Effect of *Phyllanthus* spp. against yellow-head baculovirus infection in black tiger shrimp, *Penaeus monodon*

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Abstract

Five species of the herb *Phyllanthus*; *P. amarus, P. debilis, P. pulcher, P. reticulatus,* and *P. urinaria* were tested for their ability to inactivate yellow-head baculovirus (YBV), a virulent pathogen of black tiger shrimp, *Penaeus monodon*. Extraction was done by ethanol using a soxhlet apparatus. The virucidal activities of these extracts were tested by mixing the virus with plant extracts followed by incubation and injection into healthy black tiger shrimp. Antivirucidal activity was assessed by observation of the mortality rates of the injected shrimp. The toxicity of the plant extracts was also tested against shrimp. The extracts of *P. amarus* and *P. urinaria* inhibited YBV. The minimum inhibitory concentrations for *P. amarus* and *P. urinaria* were 0.1 and 1 mg /mL, respectively while the LD_{50} of the extract to postlarvae 15 were 2471 ± 6.3 mg/mL and 2564 ± 5.8 mg/mL, respectively. The extracts also enhanced phagocytic activity in black tiger shrimp.

Introduction

Outbreaks of yellow-head disease of the black tiger shrimp, *Penaeus monodon* have been observed in Thailand since 1990. Yellow-head disease originally caused extensive losses to shrimp farmers in the eastern and central parts of the country, and later in the southern region. Affected shrimps developed a pale body and light yellow coloration of the hepatopancreas and the gills. Cumulative mortality often reached 100% in affected populations within 3-5 d from the onset of the disease. The causative agent of the disease is yellow-head baculovirus (YBV) (Boonyaratpalin *et al.*, 1993).

The genus *Phyllanthus* comprises a large and widespread group of tropical herbs belonging to the family Euphorbiceae (Unander *et al.*, 1992). They have been used widely by traditional medical practitioners for the treatment of jaundice and other diseases. Their use was described in Indian Ayurvedic literature more than 2000 years ago. *Phyllanthus* species are also used as traditional medicine in China, the Philippines, Cuba, Nigeria, Guam, East and West Africa, the Caribbean, Central America, and South America (Thyagarajan *et al.*, 1988).

Thyagarajan *et al.* (1982) reported that *Phyllanthus amarus* (=*P. niruri*), eradicated the hepatitis B virus (HBV) carrier state permanently in 59% of chronic carriers. In 1987, Venkateswaran *et al.* found that aqueous extract of *P. amarus* inhibited the endogenous DNA polymerase of HBV and bound to the surface antigen. The inhibition was quantitatively specific for DNA polymerase of HBV-like viruses and much larger quantities were required to inhibit the DNA polymerase of bacteria and mammals. These aforementioned reports indicated that members of the genus *Phyllanthus* appear to have antiviral activity. The purpose of this study was to investigate the antiviral activity of *Phyllanthus* spp. against YBV and to determine the effect of the extracts on phagocytosis by black tiger shrimp haemocytes.

Materials and Methods

Preparation of extracts

Five species of the genus *Phyllanthus: P. amarus, P. debilis, P. pulcher, P. reticulatus,* and *P. urinaria,* were used for this experiment. The whole herbs were dried, ground and extracted with ethanol in the ratio 1:10 using a soxhlet apparatus. The extracts were evaporated and prepared as complexes with polyvinyl pyrrolidone (PVP) in the ratio 1:10 (wt/wt).

Virus

Virus stock was prepared by injecting YBV into black tiger shrimps.The gills of moribund shrimps were collected and homogenized in 10 times the volume of lobster haemolymph medium (LHM). After homogenization, the mixture was centrifuged at 1000 rpm for 5 min. The supernatant was then filtered through a 0.45 μm membrane filter and stored at -80° C until use.

