Efficacy of Guava (*Psidium guajava*) Extract Against Some Fish and Shrimp Pathogenic Agents

[1]S. Direkbusarakom, [2]A. Herunsalee, [3]M. Yoshimizu [3]Y. Ezura and [3]T. Kimura

[1] *National Institute of Coastal Aquaculture, Kaosan Soi 1, Songkhla 90000, Thailand*

[2] *Division of Medical Plant Research and Development Department of Medical Science, Nontaburi 10100, Thailand*

[3] *Laboratory of Microbiology, Faculty of Fisheries Hokkaido University, Hakodate 041 Japan*

ABSTRACT

Guava (*Psidium guajava*) extract was tested for anti viral activity against the fish pathogenic viruses, infectious haematopoietic necrosis virus (IHNV), infectious pancreatic necrosis virus (IPNV) and *Oncorhynchus masou* virus (OMV) using plaque reduction in CHSE-214 cell lines. Anti viral tests against the shrimp pathogenic virus, yellow-head virus (YHV), was carried out using the injection method. The efficacy of guava extract was also determined using MIC of the extract against 24 strains of pathogenic bacteria including; *Vibrio harveyi* (9 strains), *V. splendidus* (7 strains), *V. parahaemolyticus* (2 strains) and 1 strain of each *V. mimicus*, *V. vulnificus*, *V. fluvialis*, *V. chorelae*, *V. alginolyticus* and *Aeromonas hydrophila*. A toxicity test of the extract was performed with CHSE - 214 cell lines and post larvae 15 of black tiger shrimp (*Penaeus monodon*). The efficacy of guava extract for the prevention of viral disease and bacterial disease in aquatic animals was estimated using YHV infection in black tiger shrimp and *A. hydrophila* infection in catfish, respectively. The extract of guava demonstrated anti viral activity against IHNV, OMV and YHV but was not effective for IPNV. Furthermore, the MIC of the extract ranged from 625 - 5,000 µg/ml against all pathogenic bacterial strains tested. The 50% cytotoxicity of the extract to CHSE - 214 was 1,923 µg/ml while the LD_{50} of the extract to black tiger shrimp post larvae was 2,968 ± 3.8 µg/ml. These results show that guava extract has low toxicity to salmon cell lines and black tiger shrimp. Moreover, the extract is effective for prevention of bacterial infection in catfish (*Clarias macrocephalus*) while not suitable for prevention of yellow-head virus infection in black tiger shrimp. From these results, guava can be recommended for treatment of bacterial disease in fish. The route of administration and effective dose should be determined before their efficacy can be tested in field trials.

INTRODUCTION

Diseases pose a serious problem in the development of aquaculture, especially bacterial and viral diseases that cause massive mortalities. Various chemotherapeutants have been applied for treatment of bacterial diseases, however, these may leave drug residues in the product. It may be possible that herbs can be used in treatment of bacterial and viral diseases in aquatic animals. They are natural products which are safe for consumers and many kinds of herbs may be used, including guava. Guava is a fruit which contains high levels of vitamin C.

Figure 1. Guava tree (*Psidium guajava*)

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In Thai traditional medicine guava has been used for treatment of many diseases such as diarrhoea, and herpes and for treatment of wounds. There are many scientific reports on the antimicrobial agent in guava. For example, Simon *et al.* (1961) found that guava had anti viral activity against Tobacco mosaic virus. Anti viral activity of guava extract against herpes simplex virus was also reported by Pienthong (1992). In addition, guava extract has antibacterial activity against *Salmonella typhi*, *Escherichia coli* and *Vibrio cholerae* (Julasiri *et al.* 1986; Sindermsuk *et al.* 1989).

The purpose of this study was to test the efficacy of guava against fish and shrimp pathogens in the laboratory as a preliminary step in testing the use of herbs for controlling diseases in aquaculture.

MATERIALS AND METHODS

Preparation of guava extract

Guava extract was prepared according to the method of Herunsalee (1993). Guava (*Psidium guajava*) leaves are dried at 50°C for 20 hr and extracted with ethanol using a Soxhlet apparatus.

