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Immunological and Bactericidal Effects of Turmeric (*Curcuma longa* Linn.) Extract in Pacific White Shrimps (*Litopenaeus vannamei* Boone)

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ABSTRACT

Pacific white shrimp (*Litopenaeus vannamei* Boone) has been one of the main export agricultural products of Thailand. However, culture of this marine shrimp has been retarded continuously by disease problems that have lead to a decline in production or even the collapse of the farming system in some areas of the country. To cope with the disease outbreaks, some farmers apply antibiotics, which can cause negative consequences to shrimp products involving drug residues. In this study, turmeric (*Curcuma longa* Linn.) extract containing 25.726% (w/w) curcuminoids was added to shrimp feed as an immunoenhancement. Shrimp with an average weight of 12 g were raised with feed containing turmeric extract at 0, 12.5, 25.0 and 50.0 mg/kg feed (ppm). The studied parameters included resistance against pathogenic *Vibrio harveyi*, immune functions and the total count of bacteria from the shrimp intestines. The results showed significantly ($P<0.05$) better resistance against *V. harveyi* in shrimps fed with 25 mg/kg feed of turmeric extract when compared with control. Phenoloxidase activity of shrimps fed with 25 and 50 mg/kg feed of turmeric extract was significantly ($P<0.05$) higher than the control. All turmeric extract-treated shrimps showed higher bactericidal activity than the control. However, no significant ($P>0.05$) differences were found amongst the values of total haemocyte count and percent phagocytosis. Total bacteria and the *Vibrio* spp. count from the intestines of shrimps fed with turmeric extract at all concentrations were significantly ($P<0.05$) lower than the control.

Keywords: Pacific white shrimp, turmeric *Curcuma longa* Linn. extract, immunity

INTRODUCTION

Marine shrimp products have become a major export commodity, as well as a major source of income for people involved in the shrimp culture industry. Pacific white shrimp (*Litopenaeus vannamai* Boone) is the main species used for marine shrimp culture of Thailand. It is also the primary penaeid shrimp currently being cultured in Central and South America, China, Indonesia and Taiwan (Hsu and Chen, 2007). As expected in intensive culture systems, disease outbreaks have been common in Pacific white shrimp cultures, including parasitic, bacterial and viral infections. Disease outbreak is a result of environment deterioration and stress associated

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with intensive farming and an increase in the numbers of pathogens, such as *Vibrio* spp., in the pond water (Lavilla-Pitogo *et al*., 1998). Antibiotics have been used in shrimp culture as a treatment for bacterial disease, which can cause residue problems if good practice has not been implemented as part of the drug regime. In addition, measures that are more stringent have been exercised by trade counterparts in regard to contamination with antibiotics. Thus, it is imperative for shrimp health researchers to investigate alternative approaches for effective disease prevention and treatment with minimal negative consequences to shrimp products.

Turmeric (*Curcuma longa* Linn.) is a native plant of southern Asia and is cultivated extensively throughout the tropical parts of the world (Gupta and Balasubrahmanyam, 1998). Many biological activities have been attributed to the extracts of *C. longa* and to its active compound (curcumin). These activities include antioxidant, anti-inflammatory and antiproliferative properties. Turmeric extract and curcumin have also been used widely as a hepatoprotective agent (Mesa *et al*., 2000). Little of the research on herbs has investigated matters of immuno-modulation and disease resistance in aquatic animals. Black tiger shrimp (*Penaeus monodon* Fabricius) fed on a diet containing turmeric extracts at 25 mg/kg diet showed resistance against *Vibrio harveyi* and *Vibrio* spp., while an *in vitro* study showed that 15 isolates of *Vibrio* spp. were eradicated by extracts containing 250 mg/l turmeric (Supamattaya *et al*., 2004). Turmeric has good potential for application to aquatic animals, because it is a natural product without any negative consequences to the harvest. However, due to limited information on its effective and practical application to aquatic animals, study that is more thorough should be conducted to demonstrate the benefit of this herb. The purpose of this study was to investigate the effect of turmeric (*Curcuma longa* Linn.) extract in feed of the Pacific white shrimp (*Litopenaeus vannamei* Boone) on its immunity to disease. Bactericidal activity of turmeric extract in the shrimp intestine was also studied.

MATERIALS AND METHODS

Test animal

Clinically healthy Pacific white shrimp (*Litopenaeus vannamei* Boone) with a weight range of 10-12 g were obtained from a commercial farm in Thailand and acclimated in cement tanks with dimensions 1.5 × 1.5 × 0.80 m containing 25 × 10^{-9} g/kg chlorinated sea water for two weeks before commencing the experiment. The tanks were covered with black plastic to maintain the water temperature, and the sea water was changed regularly to maintain optimum water conditions throughout the trial. There were four treatments (one control group and three concentrations of turmeric extract at 12.5, 25 and 50 mg/kg (ppm) feed) replicated in triplicate (three tanks per treatment). Each tank was stocked with 15 shrimps.

