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Influence of *Cyperus rotundus* Active Principles Inhibit Viral Multiplication and Stimulate Immune System in Indian White Shrimp *Fenneropenaeus indicus* against White Spot Syndrome Virus Infection

T. Citarasu, M. Michaelbabu V. N. Vakharia

***Abstract*—**The rhizome of Java grass, *Cyperus rotundus* was extracted different organic polar and non-polar solvents and performed the *in vitro* antiviral and immunostimulant activities against White Spot Syndrome Virus (WSSV) and *Vibrio harveyi* respectively. Based on the initial screening the ethyl acetate extract of *C. rotundus* was strong activities and further it was purified through silica column chromatography and the fractions were screened again for antiviral and immunostimulant activity. Among the different fractions screened against the WSSV and *V. harveyi,* the fractions, F-III to FV had strong activities. In order to study the *in vivo* influence of *C. rotundus*, the fractions (F-III to FV) were pooled and delivered to the *F. indicus* through artificial feed for 30 days. After the feeding trail the experimental and control diet fed *F. indicus* were challenged with virulent WSSV and studied the survival, molecular diagnosis, biochemical, haematological, and immunological parameters. Surprisingly, the pooled fractions (F-IV to FVI) incorporated diets helped to significantly ($P<0.01$) suppressed viral multiplication, showed significant ($P<0.01$) differences in protein and glucose levels, improved total haemocyte count (THC), coagulase activity, significantly increased ($P <= 0.001$) prophenol oxidase and intracellular superoxide anion production compared to the control shrimps. Based on the results, *C. rotundus* extracts effectively suppressed WSSV multiplication and improve the immune system in *F. indicus* against WSSV infection and this knowledge will helps to develop novel drugs from *C. rotundus* against WSSV.

***Keywords*—**Antiviral drugs, *Cyperus rotundus, Fenneropenaeus indicus,* WSSV.

I. 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]. As aquaculture production becomes more intensive, the incidence of disease including various infectious diseases has increased as a result of it leading to significant economic losses [2]. Among the infectious diseases, White spot syndrome virus (WSSV) is one of the most devastating viral pathogens responsible for mass mortalities resulting in extensive losses to shrimp culture industry throughout the world [3].

The current disease treatment protocols against WSSV are cost effective, less effective, rather difficult and are creating so many undesirable side effects [4]. Even though antibiotics and synthetic drugs are giving positive effects, they cannot be recommended due to their residual effects, resistant strain development and other environmental hazards [5]. 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 [6].

Considering this potential threat of disease on one hand and the environmental issues on the other hand, the disease management aspects should concentrate on eco-friendly methods like the development of vaccines, immunostimulants and herbal based antiviral disease treatment protocols [7]. Herbal plants are the storehouses and rich sources of safer and cheaper chemical compounds. These natural plant products have been reported to have various activities like antistress, growth promoters, appetiser, tonic, immunostimulants and antimicrobials [8]. Moreover, the substances are obtained from natural sources, besides possessing other interesting properties like non-toxic, biodegradable and biocompatible [4]. The present study intends to the antiviral and immunostimulant effect of *C. rotundus* against the white spot syndrome virus (WSSV) on the Indian white shrimp *Fenneropenaeus indicus.*

II. Material and Methods

A. Extraction of C. rotundus

Known weight of the dried powder of *C. rotundus* tubers was serially extracted with hexane, ethyl acetate and methanol 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 50ºC. Finally the extracts were concentrated using lyophilizer and stored at 4ºC for further study.

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B. Primary Antiviral and Immunostimulant Screening

One hundred micro grams of *C. rotundus* extract condensates which dissolved in 100 µl of NTE buffer was incubated with 100 µl of WSSV suspension (300 µg of total protein) and incubated at 29 ºC for 3 h. The incubated mixture was injected intramuscularly to *Fenneropenaeus indicus* adult in triplicates (n =10 × 3=30). Control shrimps received only WSSV without *C. rotundus* extracts incubation. Survival and external symptoms were monitored every 5 h until ten days from the injection period [9]. The haemocyte lysate fraction (HLF), which prepared from *F. indicus* by repeated freeze and thaw, was incubated with *C. rotundus* extracts 25 ± 2ºC for 1 h. One hundred micro litre of immunostimulated HLF was incubated again with 100 ml *Vibrio harveyi* bacterial culture 1 $\times 10^3$ cfu/ ml for 30 min at 25 ± 2ºC. 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 ºC for 24 h. Based on the positive immunostimulation results, the best active extract was selected for further study [10].