Bacteria

Twenty four strains of pathogenic bacteria were used in this study. These included *V. harveyi* (9 strains), *V. splendidus* (7 strains), *V. parahaemolyticus* (2 strains) and 1 strain each of *V. mimicus*, *V. vulnificus*, *V. fluvialis*, *V. cholerae*, *V. alginolyticus* and *Aeromonas hydrophila*. All strains of *Vibrio* were maintained in trypticase soy agar (TSA) supplemented with 1.5 % NaCl. TSA without added salt was used for *A. hydrophila*.

Viruses

Three fish pathogenic viruses were used: infectious haematopoietic necrosis virus (IHNV), infectious pancreatic necrosis virus (IPNV) and *Oncorhynchus masou* virus (OMV) (Kimura and Yoshimizu 1991). All viruses were inoculated onto CHSE-214 cell lines (Chinook salmon embryo) (Fryer *et al.* 1965) grown in 75 cm^2 plastic flasks containing 25 ml of Tris-MEM10 (Modified Eagle's minimum essential medium, pH 7.2). When the cytopathic effect reached a maximum, the culture fluid was removed from the flask and filtered through a 0.45 µm pore membrane filter (Millipore) and stored at -80°C until used.

One shrimp pathogenic virus, yellow-head virus (YHV) (Wongteerasupaya *et al.* 1995) was used. Virus stocks were prepared by injection of YHV into black tiger shrimps (*Penaeus monodon*). The gills of moribund shrimp were collected and homogenised in 10 times the volume of lobster haemolymph medium (LHM).

After homogenisation, the mixture was centrifuged at 1,000 g for 5 min. The supernatant was then filtered through a 0.45 µm membrane filter and stored at -80°C until required.

Antiviral tests

Antiviral activity for fish pathogenic viruses was estimated using the plaque reduction method according to Kamei *et al.* (1989). Briefly, each extract was diluted appropriately with Hank's balanced salt solution (Hank's BSS). A mixture of 0.2 ml of this diluted guava extract and an equal volume of viral suspension (approximately 200 PFU/0.1 ml) was allowed to react at 15°C for 3 hr. A 0.2 ml aliquot of the mixture was inoculated into each of 2 wells of a 24 well microplate (Falcon) containing confluent monolayers of CHSE - 214 cell lines at 15°C for 1 hr. The inocula was then removed and the cells washed 3 times with Hank's BSS. One ml of 0.8% methyl cellulose was then added to the cell culture. After 10 days incubation the cells were fixed with 10% formalin, stained with 0.1% crystal violet, and the plaques counted. The plaque reduction rate was calculated by comparison with a positive control which was inoculated with the virus suspension.

The injection method of Direkbusarakom *et al.* (1993) was used for antiviral assay of the shrimp pathogenic virus. One ml of YHV extract was diluted to 10^{-5} and mixed with an equal volume of the diluted guava extract, then incubated at 25°C for 3 hr. After incubation, 0.2 ml of the mixture was injected into each of 20 black tiger shrimp. Positive controls received YHV mixed with LHM and the negative control received only LHM. Anti-viral activity was determined by observation of shrimp mortality within 14 days of injection.

Antibacterial tests

The plate dilution method according to Tragen (1983) was used for antibacterial tests. Serial two-fold dilutions of the guava extract were prepared with concentrations ranging from 625 to 10,000 µg/ml. One millilitre of each dilution was mixed with 9 ml of Muller Hinton Agar + 2% NaCl and plated. Bacterial strains were cultured in tryticase soy broth (TSB) + 2 % NaCl and incubated at 30°C for 18 hr before use and 0.1 ml of each strain inoculated into wells holding 1 ml of the same broth.

Toxicity tests in black tiger shrimp

Toxicity of extracts was tested at concentrations of 0, 1, 10, 100, 1000 and 5000 ppm of guava extract using postlarvae 15 of black tiger shrimp. Each group of 50 postlarvae was reared in aquaria containing 10 litres of sea water at 29-31°C and pH 7.9-8.1. Mortality was observed after 24 hr and LD_{50} was calculated using probit analysis.