Turmeric extract and analysis

Turmeric (rhizome) was chopped into small pieces, dried and ground finely and then macerated in 95% ethyl alcohol for 10 h. Filtration and evaporation were used to obtain crude extract of turmeric. The amount of active content in the turmeric extract (curcuminoids) was analyzed by high performance liquid chromatography (HPLC) using the method described by Kongkathip and Kongkathip (2005). The turmeric extract was kept in a refrigerator at 4°C before use.

The turmeric extract was dissolved in 95% ethyl alcohol before being mixed with feed according to the experimental concentrations and then air-dried before coating with squid oil.

Disease resistance of Pacific white shrimp against vibriosis

Vibrio harveyi was isolated from diseased Pacific white shrimp (*Litopenaeus vannamei* Boone) and identified, as described by Buchanan and Gibbons (1974). A suspension of *V. harveyi* was prepared in sterile 1.5% NaCl and diluted to the predetermined concentration. After two weeks of the feeding trial, 10 shrimps from each treatment and the control were injected intramuscularly on a sixth segment with 0.1 mL of bacterial suspension containing 1×10^6 CFU/mL of *V. harveyi*. Dead shrimps were removed daily and the number recorded for 7 d. Bacteria were isolated from the hepatopancreas to confirm the cause of mortality. Mortality rates were statistically compared.

Immune functions

Ten shrimps from the control and treatment groups were sampled for immune analysis in the fourth week of the feeding trial.

Total haemocyte count

Haemolymph was collected from the ventral sinus of each shrimp. A syringe (3 mL) containing 1 mL of anticoagulant (K-199 + 5% L-cystein) with a needle (26 G) was used to draw 0.5 mL of shrimp blood. The total haemocyte count was recorded using a haemacytometer and calculated as the number of blood cells (total haemocytes/mm^3).

Phenoloxidase activiy (modified from Supamattaya *et al*. (2000))

Haemolymph was collected from each shrimp by plastic syringe (3mL) and the haemocytes were separated and washed three times with shrimp saline. Haemocyte lysate (HLS) was obtained by suspending the prepared haemocytes in cacodylate buffer pH 7.4, sonicated at 30 amplitudes for 5 sec, followed by centrifugation at 10,000 rpm at 4°C for 20 min. Phenoloxidase activity was measured from the HLS by adding 200 µL of 0.1% trypsin in cacodylate buffer in 200 µL HLS, followed by 200 µL of L-dihydroxyphenylalanine (L-DOPA). Enzyme activity was measured as the absorbance of dopachrome at the 490 nm wavelength. The protein concentration in the HLS was determined by Lowry's method (Lowry *et al*., 1951). The phenoloxidase activity was expressed as IU/min/mg protein (1 unit of phenoloxidase = ΔOD_{490} / min/mg protein).

Phagocytic activity

The phagocytic activity was measured as described by Supamattaya *et al*. (2000). Shrimp haemolymph (200 µL) was smeared on a glass slide and incubated at room temperature for 1 h. The non-adherent cells were removed and washed three times with shrimp saline. Baker's yeast was added to the haemolymph layer and incubated for 2 h at room temperature and then washed three times with shrimp saline, air dried and stained with Wright-Giemsa solution. The phagocytosis percentage was enumerated from the number of phagocytizing cell in 100 haemocytes.

Bactericidal activity

Shrimp serum was diluted with 2.6% NaCl by a twofold method in a multiwell-plate. A suspension of *V. harveyi* was added into the diluted serum and incubated at room temperature for 3 h before enumerating the number of bacteria by a spread plate technique on thiosulfate citrate bile sucrouse agar (TCBS). The results were recorded as the lowest dilution that killed 50% of the *V. harveyi* compared to the control.

Total bacterial count from shrimp intestine

After four weeks of the feeding trial, shrimp intestines were removed, homogenized and tenfold diluted with sterile 1.5% NaCl. A spread plate technique was used to determine the total bacterial count and total *Vibrio* spp. count on plate count agar (PCA) and TCBS, respectively.

Statistical analysis

Statistical analysis was performed using

analysis of variance (ANOVA) and Duncan's new multiple range test at $P = 0.05$ (Duncan, 1995).