C. Purification of C. rotundus Extract

Based on the better antiviral and immunostimulant activity, the ethyl acetate extract of *C. rotundus* was purified by preparative silica column chromatography (50-80 µm particle size; 30 cm column length; 0.5 ml elution flow rate and three bed volume elution) using the mobile phase of hexane/ethyl acetate and ethyl acetate/methanol. The fractions were collected, concentrated and lyophilized.

D. Secondary Antiviral and Immunostimulant Screening

Secondary antiviral and immunostimulant screening was also performed against WSSV and *V. harveyi* respectively using different *C. rotundus* extract fractions which eluted from column chromatography as per the protocols mentioned in the earlier section of primary antiviral and immunostimulant screening.

E. Diet Preparation

The artificial shrimp diet was prepared of 45.1% protein; 7.2% lipid; 14.6% ash; 7.1% moisture and 3% fibre [11]. The pooled *C. rotundus* active fractions (FIV to VI) were mixed with the basal ingredients at the concentration of 100 (CR-1), 200 (CR-2), 400 (CR-3) and 800 (CR-4) mg/ kG for the experimental diets and the control diet devoid of *C. rotundus* active fraction.

F. Experimental Set-Up and Feeding

Healthy WSSV free uniform size of *F. indicus* (9.0 ± 0.5 g) were stocked into individual experimental fibre glass tanks (1000 l capacity) of four experimental groups (CR-1 to CR-4) and a control group in triplicate (n = 50 × = 150) with continuous flow through water with a flow rate of 1 l/min and constant aeration system. The shrimps were fed three times a day at 8.00, 13.00 and 18.00 h at 10% of the body weight for 30 days. Uneaten food and waste were removed before feeding. The water quality parameters such as temperature (27 ± 1.0ºC), salinity (30 ± 1.0‰) and pH (8.4 ± 0.2) were monitored daily.

G.WSSV Challenge and Cumulative Mortality

After completion of the feeding trail, ten numbers of *F. indicus* from each replicates from experimental and control groups were challenged with virulent WSSV by intra muscular (IM) injection at the rate of 15 µl WSSV filtrate contain 300 µg of total protein. During the challenge experiment all, the shrimps were fed on the respective diets. The percentage of cumulative mortality was recorded daily and the experiment was carried out up to 10 days.

H. Molecular Diagnosis

Haemolymph samples were collected from the both challenged shrimp groups of control and experimental and checked by double step WSSV diagnostic PCR using VP 28 primers [12]. The negative samples detected in the first step were further subjected for second step PCR analysis. In each group, 10 shrimp samples were individually tested.

I. Biochemical Changes

Biochemical parameters such as the total protein and glucose were determined in haemolymph samples. The total protein was determined spectrophotometrically (O.D 595 nm) and glucose was estimated by the glucose oxidase method [13].

J. Haematological Changes

The coagulation time of the haemolymph was determined by capillary method [14]. The Total Haemocyte Count (THC) (cells/ mL) was performed using Burker haemocytometer [15]. The concentration of oxyhaemocyanin was calculated following the method of Hagerman [16].

K. Immunological Changes

Phenoloxidase activity in haemolymph samples was determined using L-dihydroxyphenylalanine (L-DOPA) as a substrate [17]. Superoxide anion was quantified by the method of [18]. The optical density of the dissolved formazan was read at 630 nm and the effects of different treatments on the generation of O_2^- .

L. Data Analysis

One and two way Analysis of Variance (ANOVA) was carried out using SPSS statistics data package and Ky plot respectively. Means were compared at 0.01 and 0.001% level.

III. Results and Discussion

F. indicus succumbed to death 100% within five days when no *C. rotundus* extracts incubated WSSV were injection. The percentage were significantly ($P<0.01$) decreased to 85.55, 35.76 and 5.23% in hexane, methanol and ethyl acetate extracts of *C. rotundus* treated groups. The immunostimulant activity also reflected the same manner as like in antiviral activity. There is no *V. harveyi* growth observed in the ethyl acetate treated groups (Table I).