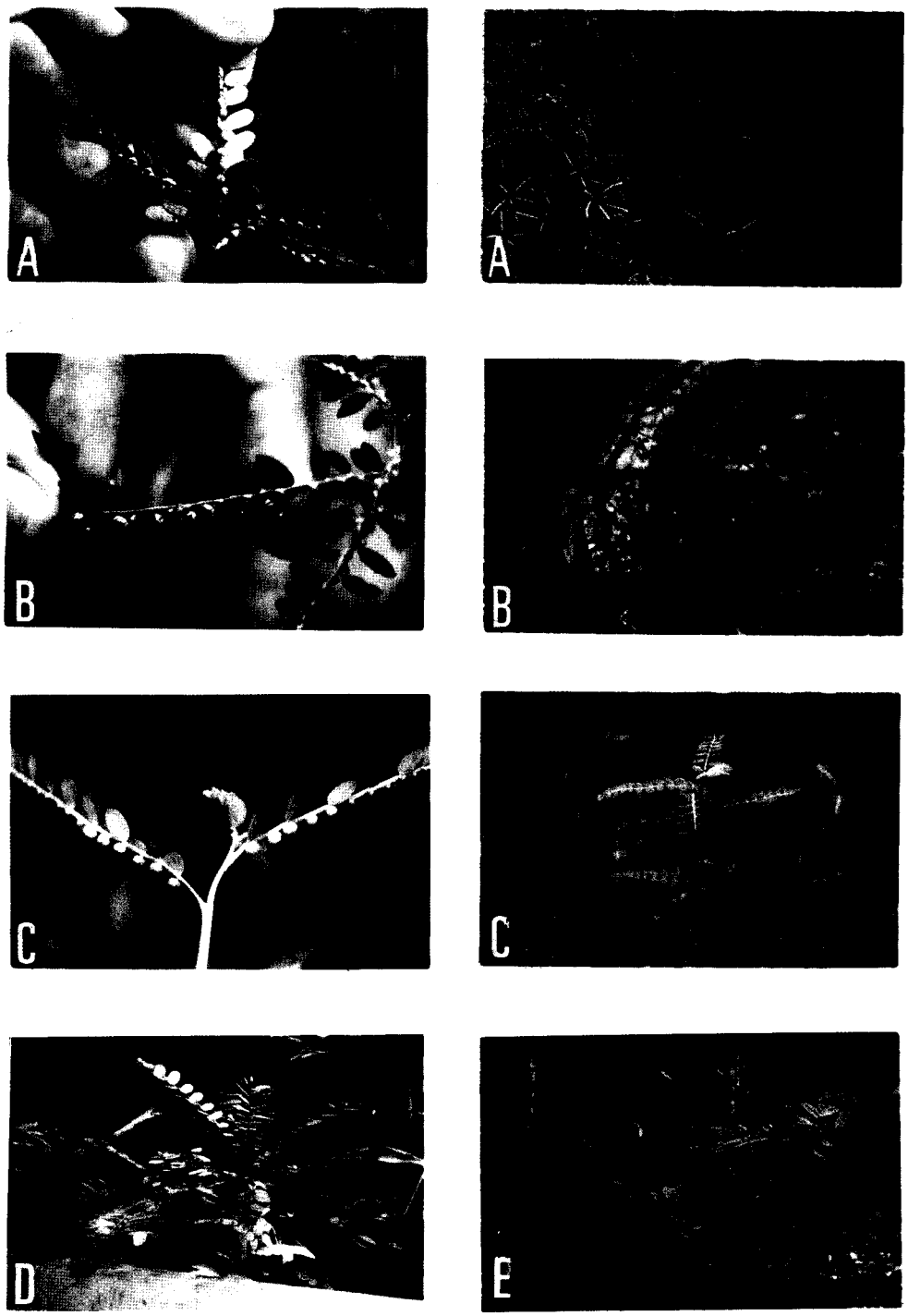

Fig. 1. *Phyllanthus species* used in this study; A: *P. amarus,* B: *P. debilis*, C: *P. urinaria,* D: *P. pulcher,* and E: *P. reticulatus.*

Antiviral activity

YBV extract diluted to 10^{-5} was mixed with 10 mg of each herb extract and incubated at 25°C for 2 h. After incubation, 0.2 mL of the mixture was injected into each group of 20 black tiger shrimps. Positive control shrimp (n=20) received YBV mixed with LHM and negative control shrimp (n=20) received only LHM by injection. Anti-viral activity was determined by observation of shrimp mortality within 14 d post-injection.

Toxicity study

Toxicity of the extracts was tested at concentrations of 0, 1,10, 100, 1000, and 5000 ppm of PVP complex using postlarvae 15 black tiger shrimps. Aquaria containing 10 L of seawater at 29-31°C and pH 7.9-8.1 were stocked with 50 postlarvae each. Mortality was observed after 24 h and LD_{50} calculations were determined using probit analysis.

