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Antiviral activity of *Cynodon dactylon* on white spot syndrome virus (WSSV)-infected shrimp: an attempt to mitigate risk in shrimp farming

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Abstract
White spot syndrome virus (WSSV) is the most contagious pathogen causing huge economic losses in the shrimp industry worldwide. Several medicinal plants are known to have antiviral activity through the inhibition of viral diseases in fish and shellfish. Hence, there is a need to investigate the ability of natural remedies like plant sources to mitigate this devastating disease in crustaceans. This study was carried out to test the antiviral activity of ethanolic extract of *Cynodon dactylon* on WSSV in tiger shrimp *Penaeus monodon* juveniles with an average weight of 13.541 ± 2.927 g. Different doses of the extract (75, 100, and 150 mg/kg) were administrated in vivo through intramuscular injection. The antiviral activity was determined by observing survival rates, and WSSV infection was confirmed at the end of the experiment through polymerase chain reaction (PCR) identification. Before the in vivo experiment, presence of antiviral compounds ((+)-catechin, vanillic acid, syringic acid, (−)-epicatechin, *p*-coumaric acid, and quercetin bioactive compounds) in *C. dactylon* was confirmed through high-performance liquid chromatography with diode-array detection (HPLC-DAD) analysis. The results obtained in this study show that a dose of 100 to 150 mg/kg body weight ethanolic extract of *C. dactylon* prevented the infection of WSSV marked by 100% survival and absence of WSSV-specific band using nested diagnostic PCR, thus demonstrating the suitability of ethanolic extract of *C. dactylon* as a possible prophylactic for the prevention of WSSV infections in shrimp culture.

Keywords *Cynodon dactylon* · Plant extract · White spot syndrome virus · Crustaceans · Survival · Polymerase chain reaction

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Introduction

Tiger shrimp *Penaeus monodon* are an important aquatic resource that supports a large industrial development in coastal Bangladesh, and because of the economic value, it is known as "white gold." Tiger shrimp, together with giant freshwater prawn *Macrobrachium rosenbergii*, is considered the second largest foreign exchange earning sector of the country (DoF 2016). Shrimp culture covers a major part of aquaculture which is an important economic activity all over the world. Today, the shrimp industry in Bangladesh, as well as other parts of the world, is facing a serious problem due to microbial diseases.

The white spot syndrome virus (WSSV) is one of the most devastating viral pathogens in the shrimp industry in Bangladesh. The WSSV is an enveloped, non-occluded DNA (300 kb) virus of the family Nimaviride, Genus *Wispovirus* (Mayo 2002). The virus has been reported to cause severe mortality in tiger shrimp in several countries with associated huge economic losses (Lightner 1996). The mortality can often reach 100% within 3 to 10 days from the onset of visible signs of infection (Flegel 1997). Despite this, no effective treatment or prophylactic has been developed to manage or reduce the effects of WSSV infections in Bangladesh. However, in other countries, several approaches have been investigated to manage this pathogen. For example, use of immunostimulants (peptidoglycan, lipopolysaccharides, ß-1,3 glucan; Itami et al. 1998; Chang et al. 2003), vaccinations (inactivated viral envelop protein VP19 and VP28; Singh et al. 2005; Namikoshi et al. 2004**)**, feeding plant extract (fucoidan from *Sargassm polycysticus*; Chotigeat et al. 2004), and antiviral drugs (cidofovir; Rahman et al. 2006). However, these control measures are expensive and complex to combat viral diseases and using chemicals and drugs in aquaculture can cause severe problems in biodiversity, environment and human health.

The use of herbs, plants or grasses and the associated bioactive chemicals to treat disease is now common in organic aquaculture, veterinary and human medicine (Direkbusarakom 2004, Sudheer et al. 2011). Plant extracts and their associates are widely used for medicinal purposes (Bandaranayake 1998). Hence, plant extracts provide unlimited opportunities for new drug discoveries because of the chemical diversity (Cos et al. 2006). Secondary metabolites of many plants act as bioactive compounds, chemotherapeutic, bactericidal as well as bacteriostatic agents (Singh and Gupta 2008; Pongsak and Parichat 2010; Syahriel et al. 2012). Therefore, in recent years, scientists are focusing their research efforts on plant-derived substances for antimicrobial agents.