TABLE I
PRIMARY ANTIVIRAL AND IMMUNOSTIMULANT SCREENING

Extractions	Antiviral activity *	Immunostimulant activity **
Control	100.00 ± 0.00 [a]	+++
Hexane	85.55 ± 6.23 [b]	+++
Ethyl acetate	5.23 ± 0.45 [c]	-
Methanol	35.76 ± 1.29 [d]	+

* Antiviral activity monitored by Cumulative mortality (%) against WSSV
** Immunostimulant screening against *V. harveyi*
–: no growth, +: minimum growth; +++: maximum growth
Means with the same superscripts (a-d) do not differ from each other ($p < 0.01$) – one way ANOVA

Direkbusarakom et al. [19] examined the ability of the ethanol extracts of *Psidium guajava* leaves against various fish pathogenic viruses such as IHNV and IPNV. Several plants including *Emblica officinale, Cynodon dactylon* and *Adathoda vasica* improved the immune system and reduced the microbial infection in the gold fish *Carassius auratus* [20].

Among the different purified fractions (FI to FVI) of *C. rotundus* tested for antiviral and immunostimulant activities, the fractions FIV to FV I had potent anti viral and immunostimulant activities. There is less cumulative mortality (8 to 10 %) and no *V. harveyi* growth found in the FIV to FVI fractions treated groups and the data are significantly ($P < 0.01$) differed (Table II).

TABLE II
SECONDARY ANTIVIRAL AND IMMUNOSTIMULANT SCREENING OF *C. ROTUNDUS* ETHYL ACETATE FRACTIONS

Fractions	Antiviral activity *	Immunostimulant activity **
Control	100.00 ± 0.00 [a]	+++
F-I	78.71 ± 2.54 [b]	++
F-II	35.50 ± 1.05 [c]	+
F-III	29.22 ± 3.15 [d]	+
F-IV	8.89 ± 0.33 [e]	-
F-V	7.15 ± 0.57 [e]	-
F-VI	10.48 ± 1.85 [f]	-
F-VII	33.45 ± 2.87 [g]	+

* Antiviral activity monitored by Cumulative mortality (%) against WSSV
** Immunostimulant screening against *V. harveyi*
–: no growth, +: minimum growth; +++: maximum growth
Means with the same superscripts (a-g) do not differ from each other ($P < 0.01$) – one way ANOVA

In the present study, various antiviral and immunostimulant characteristic compounds present in the fractions FIV to FVI are effectively suppressed the replication of WSSV and stimulate the immune system.

The control diet fed shrimps succumbed to death 100 % within four days whereas the mortality significantly ($F= 50.62;$ P <= 0.001) decreased in the experimental groups. The lowest cumulative mortality observed of 21 and 25 % in CR-3 and CR-4 fed *F. indicus* after WSSV challenge (Fig. 1).

Tinospora cordifolia, *Picrorrhiza kurrooa, Eclipta alba, Acalypha indica, Cynodon dactylon*, *Withania somnifera* and *Zingiber officinalis* extracts incorporated diets were well influenced by better survival, reduction in the viral load in *Penaeus monodon* against WSSV challenge [4], [21]. The double step PCR detection result revealed that, the control diet *F. indicus* had 100% PCR positive signals after WSSV challenge. The PCR signals were significantly ($P<0.01$) decreased to 70, 36, 6 and 7% after overall detection in CR-1 to CR-4 diets fed shrimps (Fig 2). The active compounds of the *C. rotundus* extracts interferes the WSSV envelope degradation and bind the nucleocapsid proteins and arrest the transcription and translation leading to multiplication arrest of WSSV. The WSSV infection was confirmed by using WSSV two steps PCR in *Penaeus monodon* fed with antiviral/ immunostimulant herbal active principles [4], [21].