Effect of extracts on phagocytic activity

Groups of 20 shrimps (body weight about 15-20 g) were fed with pellets containing 10 g of the extract (*P. amarus* and *P. urinaria*) per 1 kg feed, while the control group (n=20) was fed with normal pellets. Each group was fed two times a day, every day. After 7 and 14 d of feeding, 10 shrimps from each group were collected for phagocytosis testing according to Itami's method (Itami *et al.*,1989). Briefly, 0.5 mL of haemolymph was mixed with 4.5 mL of LHM containing 0.5 g of L-cystine and centrifuged at 6400 rpm (2000 G) for 2 min. The pellet was washed once with LHM then mixed with latex beads (diameter 1.09 mm), and incubated at 25°C for 45 min. Following incubation, the mixture was fixed with 0.125% glutaraldehyde for 10 min, washed with LHM, and stained with Diff-Quick (Merz & Dade). Phagocytes were then counted using a bright-field light microscope. The number of haemocytes containing engulfed latex beads was divided by the total number of haemocytes and multiplied by 100 to give a phagocytic index.

Effect of extract on water quality

Only the extract of *P. amarus* was used for this experiment. PVP complex at concentrations of 0, 1, 10, and 50 ppm was added to aquaria containing 10 L of seawater. Each aquarium was aerated through an air stone to maintain dissolved oxygen at saturation. After addition of the extract, the water was tested on days 1, 3, and 7 for pH, alkalinity, BOD, and COD.

Results

Of the five species of *Phyllanthus* tested, three species (*P. amarus, P. urinaria,* and *P. reticulatus*) had antiviral activity against YBV (Table 1). The minimum inhibitory concentrations of *P. amarus* and *P. urinaria* against YBV were 100 µg/mL and 1 mg/mL, respectively (Table 2). By contrast, the LD_{50} of *P. amarus* and *P. urinaria* was 2471 ± 6.3 and 2564 ± 5.8 mg/L, respectively.

Table 1. Virucidal effects of herbs in the genus *Phyllanthus* on YBV in *Penaeus monodon.*

Treatment	% mortality
Positive control (virus)	100
Negative control (LHM)	0
P. amarus	0
P. debilis	100
P. pulcher	100
P. reticulatus	20
P. urinaria	0

Table 2. MIC of *Phyllanthus amarus* and *P. urinaria* PVP complex against YBV in *Penaeus monodon.*

Treatment	% mortality	
	P. amarus	*P. urinaria*
Positive control (virus)	100	100
Negative control (LHM)	0	0
10 mg	0	0
1 mg	0	0
100 µg	0	0
10 µg	0	90
1 µg	80	100

Table 3. Phagocytic activity of haemocytes from *Penaeus monodon* fed with extracts of *Phyllanthus amarus* and *P. urinaria.*

Treatment	Phagocytic index (%)	
	7 days	14 days
Control	16.41	13.05
P. amarus	36.83	31.89
P. urinaria	34.76	37.89

The phagocytic indices of haemocytes from black tiger shrimps fed with 10 g of PVP complex per 1 kg feed for 7 and 14 d were higher than in the control group. For extracts of *P. amarus* and *P. urinaria,* the indices were 36.83% and 34.76% respectively, on day 7 and 31.89% and 37.89% respectively, on day 14. By contrast, the phagocytic index was 13.05 and 19.14%, respectively in the control group on day 7 and 14. These results showed that the extracts could stimulate phagocytosis in black tiger shrimps (Table 3). No histopathological changes could be observed in the gills and the hepatopancreas.

The extracts of *P. amarus* did not effect the pH and alkalinity of seawater. BOD and COD at high concentrations of extract (50 ppm) were higher than the other groups after 1 h. However, these BOD and COD values decreased after 1 d (Fig. 2).

