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In vivo screening of mangrove plants for anti WSSV activity in *Penaeus monodon*, and evaluation of *Ceriops tagal* as a potential source of antiviral molecules

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ABSTRACT

The objective of the study was to find out a natural way to fight white spot syndrome virus (WSSV) in cultured shrimps, as the present scenario necessitated an organic remedy for the devastating pathogen in crustaceans. Under this research programme seven mangrove plants were collected, identified and aqueous extracts screened for their protective effect on the giant tiger shrimp *Penaeus monodon* against WSSV. The experimental design consisted two modes of application, such as exposure of the virus to the extract and injection challenge, and oral administration of the extract coated feed followed by oral challenge. All experimental animals were monitored through a nested diagnostic PCR analysis. Of the seven mangrove extracts screened aqueous extract from *Ceriops tagal* imparted total protection to shrimp from WSSV when challenged by both methods. Shrimps administered with the aqueous extract from *C. tagal* were devoid of virions. The HPLC fingerprint of the aqueous extracts from *C. tagal* showed more than 25 peaks and 7 of them were larger and well separated. Preliminary phytochemical analysis revealed the presence of alkaloids, flavonoids, polyphenolics, cardiac glycosides, saponins and sterols. The study indicated suitability of the aqueous extract of *C. tagal* as a possible prophylaxis for WSSV infection in shrimp. This is the first report on the anti WSSV property of the mangrove plant *C. tagal*.

1. Introduction

White spot syndrome virus (WSSV), an enveloped non occluded DNA (300 kb) virus of the family Nimaviride under the new genus *Wispovirus* (Mayo, 2002), is the most devastating shrimp pathogen ever isolated and studied; it causes total mortality to a rearing stock within 3–7 days of infection in a culture system (Lightner, 1996). The virus has a wide host range and has been detected in diverse groups of crustaceans (Lo et al., 1996). Ironically till now no effective treatment or prophylactic measure could be developed to manage the virus. However, different approaches to manage the pathogen in culture systems have been experimented, such as oral administration of peptidoglycan, lipopolysacharides, β-1,3 glucan (Itami et al., 1998; Takahashi et al., 2000; Chang et al., 2003), vaccination with inactivated viral preparation and viral envelop protein VP19 and VP 28 (Singh et al., 2005; Namikoshi et al., 2004; Witteveldt et al., 2004), feeding with fucoidan extracted from *Sargassm polycysticus,* (Chotigeat et al., 2004), and Cidofovir an anti viral drug supplemented with *Spirulina platensis* (Rahman et al., 2006). The protective effect of *Cynodon dactylyon* (a terrestrial plant) in *Penaeus monodon* from WSSV has also been documented (Balasubramanian et al., 2007;2008).

For centuries, mangrove plants found in the tropics have been in focus as the source of bioactive molecules (Bandaranayake, 2002), having different dimensions of activities. They have been used in folklore medicine for treatment of several diseases (Kirtikar and Basu, 1935; Chopra et al., 1956; Datta and Datta, 1982). Extracts from different parts of the plant and their associates are being widely used worldwide for medicinal purposes (Bandaranayake, 1998). The most important reports available are the anti Tobaco Mosaic Virus activity from 16 species of mangrove plants (Padmakumar et al., 1993), anti Newcastle Disease Virus, anti Encephalomyocarditis Virus, anti Semliki Forest Virus, anti Human Immunodefficiency Virus, anti Vaccinia Virus, and anti Hepatitis B Virus activities as detected in the leaves of *Bruguira cylindrica,* and the broad spectrum antiviral activity in the bark of *Rhizophora mucuronata* (Premanathan et al., 1999a,b). However, there has never been any report on the anti WSSV activity in mangrove plants. This prompted us to take up the present investigation.

2. Methods

2.1. Collection and identification of mangrove plants

Mangrove plants such as *Excoecaria agallocha*, *Acanthus ilicifolius*, *Avicennia* sp., *Rhizophora mucronata*, *Rhizophora apiculata, Sonneratia* sp. and *Ceriops tagal* were collected from different localities in South India (9° 58′ 1.20″N, 76° 15′ 0.00″E). The plants were identified

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following Naskar and Mandal (1999), coded and voucher specimens deposited in the herbarium collection of the National Centre for Aquatic Animal Health, Cochin University of Science and Technology.

2.2. Aqueous extract from mangrove plants

Leaves were shade dried, powdered and used for the preparation of the aqueous extract using a protocol developed here. Accordingly, 50 g of mangrove plant leaf powder was soaked in a minimum quantity of double distilled water and frozen to −20 °C, thawed and frozen repeatedly for three times and extracted to 500 ml final volume of double distilled water in a Warring blender at an ambient temperature. The extract was sieved through a fine meshed (100 μm) cloth, centrifuged at 10,000 g for 20 min and the supernatant maintained at −20 °C till used. These preparations were examined for their virucidal activity and for their protective effects from WSSV on oral administration in *P. monodon*.