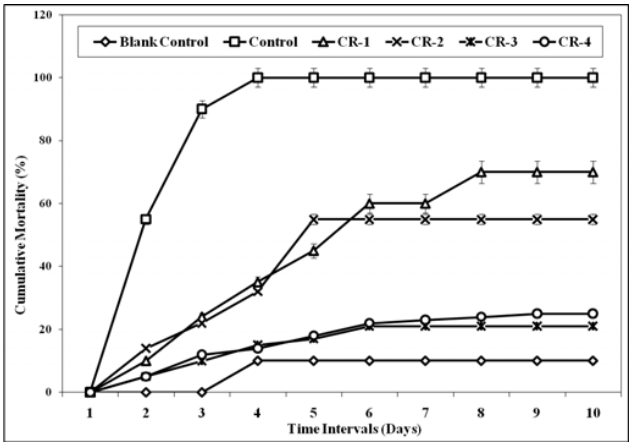

Fig. 1 Cumulative mortality of *F. indicus* fed with *C. rotundus* extract fractions (FIV-VI) enriched diets after WSSV Challenge. The values are significantly differed each other's (Column: $F= 50.62;$ P <= 0.001 and Row: $F= 50.70;$ P <= 0.001)

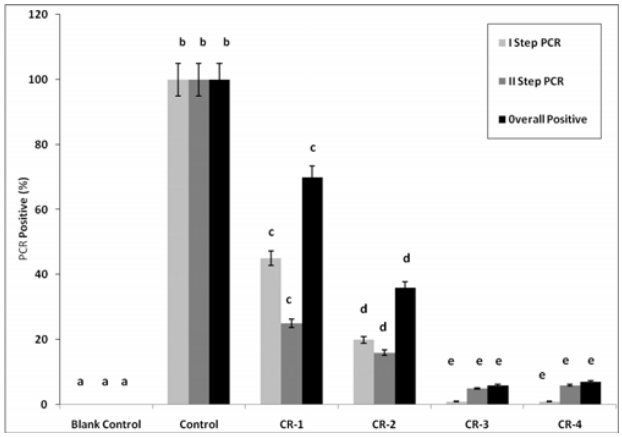

Fig. 2 PCR amplification of the WSSV VP28 gene from the genomic DNA of *F. indicus* fed with *C. rotundus* extract fractions (FIV-VI) enriched diets after WSSV Challenge. Means with the same superscripts (a-e) do not differ from each other ($P < 0.01$)

The protein level was higher (119.5 µg / mL) in control diet fed *F. indicus* due to heavy WSSV loads and this was significantly ($P < 0.001$) decreased in the experimental diet fed *F. indicus*, the minimum level observed of 95.1 µg ml^{-1} in CR-4 fed groups. The carbohydrate level was significantly ($P < 0.001$) increased in the experimental diet fed *F. indicus* (Table III).

TABLE III
BIOCHEMICAL CHANGES IN THE HAEMOLYMPH OF *F. INDICUS* FED WITH *C. ROTUNDUS* EXTRACT ENRICHED DIETS

Treatments	Biochemical parameters	
	Protein (µg/ mL)	Carbohydrate (µg/ mL)
Blank Control	94.54 ± 0.12 [a]	105.45 ± 0.32 [a]
Control	119.5± 1.06 [b]	96.5± 0.17 [b]
CR-1	114.5 ± 0.52 [c]	97.1 ± 2.12 [b]
CR-2	108.4± 0. 87 [d]	105.6 ± 1.49 [c]
CR-3	97.3 ± 0.98 [e]	121.9 ± 1.56 [d]
CR-4	95.1 ± 1.34 [f]	128.5 ± 2.54 [e]

Means with the same superscripts (a-e) do not differ from each other ($p < 0.001$)

Lo et al. [22] reported the high concentrations of protein, amino acids in the haemolymph of crustaceans due to WSSV heavy load. The possibility of high levels of glucose and total carbohydrate in haemolymph might be due to the transport from hepatopancreas and muscle to haemolymph. Hall and Van Ham [23] showed a significant elevation of blood glucose in *P. monodon* in stress condition.

The haemolymph took coagulate 163 seconds due to the heavy WSSV load in the control diet fed *F. indicus* and it was significantly ($P < 0.0\ 01$) decreased to the experimental diet fed *F. indicus.* The total haemocyte count (THC) also significantly ($P < 0.001$) increased in the experimental groups and the maximum count observed of 45.22 ($\times 10^5$ cells/ mL in the CR-3 diet fed group (Table IV).

