence, there was

no erosion observed in any of the tissues and there were no traces of pathogen infection (Figures 5, 6 and 7).

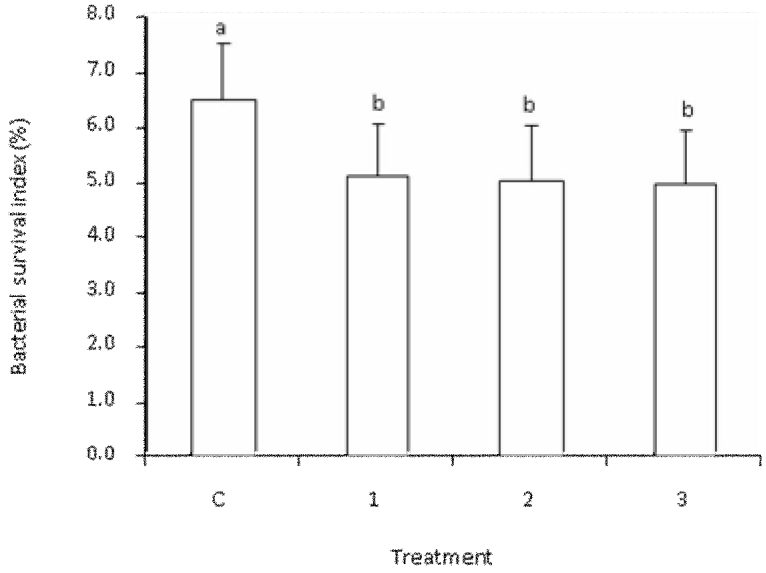

Figure 4. Bacterial growth inhibitory activities of *P. monodon* juveniles fed the control diet (C) (commercial starter diet fed three times daily) and diets with methanolic twig extracts of *S. caseolaris* fed twice daily (1), three times daily (2), and fed four times daily (3). Error bars indicate ±1 standard deviation. Values not sharing the same superscript are significantly different ($P<0.05$) according to Tukey's Honestly Significant Difference Test.

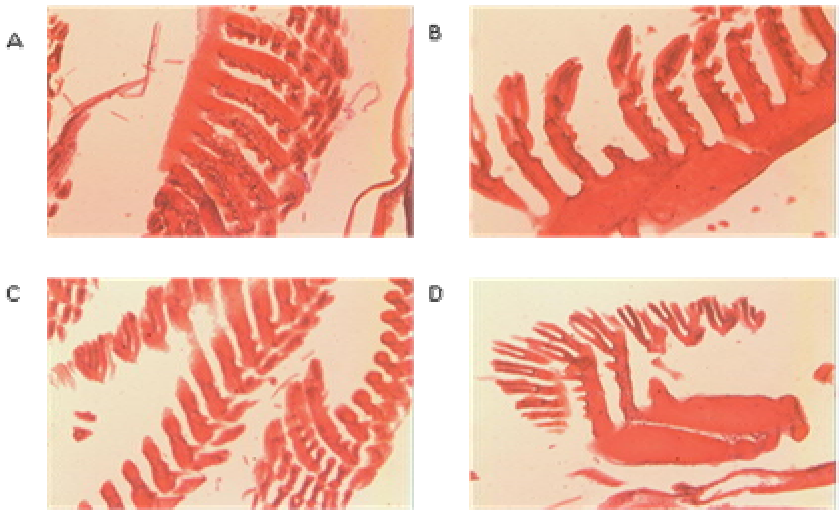

Figure 5. Histological examination of the gills of *P. monodon* juveniles fed the control diet (A) (commercial starter diet fed three times daily) and diets with methanolic twig extracts of *S. caseolaris* fed twice daily (B), three times daily (C), and fed four times daily (D) for 75 days.

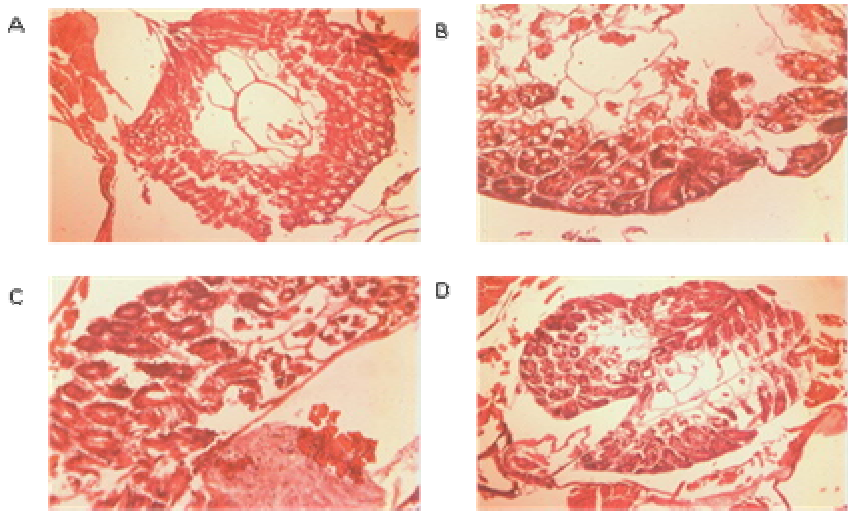

Figure 6. Histological examination of the hepatopancreas of *P. monodon* juveniles fed the control diet (A) (commercial starter diet fed three times daily) and diets with methanolic twig extracts of *S. caseolaris* fed twice daily (B), three times daily (C), and fed four times daily (D) for 75 days.