Cynodon dactylon is a perennial, pan-tropical species of grass which belongs to the family Poaceae. This grass is found frequently in tropical, subtropical and even in semi-arid climates (Watson and Dallwitz 1992), and because of its widespread distribution, it is also commonly used by humans as a medicinal plant, particularly during the earlier period. *C. dactylon* has many beneficial properties, including antidiabetic effect, diuretic activity, antioxidant, anticancer potential, antiulcer activity, right heart failure protective activity and allergic effect (Singh et al. 2007; Albert-Baskar and Ignacimuthu 2010). To date, very few reports have described the anti-WSSV activity of *C. dactylon* in *P. monodon* (Balasubramanian et al. 2008). Although *C. dactylon* has diverse medicinal activities, the antiviral effects against WSSV are poorly known.

Therefore, the aim of this research was to understand the anti-WSSV activity of *C. dactylon* to combat the WSSV infection in *P. monodon*.

Materials and methods

Detection of WSSV from collected shrimp samples

The WSSV-infected *P. monodon* were collected randomly from three tiger shrimp farms of two coastal Districts, Khulna and Bagerhat, Bangladesh. The genomic DNA was extracted using AccuPrep® Genomic DNA Extraction Kit (Cat No. K-3032) following the manufacturer's protocol. About 25~50-mg samples were homogenized with sterile mortar and pestle. In the homogenate, 200 μl of tissue lysis buffer (TL), 20 μl of proteinase K, and 200 μl of binding buffer (GC) were added. The mixtures were centrifuged and incubated at 60 °C for 1 h. After incubation, 100 μl of isopropanol was added to the mixtures and centrifuged at 8000 rpm for 1 min. The solutions were poured into disposal bottles, and 500 μl of washing buffer 2 (W2) was added and centrifuged at 12,000 rpm for 1 min. The eluted genomic DNA was stored at 4 °C for later analysis. For qualitative and quantitative determination, the integrity of extracted DNA samples was studied by agarose gel electrophoresis as described by Sambrook et al. (1989). The concentration of DNA samples was determined at A_{260} using a double-beam spectrophotometer (Hitachi U-2910 spectrophotometer, Japan) against an Elution buffer blank. The DNA purity was found by using the following formula:

$$\text{Purity} = \text{Absorbance at } A_{260}$$

Confirmation of WSSV using polymerase chain reaction

WSSV infection was confirmed by the polymerase chain reaction (PCR) technique using the primer, 146F2/146R2 (Lo et al. 1996). After qualitative and quantitative determination of DNA, the required (4 ng/μl) concentration of DNA template was diluted for the PCR program. PCR amplification was performed in a thermal cycler (MyGene96 Thermal Block, Bioneer, Korea). PCR product was checked electrophoretically by loading 10 μl of PCR product on a 1.5% agarose gel. During the evaluation of PCR products, at first, a specific band for 146F2/146R2 (941 bp) was screened out on gel to determine the presence of WSSV in the sample. Then, a nested PCR was performed using IK 3–4 (298 bp) primer to find out the lowest intensity of DNA.

Preparation of plant extract from *C. dactylon*

Fresh whole plant of *C. dactylon* was collected from the field of Khulna University campus, Bangladesh, washed with distilled water, and dried in a shaded environment. The plant was further dried in an oven at 50 °C for 10 min and grinded by using a mixer grinder (Capacitor start motor, WUHU motor factory, China). The sample was then macerated with 100% ethanol at a ratio of 1:10 (plant/ethanol) and kept at room temperature for 72 h. During this time, the mixture was stirred every 24 h using a sterile glass rod. After 72 h, the mixture was filtered

through Whatman® No.1 filter paper. The filtrate was collected in a separate beaker and concentrated by evaporating the solvent. The extracts were re-suspended in the ethanol solvent before testing it for antiviral activity. The crude extract was kept at − 20 °C before use (Khan et al. 1988; Jayanthi et al. 2013).