Cytotoxic assay

Cytotoxicity of guava was estimated using CHSE -214 cells according to Fernandez *et al.* (1993). Briefly, wells of a 96 well plate were seeded with 0.1 ml of CHSE - 214 ($1x10^5$ cell/ml) and cultured in MEM containing the extract at a final concentration of 0, 10, 100, 1000, 10,000 and 50,000 µg/ml. After 5 days incubation at 15°C, cells in the microplate were fixed with 10% formalin for 30 min and washed with tap water. Cells were then stained with 0.1% crystal violet for 1 hr and rinsed several times. Rinsed microplates were then thoroughly air dried. Absorbance of stained microplates was measured using a microplate spectrophotometer (Corona MTP-22) at 600 nm. The 50% cytotoxic dose of each extract was analysed using probit analysis.

Efficacy against YHV infection in black tiger shrimp

Groups of 20 black tiger shrimp (body weight about 15-20 g) were fed with pellets containing 1 g of guava extract per 1 kg of feed, while the control group was fed with normal pellets. Each group was fed 2 times per day, every day. After 14 days of feeding, the shrimps were immersed in YHV suspension in sea water (1 g/10 L of seawater) for 3 hr. The clinical signs and the mortality of each group were observed up to 14 days after infection. Three replications of each trial were performed. The difference between experimental and control groups was analysed using one way analysis of variance (ANOVA).

Efficacy against *Aeromonas hydrophila* infection in catfish

Groups of 10 catfish (*Clarias macrocephalus*) (body weight about 35 g) were fed with pellets containing 1 g of guava extract per 1 kg of feed, while the control group was fed with normal pellets. Each group was fed 2 times per day, every day. After 7 days of feeding, the fish was injected with 0.1 ml *Aeromonas hydrophila* (10^9 CFU/ml). The clinical signs and the mortality of each group were observed for 14 days after infection. Three replications of each trial were performed. The difference between experimental and control groups was analysed by one way analysis of variance (ANOVA).

RESULTS

Antiviral tests

The extract of guava showed antiviral activity against IHNV, OMV and YHV but did not affect IPNV. The minimal inhibitory concentration of guava extract against IHNV, OMV and YHV was 0.8, 30.74 and 1000 µg/ml respectively (Table 1).

Table 1 Antiviral activity of *Psidium guajava* against fish and shrimp pathogenic virus

Virus	Antiviral	MIC (µg/ml)
IHNV	+	0.80
IPNV	-	N
OMV	+	30.74
YHV	+	1000.00

N = Not done

Antibacterial tests

The minimal inhibitory concentration (MIC) of the extract against the 24 strains of pathogenic bacteria ranged from 625-5,000 µg/ml. Twenty-two strains of the tested bacteria were inhibited at a concentration of 1250 µg/ml. One strain of *V. splendidus* had the highest MIC at 5,000 µg/ml while the MIC of guava against *A. hydrophila* was lowest at 625 µg/ml (Table 2).

Toxicity test in black tiger shrimp

The guava extract exhibited very low toxicity to postlarvae of black tiger shrimp. The LD_{50} of the extract to postlarvae was 2,968 ± 3.8 µg/ml at 24 hr.

Cytotoxic assay

Extracts were found to have low toxicity to CHSE - 214 cell line because the cytotoxic 50% value of guava extracts was very high at about 1,923 µg/ml.

Efficacy against YHV infection in black tiger shrimp

Mortality was first observed at day 4 in the positive control group and day 5 for the group that was fed with guava. Within 14 days the accumulated mortalities after infection of the control group and those fed with guava were 80 and 66% respectively (Table 3). ANOVA comparison showed no significant difference ($P<0.05$) for mortalities of the control group and the test group (fed with guava).

Table 2. MIC of guava extract against fish and shrimp pathogenic bacteria

Bacteria	MIC (mg/ml)
Aeromonas hydrophila	625.00
Vibrio alginolyticus	1250.00
V. cholerae	1250.00
V. fluvialis	1250.00
V. mimicus	1250.00
V. parahaemolyticus (N112)	1250.00
V. parahaemolyticus (N 119)	1250.00
V. vulnificus	1250.00
V. harveyi (N97)	1250.00
V. harveyi (N101)	1250.00
V. harveyi (N148)	1250.00
V. harveyi (N151)	1250.00
V. harveyi (N152)	1250.00
V. harveyi (N153)	1250.00
V. harveyi (N154)	1250.00
V. harveyi (N156)	1250.00
V. harveyi (N157)	1250.00
V. splendidus (N142)	1250.00
V. splendidus (N155)	5000.00
V. splendidus (N157)	1250.00
V. splendidus (N158)	1250.00
V. splendidus (N159)	1250.00
V. splendidus (N161)	1250.00
V. splendidus (N162)	1250.00

