Fish & Shellfish Immunology 41 (2014) 482–492

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Influence of *Agathi grandiflora* active principles inhibit viral multiplication and stimulate immune system in Indian white shrimp *Fenneropenaeus indicus* against white spot syndrome virus infection

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ARTICLE INFO

Article history:
Received 11 June 2014
Received in revised form
21 September 2014
Accepted 29 September 2014
Available online 7 October 2014

Keywords:
Agathi grandiflora
Antiviral
Immunostimulants
Quantitative real time PCR (*q*RT-PCR)
White spot syndrome virus (WSSV)

ABSTRACT

Five herbs including *Adathoda vasica*, *Agathi grandiflora*, *Leucas aspera*, *Psoralea corylifolia*, and *Quercus infectoria* were selected to screen the antiviral and immunostimulant activity against white spot syndrome virus (WSSV) and *Vibrio harveyi* respectively using different organic polar and non-polar solvents. Based on the initial screening results, ethyl acetate and methanolic extracts of *A. grandiflora* had strong antiviral and immunostimulant activities. Those extracts incubated with WSSV injected *Fenneropenaeus indicus* got only 20% mortality and no PCR positive signals were seen in two step PCR amplification. The methanolic extracts of *A. grandiflora* were further purified through silica column chromatography and the fractions screened again for antiviral and immunostimulant activity. The secondary screening results revealed that, the fractions of F5 to F7 had effectively controlled the WSSV multiplication and *V. harveyi* growth. The pooled fractions (F5 to F7) was structurally characterized by gas chromatograph-mass spectrometry (GC-MS) analysis and few compounds were identified including 3,7.11,15-Tetramethyl-2-Hexane-1-ol, pytol and 1,2-Benzenedicarboxylic acid, diisooctyl ester. The pooled fractions were mixed with the basal feed ingredients at the concentration of 100 (D-1), 200 (D-2), 300 (D-3) and 400 (D-4) mg kg^{-1} and the diets fed to the *F. indicus* (9.0 ± 0.5 g) for 30 days. After the completion of feeding trail, they were challenged with virulent WSSV and studied the cumulative mortality, molecular diagnosis by quantitative real time PCR (*q*RT-PCR), biochemical, haematological and immunological parameters. The control diet fed *F. indicus* succumbed to death 100% within 3 days whereas the D-3 and D-4 helped to reduced the cumulative mortality of 60–80% respectively. The *q*RT-PCR revealed that, the WSSV copy number was gradually decreased when increasing concentration of *A. grandiflora* extract active fraction in the diets. The diets D-3 and D-4 helped to reduce the protein and carbohydrate levels significantly ($P < 0.01$) from the control diet fed groups. Moreover these diets help to decrease the coagulation time of maximum 61% from control groups and improve the total haemocyte count of maximum 51.82×10^5 cells ml^{-1} in D4 diet fed *F. indicus*. Finally immunological parameters including prophenol oxidase (proPO) activity, intracellular superoxide anion production and intra-agar lysozyme activity was significantly ($P \leq 0.001$) improved in the D-3 and D-4 fed *F. indicus* after WSSV challenge.

1. Introduction

Shrimp aquaculture is one of the lucrative foods producing industry due to its delicious taste and high incoming generation in the international market [1]. Shrimp production from capture and culture has expanded over the past decade from 2.4 million MT in 1987 to 4.2 million MT in 2000 to reach a record high of 6.6 million MT in 2006 [2]. Microbial diseases are one of the important crucial factors which inhibit the expansion of shrimp aquaculture leading to massive disease outbreaks. For more incoming generation from aquaculture operations, farmers are doing different types of culture practices including intensive and super intensive methods. As aquaculture production becomes more intensive, the incidence of disease including various infectious diseases has increased as a result of it leading to severe damages and significant economic losses [3].

White spot syndrome virus (WSSV) is one of the most devastating viral pathogens responsible for mass mortalities resulting in

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http://dx.doi.org/10.1016/j.fsi.2014.09.034
1050-4648/

extensive losses to shrimp (Penaeus sp.) culture industry throughout the world [4,5]. The clinical symptoms of the infected shrimp are white spots in the exoskeleton and epidermis [6]. The WSSV has a wide host range and it has been observed not only in shrimps but also in crabs and other arthropods such as copepods, insects and pest prawns [7].

Current disease control protocols in the aquaculture practices are often difficult to administer, not especially effective, costly and sometimes even environmentally hazardous. Even though antibiotics and synthetic drugs have positive effects on aquaculture operations [8], they cannot be recommended in commercial aquaculture operations for a variety of reasons. Their negative aspects in aquaculture include their relatively high cost, prohibited or uncertain regulatory status, unfeasible administration routes, poor absorption, toxicity, biomagnification, certain effects on the environment, possibility of consignment rejection and the fact that resistant bacteria may be transferred to humans through food handling and consumption [9].

The failure of synthetic chemicals to cure a wide range of viral diseases in aquaculture, the frequency of viral resistance has increased, and only a small number of antiviral drugs are currently used [3]. In the search for alternative antiviral remedies that can be used in place of synthetic drugs, recent interest has focused on medicinal herbs [10]. The plant antiviral metabolites provide the prototypes for designing potentially superior new chemotherapeutic drugs to combat major aquatic viruses. Recently, various herbal extracts have been shown to successfully control shrimp viruses such as YHV and WSSV both *in vitro* and *in vivo* [11–15].