RESULTS

Turmeric extract analysis

Analysis of turmeric extract by HPLC identified 25.726 % (w/w) of curcuminoids that contained three active ingedients, namely curcumin, desmethoxycurcumin and bisdesmethoxycurcumin, and volatile oil.

Disease resistance against *V. harveyi*

After challenging with *V. harveyi* for 7 d, there were significant ($P<0.05$) differences in the mortality rates. Shrimps fed with 25 mg/kg turmeric extract had the lowest mortality rate of 13.333 ± 0.577 %, while the control had the highest mortality at 50.00 %. However, the significant difference was found only between the 25 mg/kg turmeric group and the control (Figure 1).

Immune functions

Total haemocyte count

The total haemocyte counts of the control and treatment groups (concentrations of turmeric at 12.5, 25, 50 mg/kg) were 7.183 ± 4.082 × 10^6, 6.817 ± 3.480 × 10^6, 5.267 ± 2.768 × 10^6 and 5.633 ± 2.275 × 10^6 cells/mL, respectively, which were not significantly ($P> 0.05$) different (Figure 2).

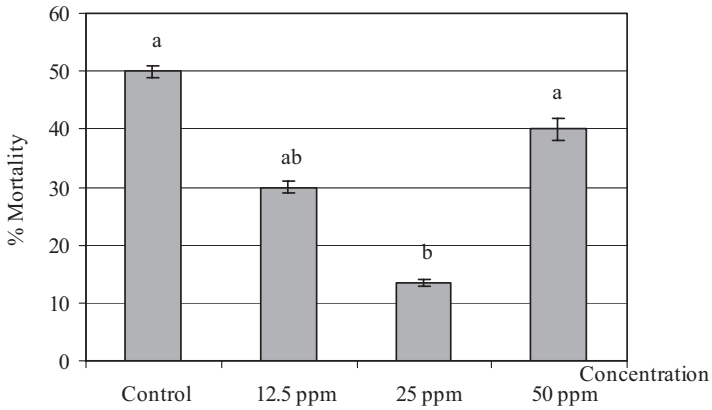

Figure 1 Mortality rate of *Litopenaeus vannamei* raised with feed containing different concentrations of turmeric extract and challenged with *Vibrio harveyi*.

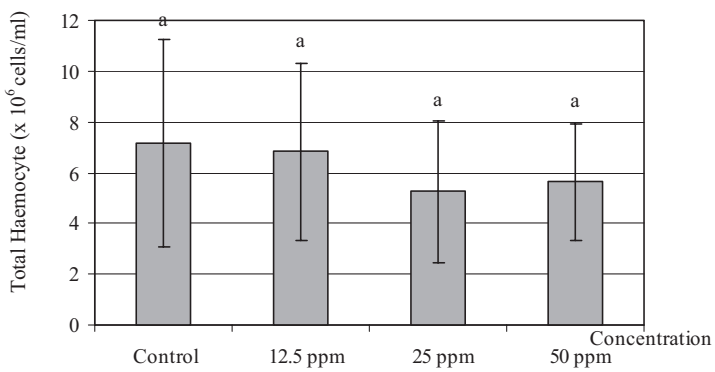

Figure 2 Total haemocytes count of *Litopenaeus vannamei* raised with feed containing different concentrations of turmeric extract.

Phenoloxidase activity

Shrimps that were fed with 25 and 50 mg/kg turmeric extract had significantly (*P<0.05*) higher activity of phenoloxidase than the control and the shrimps that were fed with 12.5 mg/kg turmeric extract, with values of 232.7592 ± 145.223, 250.863 ± 96.713, 100.119 ± 79.591 and 95.889 ± 62.853 unit/min/mg protein, respectively (Figure 3).

Phagocytic activity

There were high percentages of phagocytosis in the shrimps fed with 12.5 and 25 mg/kg of turmeric extract at 36.319 ± 24.210 and 36.364 ± 15.025%, respectively, but these values were not significantly (*P> 0.05*) different from the control, which had a value of 27.294 ± 9.986%. Shrimps that were fed with turmeric extract at 50 mg/kg had the lowest percentage of phagocytosis, which was not significantly (*P> 0.05*) different from the control, but was significantly (*P<0.05*) different from the other turmeric-treated groups (Figure 4).