TABLE IV
HEMATOLOGICAL CHANGES IN THE HAEMOLYMPH OF *F. INDICUS* FED WITH *C. ROTUNDUS* EXTRACT ENRICHED DIETS AFTER WSSV CHALLENGE

Treatments	Haematological Changes	
	Coagulase activity (Sec)	Total Haemocyte Count ($\times 10^5$ cells/ mL)
Blank Control	110.14 ± 1.09 [a]	33.54 ± 0.12 [a]
Control	163 ± 1.05 [b]	20.16 ± 1.54 [b]
CR-1	141.32 ± 2. 38 [c]	23.53± 0.36 [c]
CR-2	110.16 ± 1.26 [a]	38.87± 1.54 [d]
CR-3	98.7 ± 0.51 [d]	45.22 ± 0.88 [e]
CR-4	100.06.40 ± 0.76 [e]	44.23± 1.34 [e]

Means with the same superscripts (a-e) do not differ from each other ($p < 0.001$)

Maeda et al. [24] observed that total haemocyte count decline in shrimp infected with penaeid rodshaped DNA virus. Yoganandhan et al. [25] observed significant differences of the coagulative time and THC between the WSSV and non-infected *F. indicus* shrimps. The prophenol oxidase activity gradually decline after WSSV control diet fed *F. indicus* after WSSV challenge whereas the activity was significantly ($P < 0.001$) increased in all experimental diet fed groups. The intracellular superoxide anion production also reflected the same way (Figs. 4 and 5).

The *C. rotundus* active fractions (FIV to FVI) in the experimental diets influence the immunostimulation against the WSSV response. This reflects the pro PO and intracellular superoxide anion production. The *Agathi grandiflora* extract active fractions enriched diets help to induce the immunological parameters including prophenol oxidase (proPO) activity, intracellular superoxide anion production [26]. The proPO activating system stimulates several cellular defense reactions, including phagocytosis, nodule formation, encapsulation and haemocyte locomotion [27].

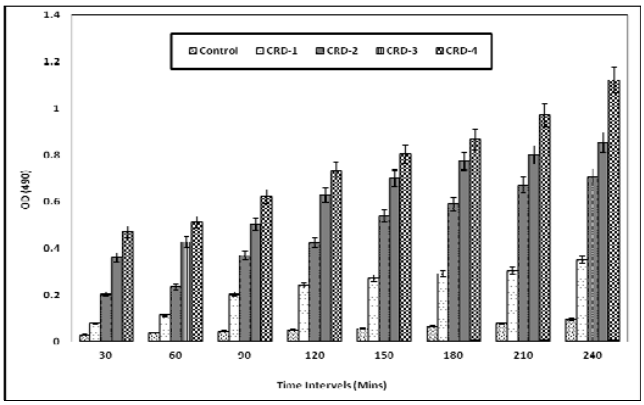

Fig. 3 Prophenol Oxidase (proPO) activity of haemocytes of *F. indicus* fed with *C. rotundus* extract enriched diets after WSSV challenge. The values significantly differed from each other (*F= 91.09*; P <= 0.0001)

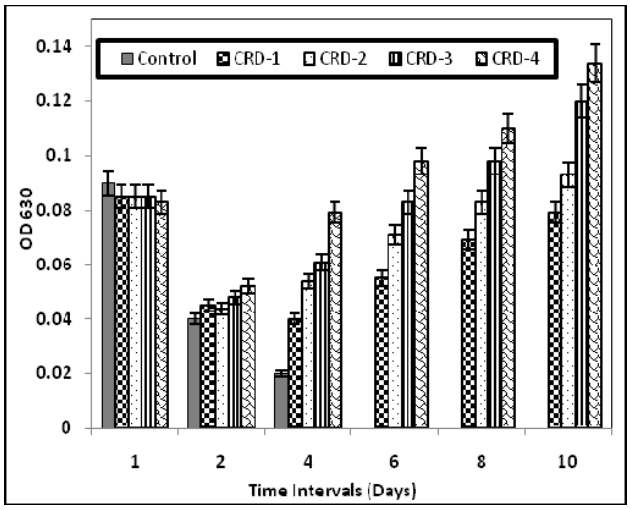

Fig. 4 Intracellular superoxide anion production (NBT assay) of *F. indicus* fed with *C. rotundus* extract enriched diets after WSSV challenge. The values significantly differed from each other (*F= 10.94*; P <= 0.0001)

V. CONCLUSION

The active principles of *C. rotundus* were highly influenced to control the WSSV and boost the immune system in *F. indicus* against WSSV infections. This approach will be highly useful to developing novel antiviral and immunostimulant drugs from *C. rotundus* against WSSV.

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