2.3. Virus inoculum

A composite sample of gills and soft parts of cephalothorax (500 mg) from freshly infected *P. monodon* was macerated in 10 ml cold PBS (NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.15 g, KH_2PO_4 0.2 g, double distilled water 100 ml) with glass wool to a homogenous slurry using mortar and pestle in an ice bath. The slurry was centrifuged at 8200 g in a refrigerated centrifuge at 4 °C and the supernatant filter sterilized using a 0.22 μ pore size PVDF membrane filter. The preparation was streaked on ZoBell's agar plates and incubated at 28 ± 2 °C for 72 h to confirm the absence of bacterial contamination. Viral infectivity titre was determined as the extractable virus and expressed as LD_{50} in shrimp following Reed and Muench (1938). The virus stock thus prepared for the experiment was stored in 500 μl aliquots at −80 °C. The viral titter determined was $1 \times 10^{13.69}$/ml inoculum as experimented by Singh et al., 2005. Viability of WSSV in suspension was checked by injecting 10 μl to a batch of apparently healthy shrimp (6 nos) and mortality confirmed over a period of 3 to 7 days.

2.4. Preparation of mangrove extract coated feed

The aqueous extracts were lyophilized and the dry mass re suspended in the required quantity of distilled water and coated onto feed pellets to arrive at a concentration of 1% w/w. As a binder, 4% aqueous gelatin in distilled water, was surface coated at a ratio 5:40 (v/w) to immobilize the plant extract. The above preparation was dried under vacuum and used for oral administration.

2.5. Experimental animals

All animals used in this study were single spawner bred, WSSV free juveniles of *P. monodon* grown in a recirculating aquaculture system at the National Centre for Aquatic Animal Health. The WSSV free status was confirmed through frequent Nested PCR analysis using a commercial kit (Bangalore Genei, Bangalore, India) of the shrimps during the culture operation in the recirculation system and also before the start of the experiment. The shrimps weighing 4–5 g were maintained in 30 liter capacity fiber reinforced plastic (FRP) tanks with diluted sea water at salinity 15. The experiment was repeated 4 times having 5 shrimps in a batch (5 × 4 = 20). Uniformly 10% water was exchanged every day.

2.6. Virucidal activity of the aqueous extract

The aqueous plant extracts (0.5 ml) were mixed with equal volumes of viral suspension and incubated for 3 h at 25 °C. The controls included mixtures of WSSV and PBS (positive control) and PBS alone (negative control). From each of the preparation, aliquots of 10 μl each were intramuscularly administered to the animals (5 × 4 = 20 animals each) and monitored for 15 days. Gill tissue was extracted from moribund animals and the controls, which survived the 15 day period of experimentation during the course of the experiment. The samples were preserved in 70% ethanol for diagnostic PCR to detect WSSV.

2.7. Oral administration of the plant extracts along with diet and challenge with WSSV

All test animals (5 × 4 = 20 animals each) were fed with the plant extract coated feed at a rate of 10% of the body weight two times a day. The control animals were fed with the diet coated with 4% gelatin. Feeding continued for 15 days, and the animals were challenged by feeding with freshly generated WSSV infected tissue at a rate of 10% of the body weight, and kept under observation for 15 days on the respective diet (normal diet for the positive and negative controls and the diet coated with the plant extract in the test group). Gill tissue was extracted from moribund/dead animals and from those which survived the challenge with WSSV, and was preserved in 70% ethanol for diagnostic PCR.

2.8. Confirmation of anti WSSV activity

To confirm the antiviral activity detected in the segregated plant species (*C. tagal*) intramuscular administration of virus suspension exposed to the plant extract, and oral administration of the plant extract and subsequent oral challenge were repeated in a batch of 24 animals and assayed by way of nested PCR. On completion of the experiment, tissue homogenates were prepared from the test and control animals and passaged to a fresh batch of nested PCR negative animals as bio assay to check the presence of virions in the survived animals. The presence of WSSV DNA was further examined by way of nested PCR.

2.9. Diagnostic PCR of the extracted tissue samples

For diagnostic PCR, DNA from the gill tissue was extracted in DNAzol according to the manufacturer's protocol. A WSSV nested PCR detection kit (Bangalore Geni) that yielded 650 and 300 bp WSSV specific amplicons was used for amplification of the viral DNA. Following the instructions given with the kit, the amplified product was generated in a thermal cycler (Eppendroff). The PCR products were then analyzed on 1% W/V agarose gels using TAE (1X) buffer (Tris–HCL 0.04 M, EDTA 0.0001 M, Glacial acetic acid 5.71%), stained with ethidium bromide and visualized on a gel documentation system, Dolphin-Doc (Weal Tec, USA).

2.10. HPLC analysis of the crude aqueous extract from C. tagal

On realizing the aqueous extract of *C. tagal* as the most effective preparation to protect shrimp from WSSV, it was subjected for HPLC analysis to generate HPLC fingerprint. The semi preparative HPLC system employed consisted of a Dionex Ultimate 3000 high performance liquid chromatograph coupled with a UV–Visible variable detector (VWD). Lyophilized crude aqueous extract from *C. tagal* was prepared in double distilled water (100 mg/10ML) and the separation was achieved on a 4.6 × 250 mm i.d Acclaim 120 Å C18 5 μm (Dionex) column at an ambient temperature. The mobile phase consisted of water (solvent A) and acetonitrile (solvent B). A linear gradient from 0 to 60% (B) was used for 85 min followed by an increase from 60 to 90% (B) for 10 min finishing at an isocratic mode at 90% (B) for 10 min. The total run time was 105 min with a flow rate of 1 ml/min, and the injection volume adjusted to 20 μl under a monitoring wavelength of 260 nm. Subsequently, the system was brought back to the initial conditions and equilibrated for 10 min with water. The process was repeated 3 times.