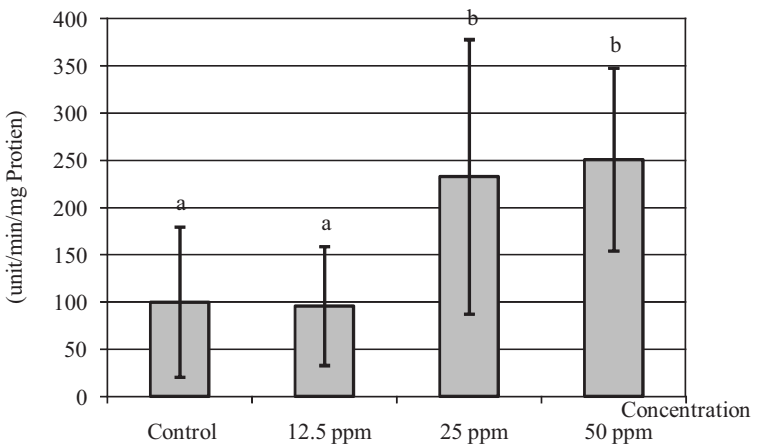

Figure 3 Phenoloxidase activity of *Litopenaeus vannamei* raised with feed containing different concentrations of turmeric extract.

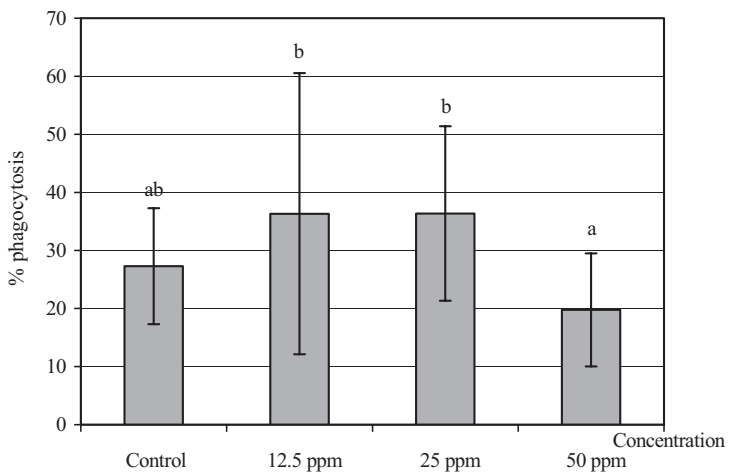

Figure 4 Percent phagocytosis of *Litopenaeus vannamei* raised with feed containing different concentrations of turmeric extract.

Bactericidal activity

Bactericidal activity (Table 1) at a serum dilution of 1:256-1:512 was found in shrimps fed with 25 mg/kg turmeric extract, which was the highest level when compared with other treatments. Shrimps fed with 12.5 and 50 mg/kg had the same bactericidal activity levels (1:128 - 1:256), while the control had the lowest bactericidal activity (1:64-1:128).

Total bacterial count from shrimp intestine

The total bacterial count of the control was $1911.00 \pm 1756.59 \times 10^9$ CFU/gm, which was significantly ($P< 0.05$) higher than in the turmeric-treated groups. The total *Vibrio* spp. count of the control was $10.67 \pm 2.65 \times 10^9$ CFU/gm, which was significantly ($P< 0.05$) higher than in the turmeric-treated groups (Table 2).

DISCUSSION

Turmeric extract

In this study, extract from turmeric using 95% ethyl alcohol contained 25.726% (w/w) curcuminoids that consisted of curcumin, desmethoxycurcumin and bisdesmethoxycurcumin mixed with volatile oil. According to Supamattaya *et al*. (2005), extraction of turmeric with ethyl alcohol yielded a higher level of active ingredient than from other solvents. Supamattaya *et al*. (2004) found 21.57% curcuminoids from turmeric extracted by alcohol, which was lower than in the current study. The source of the turmeric plant and the extraction process are key factors that determine the curcuminoid content. The amount of curcuminoids indicated the degree of bactericidal activity and immunostimulant effects in the tested animals.

Disease resistance against *V. harveyi*

Resistance against experimental infection with *V. harveyi* was clearly elevated in shrimps that were fed with turmeric extract. All turmeric-treated groups had less mortality than the control, even though the differences were significant only between the 25 mg/kg turmeric group and the control. This finding was similar to a previous report on resistance to vibriosis in black tiger shrimps fed with turmeric extract (Vanichkul *et al*., 2007). Supamattaya *et al*. (2005) compared

Table 1 Bactericidal activity of *Litopenaeus vannamei* raised with feed containing different concentrations of turmeric extract.

Treatment	Bactericidal activity
Control	1:64 - 1:128
Turmeric extract 12.5 mg/kg	1:128 - 1:256
Turmeric extract 25 mg/kg	1:256 - 1:512
Turmeric extract 50 mg/kg	1:128 - 1:256

Table 2 Total bacteria and *Vibrio* spp. counts (mean±standard deviation) from intestines of *Litopenaeus vannamei* raised with feed containing different concentrations of turmeric extract ($\times 10^9$ CFU/g).

