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Antiviral activity of 9(10H)-Acridanone extracted from marine *Streptomyces fradiae* strain VITMK2 in *Litopenaeus vannamei* infected with white spot syndrome virus

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ABSTRACT

The aim of the present study was to screen the antiviral activity of the secondary metabolite 9(10H)-Acridanone extracted from *Streptomyces fradiae* strain VITMK2 in *Litopenaeus vannamei* shrimp infected with white spot syndrome virus (WSSV). The strain was isolated from marine soil sediment sample collected from the mangrove forest region of Pichavaram, Tamil Nadu, India. Among the 31 isolates, the isolate VITMK-2 exhibited strong antiviral activity against the WSSV. The potential isolate was subjected to morphological, biochemical and molecular taxonomic characterisation. It was identified as *Streptomyces* species and designated as *S. fradiae* strain VITMK2. Purification of ethyl acetate (EA) extract by silica gel column chromatography yielded two compounds, C1 and C2. The shrimp infected with WSSV and treated with C1 compound (500 µg, 250 µg, and 125 µg) showed survival rates of 88.89%, 83.33% and 55.56% respectively. The survival of shrimp treated with the EA extract of *S. fradiae* strain VITMK2 (500 µg/animal) was 83.33%. The C1 compound (250 µg/animal) showed better antiviral activity when compared to EA extract. The chemical nature of the C1 compound was identified by FTIR, ^{1}H and ^{13}C NMR, and HRMS analysis. Based on the spectral data, the C1 compound was identified as 9(10H)-Acridanone with a molecular formula of $C_{13}H_9NO$ and a molecular mass of 195.1048 Da. Docking of the lead compound with VP26 and VP28 of WSSV drug target proteins showed the least binding energy of −5.71 kcal/mol and −5.21 kcal/mol respectively predicting the strong interaction of the compound with VP26 and VP28. This is the first report on anti-WSSV activity of 9(10H)-Acridanone derived from marine *S. fradiae* strain VITMK2 and this compound can be probed further to be used as an effective antiviral agent for better control and management of WSSV infection in aquaculture farms.

1. Introduction

Aquaculture is the fastest growing food industry which meets a major portion of the global food demand. Globally shrimp aquaculture was a profitable segment of aquaculture until disease outbreaks caused socio-economic problems. Since 1993 white spot syndrome virus (WSSV), a major pathogen of cultured shrimp has caused huge economic losses to the shrimp farming industry worldwide. WSSV is a rod shaped virus with a double stranded genome belonging to the *Nimaviridae* family. It has a wide host range such as shrimp, crab and crayfish (Sarathi et al., 2008). Nearly 100 species of arthropods have been reported as carriers or host of WSSV (Hameed et al., 1998). It was revealed that the cells of stomach, gills and integument are the possible routes of viral entry into the cells. The clinical symptoms are presence of white spots on the inner surface of the carapace and cuticle over the abdominal segments especially in Asian shrimp species such as *Penaeus monodon*. Other symptoms include reduction in food consumption, lethargy and reddening of appendages (Takahashi et al., 1994). WSSV causes 100% mortality within a few days after the onset of infection when the virus is administered by immersion, oral or intramuscular injection (Lightner, 1996).

In India, the loss of shrimp due to WSSV infection has been estimated to be about USD 150 million per year (CIBA Report 2008). Use of antibiotics as therapeutic agents even against viral pathogens associated with secondary bacterial infection is prevalent (Selvin et al., 2009). The problems and toxicity associated with the use of antibiotics as therapeutic agents to prevent viral infections are overcome by the use of probiotics and disinfectants (Li et al., 2016). One of the best

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adapted mechanisms to control aquatic diseases is the use of 'green-water technology'. However this requires routine monitoring of the morphological features of the cultured stock and the presence of viral pathogens in the cultured shrimp (Corre et al., 2000). Natural products have been reported for varied biological activities such as anti-microbial, immunostimulatory, anti-stress and growth promotion (Citarasu et al., 2002). Natural products also have an advantage as they are non-toxic, biocompatible and biodegradable (Citarasu et al., 2003). The use of natural products to control viral infection in aquaculture is considered as a promising approach and this has generated interest among researchers in the recent past. Various medicinal plants have been screened for antiviral activity against WSSV in shrimp with positive results (Balasubramanian et al., 2007).

Actinomycetes are a group of gram positive, unicellular, branching microorganism with a high G + C content in their genome. They are known for their ability to produce a wide variety of secondary metabolites with varied biological activities (Baltz et al., 2005; Bérdy, 2005). Among actinomycetes, the genus *Streptomyces* is widely distributed in marine and terrestrial habitats and account for 75% of the commercially available antibiotics (Sujatha et al., 2005). Marine actinomycetes have been reported to be antagonistic to *Vibrio* spp. pathogenic to shrimps (You et al., 2005). Das et al. (2006) reported that supplementing *Streptomyces* as a probiotic in the feed aids growth of the shrimp and prevents disease outbreaks in black tiger shrimp, *Penaeus monodon* (Fabricius). You et al. (2007) recommended the use of actinomycetes to prevent the disease caused by *Vibrio* spp. Administration of actinomycetes containing feed additives for two weeks to *P. monodon* challenged with WSSV increased the survival rate from 11 to 83% (Kumar et al., 2006). The use of actinomycetes as probiotics to control white spot disease in shrimps and vibriosis was reported (Velmurugan et al., 2015). It was reported that probiotics could be an effective alternative to antibiotics in aquaculture, which is beneficial not only for combating diseases and maintaining growth but also for stimulating immune responses in the host (Hai, 2015). The viral envelope protein VP28 acts as an important virulent factor in the initial phase of WSSV infection in shrimp.