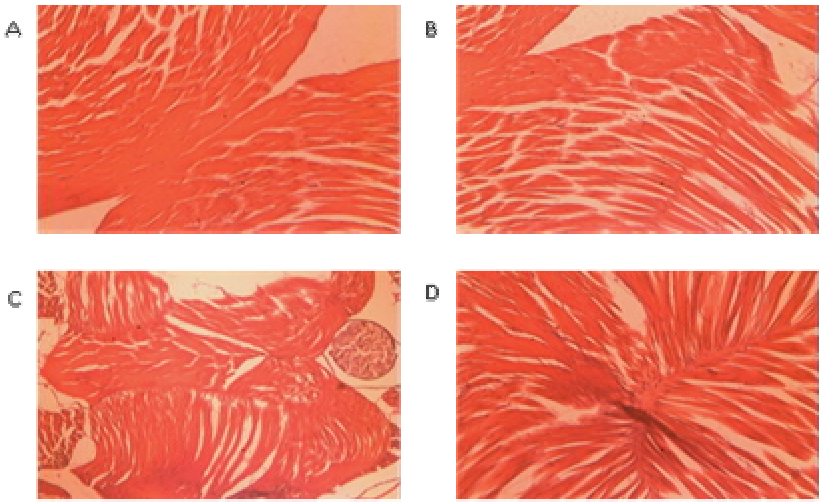

Figure 7. Histological examination of the muscles of *P. monodon* juveniles fed the control diet (A) (commercial starter diet fed three times daily) and diets with methanolic twig extracts of *S. caseolaris* fed twice daily (B), three times daily (C), and fed four times daily (D) for 75 days.

Discussion. It is apparent that feeding frequency did not affect the efficacy of the dietary apple mangrove extracts. This is may indicate that the palatability of the diet containing the plant extract were uniform among the shrimp fed the experimental diet regardless of how many times they were fed in one day.

In the present study, methanolic and hexane extracts gave the highest antibacterial activities, but these extracts were not significantly better than the aqueous extracts at the lowest concentration of 10 µm mL^{-1}. This could indicate that at low concentration, the assay was not sensitive enough to detect differences in their effects. Also, the character of the active compound seemed to be a combination of polar and nonpolar, since methanol is a very polar solvent while *n*-hexane is a nonpolar solvent.

Crustacean haemocytes play an important role in the host immune response including recognition, phagocytosis, melanization, cytotoxicity and cell to cell communication (Johansson et al 2000). The immune system of *Penaeus chinensis* could apparently be activated by oral administration of immunodrugs derived from the land and marine plants (Wang et al 1995). In the present study, feeding the shrimp medicated

diets promoted respiratory burst, phagocytic, phenoloxidase activity and bacterial growth inhibitory activities. It seemed that just like *P. chinensis*, the immune system of *P. monodon* could be activated by the principles in the mangrove twig methanolic extract. This led to the enhanced resistance in a perhaps much similar way as those of administering glucans, peptidoglycans or lipopolysaccharide described in other shrimps (Chang et al 2000; Henning et al 1998; Karunasagar et al 1997).

Gills of aquatic organisms are a vital organ; they play an important role in transportation of respiratory gases and regulation of osmotic and ionic balance. Toxic substances may damage gill tissues, thereby reducing the oxygen consumption and disrupting the osmoregulatory function of aquatic organisms (Ghate & Mulherkar 1979). Trichloroform results in the tips of the gill lamellae in *M. rosenbergii* appearing abnormal in addition to signs such as malformations, necrosis, and hyperplasia that result in the formation of clavate–globate (clubbing) lamellae (Chang et al 2005). The gills of control *M. rosenbergii* show uniform arrangement of lamellae with uniform interlamellar spaces. In the present study, gills of shrimps fed any of the dietary treatments (Fig. 5) resembled that of the gills of the control *M. rosenbergii* in the experiment of Chang et al (2005). That is, no structural abnormalities or abnormal gill lesions were observed in the gills and that there was uniform arrangement of lamellae with uniform interlamellar spaces. Thus, the apple mangrove extract did not have any adverse effect on the histology of the gills of *P. monodon*.

Foreign substances introduced into the shrimp may stimulate the body to excrete them (Burgos-Hernandez et al 2005) and the mechanism for this is not clearly understood in shrimps. Boonyaratpalin et al (2001) suggested that the hepatopancreas has a role in the elimination of toxins. Consistent with this notion, necrosis and atrophy of the hepatopancreas have been observed in aflatoxin-treated shrimp (Bintvihok et al 2003). In the present study, histological analysis of the hepatopancreas did not show any sign of necrosis or atrophy in shrimps fed medicated diets with the mangrove extract. In the muscle, there was also no sign of atrophy as an indication of toxicity. It would indicate that the mangrove extract was not at all toxic to *P. monodon*.

Conclusions. In conclusion, the methanolic extract of the apple mangrove did not affect respiratory burst activity, but enhanced the immune resistance of shrimp by promoting both phagocytic and phenoloxidase activities and lowering bacterial survival. The apple mangrove extract could be employed in shrimp culture as a prophylactic/therapeutant as well as an immunostimulant.

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