Discussion

In this study, we observed that ethanol extracts from whole plants of *P. amarus* and *P. urinaria* had potent anti-YBV effects. The effects appeared to involve viral inactivation or inhibition that resulted in reduced mortality in extract-injected black tiger shrimps, which was according to the concentration of the extract. This reaction against YBV might be similar to that seen in hepatitis virus infection (Venkateswaran *et al.*, 1987). They reported that aqueous extracts of *P. niruri* inactivated hepatitis B virus and woodchuck hepatitis virus by binding to the surface antigen of the virus *in vitro.*

Preparations of *P. reticulatus* are recommended for the treatment of smallpox and syphilis in Indo-Chinese traditional medicine (Unander *et al.*, 1988). In this study, the extract of *P. reticulatus* also showed some antiviral activity. However, its activity was lower than those of *P. amarus* and *P. urinaria.*

In acute and chronic toxicity studies of *P. niruri* (=*P. amarus*) in mice, Venkateswaran *et al.* (1987) and Jayaram *et al.* (1987) showed that the plant was non toxic. In addition, non-toxic side effects of *P. niruri* were found in treated patients given 200 mg of dried powdered, sterilized plants in a capsule three times a day for 30 d (Thyagarajan *et al.*, 1988). The toxicity of extracts of these plants to black tiger shrimps postlarvae was also quite low. Extracts of *P. amarus* appear to be safe not only for humans but also for shrimp; LD_{50} was very high and no pathological signs were observed during feeding with extracts (10 g/1kg feed) for 7 and 14 d. However, further studies are needed to develop an antiviral substance from these herbs for prevention and control of YBV.

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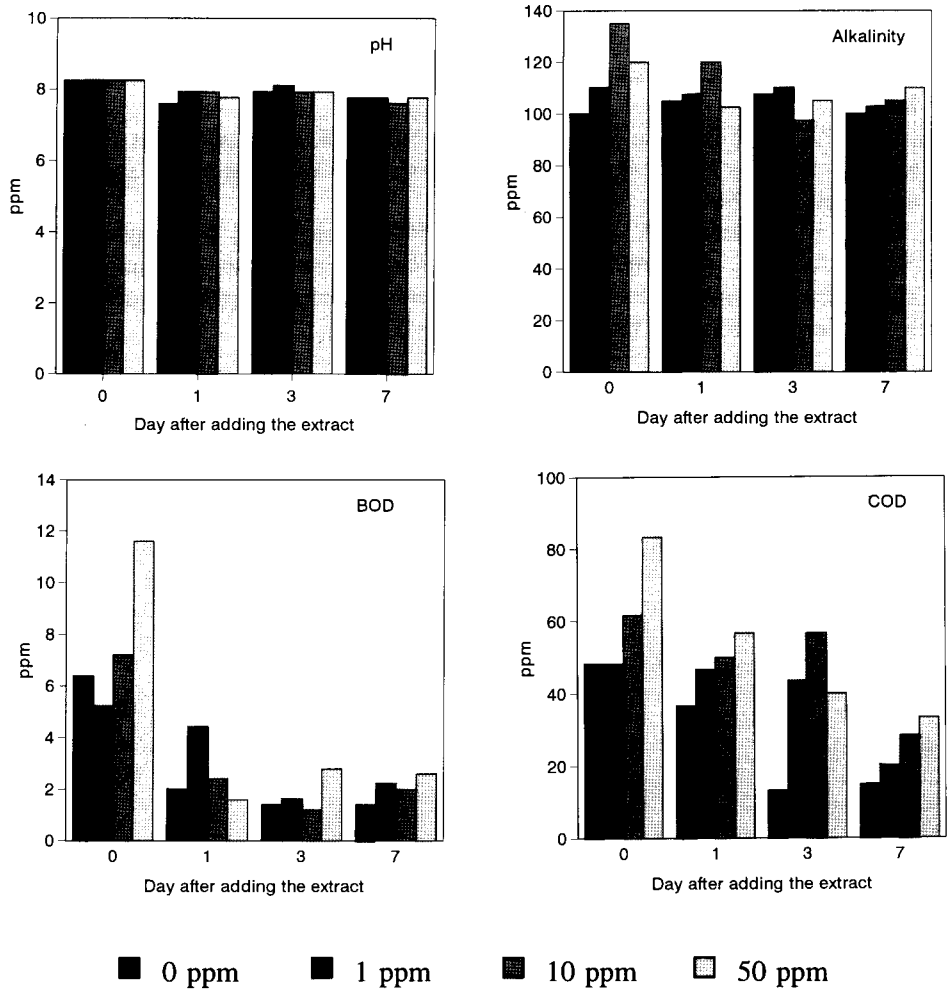

Fig. 2. Effect of *Phyllanthus amarus* extract on some water quality parameters.

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