The nucleocapsid protein VP26 is one of the proteins that is involved in the viral infection along with VP28 and VP19 (Youtong et al., 2011). Proteins V28, VP 26 and VP19 are considered as vital antiviral drug targets for the prevention of WSSV infection in shrimp (Verbruggen et al., 2016). Hence, finding a lead compound for targeting these viral proteins is considered as an important step towards the prevention of WSSV infection in shrimp. In the present study the antiviral potential of a bioactive secondary metabolite extracted from *S. fradiae* strain VITMK2 against WSSV in shrimp is reported. The mechanism of action of the lead compound on WSSV was studied using the *in Silico* molecular docking analysis.

2. Materials and methods

2.1. Isolation of actinomycetes from sediment sample

Marine soil sediment sample was collected from the mangrove forest region of Pichavaram, Tamil Nadu, India. The collected soil sediment was pre-treated at 45 °C for two days in a hot air oven. The pre-treated soil sediment sample was serially diluted and was spread plated on actinomycetes isolation agar (AIA) and starch casein agar (SCA). The AIA and SCA plates were observed periodically for actinomycetes growth. Single colonies of actinomycetes were picked and streaked on to a fresh AIA plate and stored for further use.

2.2. Collection and maintenance of experimental animals

Whiteleg shrimp, *L. vannamei* (12–15 g body weight) were collected from grow-out ponds located near Nagapatinam, Tamil Nadu, India. The animals were transported to the laboratory in live condition using continuous aeration. The animals were maintained in 1000-l fibre glass tank with airlift biological filters at an ambient temperature of 27–30 °C and salinity between 20 and 25 ppm. Natural sea water from the Bay of Bengal, India was used for the experiments. Sand and other suspended particles from the pumped sea water were removed. The sea water was treated with sodium hypochlorite at a concentration of 25 ppm and was dechlorinated by vigorous aeration followed by passing it through a sand filter. The animals were fed with artificial pellet feed (CP feed, Thailand). The temperature and pH were recorded, salinity was measured using a salinometer (Aquafauna, Japan) and dissolved oxygen concentration was estimated by Wrinkler method. The shrimp was acclimatized in the tanks for a week before starting the experiment. Shrimp were randomly selected and tested for WSSV, hepatopancreatic parvovirus (HPV) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) using specific set of viral primers (Yoganandhan et al., 2004). Healthy and virus-free shrimp were used for experimental purpose.

2.3. Preliminary screening of the actinomycete isolates

The single colonies of actinomycetes were subjected to submerged fermentation in tryptone yeast extract medium (ISP-1) (Casein enzymatic hydrolysate - 5.0 g/l, yeast extract - 3.0 g/l adjusted to a final pH of 7.0 ± 0.2) and were allowed to grow for 7 days. The cell free supernatant obtained by filtration using Whatman No. 1 filter paper was added with equal volume of ethyl acetate for extraction and placed in a rotary shaker overnight. The solvent phase was separated using a separating funnel and was concentrated using a rotary vacuum evaporator. The obtained EA extract was used for screening of antiviral activity. The viral inoculum was prepared according to the method given below. WSSV infected shrimp were collected from farms near Nellore, India. Cephalothoraxic tissues (including gills) were homogenized in NTE buffer (0.2 M Nacl, 0.02 M Tris HCl, 0.02 M EDTA, pH – 7.4) and centrifuged at 3000 ×*g* (4 °C for 20 min). The supernatant obtained was centrifuged at 8000 ×*g* (4 °C for 30 min). The resulting supernatant was passed through a 0.4 μm filter and the filtrate was stored at −20 °C. The WSSV presence was confirmed by PCR using primers designed by Yoganandhan et al., 2003. The shrimp (15 animals per group) were injected intramuscularly with a mixture of viral inoculum, EA extract of actinomycetes and NTE buffer (0.2 M NaCl, 0.02 M Tris–HCl and 0.02 M EDTA, pH 7.4). A volume of 30 μl per animal (5 μl (2.4 × 10(Citarasu et al., 2002) copies of WSSV) of viral inoculum, 10 μl of actinomycetes extract with varying concentration (500 μg/animal and 15 μl of NTE buffer) was injected to the experimental groups (Kulabhusan et al., 2017). The positive control consisted of a mixture of 25 μl NTE buffer and 5 μl of viral inoculum.

2.4. Characterisation of the potential isolate

The actinomycetes isolate exhibiting strong antiviral activity against WSSV were characterized by morphological, biochemical, and molecular methods. The isolate was inoculated on AIA and its morphological characteristics such as size of the colonies, presence of aerial and substrate mycelium, production of diffusible and reverse side pigments were studied. The isolate was inoculated onto International Streptomyces Project (ISP) media 1–7, ISP 9 (supplemented with a suitable sugar source), AIA and SCA to study the cultural characteristics of the potential isolate (Shirling and Gottlieb, 1966). The isolate was examined under a scanning electron microscope to study the spore chain morphology. The chemotaxonomy of the isolate was studied by determining the amino acids of the cell wall. The whole cell hydrolysate was spotted and run on a thin layer chromatography (TLC) plate and the spots were visualized by spraying 0.2% ninhydrin solution in acetone. The ability of the potential isolate to utilise and ferment various carbohydrates such as xylose, D-Maltose, D-Fructose, dextrose, galactose, raffinose, sucrose, L-arabinose, salicin, glucose, mannitol were