Table 3. The mortality of black tiger shrimp after infection with YRV

Treatment	Mortality (%)
Control	80 ± 8.16
Guava	66 ± 9.41

Efficacy against *Aeromonas hydrophila* infection in catfish

A wound appeared at the injection site in both groups 24 hr after injection. By day 3 the wound in the positive control group had progressed and 80% of the fish died. The size of the lesion in the control fish was bigger than the group that received guava extract (Fig 2). The wound in the group that was fed with guava extract began to recover 4 days after injection and had completely recovered by day 10. Within 14 days post injection no mortalities were observed in the group that was fed with guava extract (Table 4). ANOVA comparison showed a significant difference ($P<0.05$) for the mortalities in the control group and test group (fed with guava).

Table 4. The mortality of hybrid catfish after infection with *A. hydrophila*

Treatment	Mortality (%)
Control	80 ± 4.08
Guava	0.00

DISCUSSION

This study indicated that guava extract had antibacterial and antiviral activity against fish and shrimp pathogens. With the exception of IPNV, all the viruses (i.e., OMV, IHNV and YHV) were enveloped viruses (Kimura *et al.* 1991 and Boonyaratpalin *et al.* 1993). This suggests that viral inactivation by guava extract might be due to a reaction with the viral envelope. On the other hand, Pienthong *et al.* (1992) found that the main effect of the extract was on viral replication.

The MIC of guava extract against YHV seems to be higher than with OMV and IHNV. It is also higher than the MIC of extracts from the medicinal plants *Clinacanthus nutans* and *Phyllanthus amarus* by about 1000 and 100 times, respectively

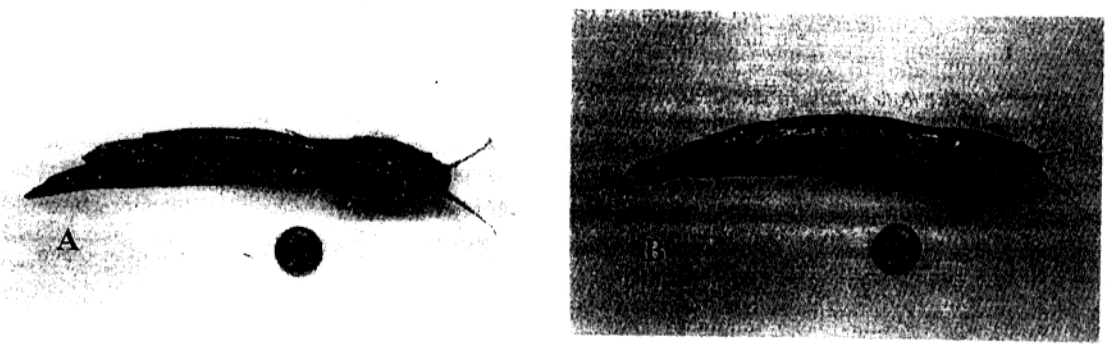

Figure 2. The lesion 3 days after injection.
A = Control group, B = Fed with guava extract.

(Direkbusarakom *et al.* 1993a,b). Therefore, the efficacy of guava extract against YHV is much less than that of *Clinacanthus nutans* and *Phyllanthus amarus*.

This herb had low toxicity to both the CHSE - 214 cell line and postlarvae 15 of black tiger shrimp. The extract was effective for prevention of bacterial infection in hybrid catfish while not suitable for prevention of yellow-head virus infection in black tiger shrimp. This might have been due to loss of the extract before feeding, as shrimp are slow feeding animals.

Thanangkol and Chaichangptipayut (1987) found that guava leaf is better than oxytetracycline for treatment of acute diarrhoea in humans. For this reason, it might be possible to use guava extract for prevention of bacterial disease in fish. However, before guava can be used for prevention of either bacterial or viral diseases in black tiger shrimp, further study is required.

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