Bacteria \ Treatment	Control	12.5 mg/kg	25 mg/kg	50 mg/kg
Total count	1911.00±1756.59[a]	387.33±329.27[b]	369.16±237.97[b]	261.16±196.43[b]
Total *Vibrio* spp. *Count*	10.67±2.65[a]	1.09±1.04[b]	0.63±0.24[b]	5.56±2.07[b]

Row values with different superscript letters are significantly different at ($P<0.05$).

the viral and bacterial inhibition activities of three herbs: turmeric (*Curcuma longa*), *Andrographis paniculata* and *Clinacanthus mutans*. They found by *in vitro* study that extracts of all the herbs could inhibit, as well as eradicate, the shrimp pathogenic bacteria, *Vibrio* spp. and white spot virus, for which the turmeric extract showed the highest efficacy. By *in vivo* study, Supamattaya *et al*. (2004) prepared feed containing different concentrations of turmeric that was then fed to black tiger shrimps (*Penaeus monodon* Fabricius) for two weeks. They found that 5 and 25 mg/kg turmeric extract could enhance the survival rate of black tiger shrimps infected experimentally with *Vibrio harveyi*. In addition, volatile oil in the turmeric extract has been reported to show bactericidal effects (Lutomoski *et al*., 1974; Bhavanishankar and Murthy, 1986). There have been very limited studies on the effects of turmeric on aquatic animals. Dey and Chandra (1995) reported the production of disease-resistant fry of Indian major carp (*Catla catla*) by spawn treatment with turmeric, neem leaves and garlic powder.

Immune functions

The study on the effects of turmeric extract on Pacific white shrimp immunity showed a positive effect with three of the four parameters studied. Total haemocyte count was not affected by the herb extract. The bactericidal activity, a parameter that showed the ability of shrimp haemolymph to reduce certain amounts of bacteria, of all turmeric-treated shrimps had a higher range than in the control. This activity is believed to be an important defense mechanism against bacterial systemic infection in shrimps. Phenoloxidase and phagocytic activity was also significantly different between the treated and the control group. Interestingly, the most effective concentration of turmeric extract related to these parameters was 25 mg/kg. There have been very few studies on turmeric with regard to enhancing immunity in invertebrates. Supamattaya *et al*. (2004) reported the negative effects of feed supplemented with 50 and 200 mg/kg of turmeric extract (with 21.57% curcuminoids) on the immune functions of black tiger shrimp (*P. monodon* Fabricius). After 8 weeks of the feeding trial, shrimps that were fed with turmeric extract had lower total haemocyte counts and phenoloxidase activity than the control. These findings might have been caused by poor feed consumption and the rate of feed uptake of the treated shrimps.

Total bacterial count from shrimp intestine

The current study indicated clearly the bactericidal activity of turmeric extract in the intestines of Pacific white shrimp, in which the total bacterial counts and total *Vibrio* counts of all turmeric-treated groups were significantly lower than for the control. This result was different from Supamattaya *et al*. (2004), as they did not find any significant changes in the bacterial count in the hepatopancreas and intestines of black tiger shrimps fed with turmeric extract at 50 and 200 mg/kg, which might have been related to the low curcuminoids content in the extract and poor feed consumption by the experimental shrimps.

The mechanism of the immunostimulant effect of herbs in animals is not clearly understood. The immunostimulant effect can be achieved by four mechanisms: activation of phagocytosis, stimulation of the fibroblasts, increasing respiratory activites and increased mobility of leucocytes (Gurib-Fakim, 2006). Extracts from roots and aerial parts from various species of herbs have been assessed for their phagocytic potential in animals and all ethanolic root extracts increased phagocytosis by *in vitro* study (Gurib-Fakim, 2006). It is also useful to understand how animal cells react to herbs and their extracts. Gupta and Balasubrahmanyam (1998) reported an *in vitro* analysis of the effect of turmeric on endothelial cells from the human umbilical vein. They found that cells cultured in media with turmeric showed a proliferative response.

CONCLUSION

The results from the current study showed that the application of turmeric extract at 25 mg/kg feed for two weeks, and one month feeding of *Litopenaeus vannamei* Boone showed positive effects on: resistance to vibriosis; immune functions; and bactericidal activity in shrimp intestines. Thus, the extract of this herb can be used as an effective immunoenhancement that can be applied for the prevention of vibriosis in marine shrimp. The extract of this herb also showed an effective bactericidal effect in shrimp intestines. Caution will be required in the extraction process and in determining the most effective amount of the active components in the turmeric extract.

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