Medicinal plants have been reported as effective anti-stress agents, growth promoters, appetizers, immunostimulants, aphrodisiac, and antimicrobials in finfish and shellfish species due to their active principles of alkaloids, flavanoids, pigments, phenolics, terpenoids, steroids, and essential oils [16]. Their versatile characteristics are effective, eco-friendly, easily available, economically attractive and non-biomagnifiable, and have no harmful side effects. Antiviral herbal compounds are able to not only control viral pathogens, but also boost the immune system in fish and shrimps against pathogenic infections [3]. It has been proven that several herbal extracts were boosting the immune system in fin and shellfishes against bacterial and viral pathogens [17–19]. The present study was focus on the antiviral and immunostimulant effect of *Agathi grandiflora* active principles fed on the Indian white shrimp *Fenneropenaeus indicus* for 30 days and challenged with virulent WSSV. Further the biochemical, haematological, immunological improvements and WSSV diagnosis by *q*RT PCR were studied after WSSV challenge.

2. Materials and methods

2.1. Herbal extracts

Known weight of antiviral and immunostimulant characteristic herbals including *Adathoda vasica* (leaves), *A. grandiflora* (bark), *Leucas aspera* (leaves), *Psoralea corylifolia* (seeds), and *Quercus infectoria* (boll) collected from the Keeriparai Western Ghats region of Tamilnadu, India (8° 23′ 35.76″N; 77° 24′ 35.64″E) were serially extracted with hexane, ethyl acetate and methanol. They were shade dried, individually ground and one hundred grams of fibre free powders (sieved by the 50 μm nylon cloth and removed the fibres) serially extracted with 100 ml of respective solvents by percolation extraction method. The extracts were filtered by Whatman no.1 filter paper and the filtrate was condensed by rotary evaporator under reduced pressure of 50 °C. Finally the extracts were concentrated using lyophilizer and stored at 4 °C for further study.

2.2. In vitro antiviral activity

The *in vitro* antiviral activity was performed following the method of Balasubramanian et al. [20] using different extracts of all herbals. Five hundred milligram of plant extract condensate was dissolved in 100 ml of NTE buffer (0.2 M NaCl, 0.02 M Tris–Hcl and 0.02 M EDTA, pH 7.4) as stock for antiviral assay. Ten micro litres of individual herbal extracts was mixed with 5 μl of WSSV suspension (300 μg of total protein) and incubated at 29 °C for 3 h. The incubated mixture was injected intramuscularly to *F. indicus* weighed of 8 ± 0.5 g in triplicates ($n = 10 \times 3 = 30$). Control shrimps were injected with a mixture of 10 μl of NTE buffer and 5 μl WSSV suspensions. Survival and external symptoms were monitored every 5 h until ten days from the injection period. Haemolymph samples were collected from the both challenged shrimp groups and checked by double step WSSV diagnostic PCR [21] using VP 28 primer designed by Namita et al. [22].

2.3. In vitro immunostimulant activity

In vitro immunostimulation was carried out following the method of Sritunyalucksana et al. [23] with slight modification. Haemolymph was bled from the ventral-sinus cavity of *F. indicus* adult and left to clot at 25 ± 2 °C for 1 h and stored in −80 °C deep freezer for 5 min and thawing to induce lysis. Repeated freeze and thaw cycle resulted in complete haemocyte lysis. The liquid fraction was collected by centrifuge at 10,000× *g* for 10 min and designated as the haemocyte lysate fraction (HLF). This comprised plasma and substances released from haemocytes. Five micro litres of each herbal extracts was incubated with 100 μl of HLF at 25 ± 2 °C for 1 h. The herbal extract incubated HLF was used for testing the antibacterial assay with *Vibrio harveyi* [24]. One hundred micro litre of immunostimulated HLF was incubated again with 100 μl *V. harveyi* bacterial culture 1×10^3 cfu ml^{-1} for 30 min at 25 ± 2 °C. Control experiments were performed for *V. harveyi* incubated with HLF without incubation of herbal extracts. Triplicate samples of 20 μl each were drop-transferred to TCBS agar (Hi media, India) to obtain bacterial counts (CFU) after incubation at 37 °C for 24 h. Based on the positive immunostimulation results, the best active extract was selected for further study.

2.4. Purification of A. grandiflora extract

The ethyl acetate extract of *A. grandiflora* had higher antiviral and immunostimulant activities among the herbals which were screened. *A. grandiflora* ethyl acetate extract condensate was further purified through preparative silica column chromatography (50–80 μm particle size; 30 cm column length; 0.5 ml elution flow rate and three bed volume elution) and collected different fractions. Different proportions of the mobile phases such as hexane/ethyl acetate and ethyl acetate/methanol were used for eluting the compounds. The fractions were concentrated in a rotary evaporator and spotted on silica gel plates GF254 (Merck), 20 cm, 1 mm thick and the chromatogram was developed using, hexane:ethyl acetate (8:2) as mobile phase. The plates were visualized under short UV wavelength.

2.5. Secondary antiviral and immunostimulant screening

Secondary antiviral and immunostimulant screening was also performed against WSSV and *V. harveyi* respectively using different *A. grandiflora* extract fractions which eluted from column chromatography as per the protocols mentioned in the Sections 2.2 and 2.3 respectively.