Plant extract dose preparation

Three different doses were prepared to check the anti-WSSV activity of *C. dactylon* ethanolic extract. For treatment 1, 150 mg of crude ethanolic extract was dissolved in 0.2 ml of absolute ethanol and mixed with 0.8 ml of distilled water, before being triturated in a unidirectional manner using a vortex mixer to get a concentration of 150 mg/kg of shrimp body weight. For the remaining treatments, 100 mg/kg and 75 mg/kg of body weight of crude ethanol extract of *C. dactylon* were prepared for treatment 2 and treatment 3, respectively, in the same manner as treatment 1.

HPLC detection and quantification of polyphenolic compounds of *C. dactylon*

Detection and quantification of selected phenolic compounds in the ethanolic extract were determined by high-performance liquid chromatography with diode-array detection (HPLC-DAD) analysis as described by Jahan et al. (2014) with some modifications. It was carried out on a Dionex UltiMate 3000 system equipped with quaternary rapid separation pump (LPG-3400RS) and photodiode array detector (DAD-3000RS). Separation was performed using Acclaim® C_{18} (5 μm) Dionex column (4.6 × 250 mm) at 30 °C with a flow rate of 1 ml/min and an injection volume of 20 μl. For the preparation of calibration curve, a standard stock solution was prepared. The HPLC was performed at the Chemical Research Division, Bangladesh Council of Scientific and Industrial Research (BCSIR) Laboratories, Dhaka.

Investigation of anti-WSSV activity of *C. dactylon*

Viral inoculum preparation

The viral inoculum was prepared according to the method of Singh et al. (2005). In brief, a composite sample of gills and soft parts of the cephalothorax (500 mg) from WSSV-positive (previously confirmed) *P. monodon* was macerated in 10-ml cold phosphate-buffered saline (PBS) with glass wool to a homogenous slurry using a mortar and pestle in an ice bath. The slurry was centrifuged at 12,000 rpm in a refrigerated centrifuge at 4 °C, and the supernatant was filter-sterilized using a 0.45-μm-pore-size Millex®-GS MF Millipore™ MCE membrane (Millipore Corp., Ireland). The inoculum was stored at − 20 °C before use.

In vivo challenge test

For the survival challenge test, 110 juvenile *P. monodon* shrimps (Specific Pathogen Free (WSSV Free)) were collected from a tiger shrimp farm of Batiaghata, Khulna, Bangladesh, acclimatized, and transferred to the aquariums (1.220 × 0.305 × 0.305 m^3) at the wet laboratory of Fisheries and Marine Resource Technology Discipline, Khulna University. The average mass of shrimps were 13.541 ± 2.927 g. The shrimps were fed with artificial pellet feed and acclimatized for 5 days before injection. The shrimp juveniles were stocked into five different groups: negative control (NC), positive control (PC), and the three different treatments (T1,

Table 1 Purity of the DNA samples

Sampling area		Sample no.	A_{260}	A_{280}	A_{260}/A_{280}
Batiaghata, Khulna	Gher-1	1	0.019	0.052	0.66
		2	0.029	0.046	0.63
		3	0.047	0.076	0.66
	Gher-2	4	0.019	0.055	0.34
		5	0.024	0.035	0.68
		6	0.049	0.081	0.60
	Gher-3	7	0.069	0.080	0.86
		8	0.048	0.077	0.62
		9	0.062	0.076	0.82
Rampal, Bagerhat	Gher-1	10	0.071	0.084	0.84
		11	0.061	0.078	0.78
		12	0.047	0.076	0.66
	Gher-2	13	0.074	0.089	0.83
		14	0.021	0.046	0.46
		15	0.060	0.079	0.76
	Gher-3	16	0.056	0.071	0.79
		17	0.071	0.083	0.86
		18	0.039	0.054	0.72

T2, T3). For PC, 20 shrimps were injected intramuscularly with a mixture of 25-μl PBS buffer and 10-μl viral suspension per shrimp and kept in the tanks. The animals under the NC were injected with 35-μl PBS buffer solution. The treatment group was divided into three experimental subunits according to different doses of plant extracts, for example, 150 mg/kg (T1), 100 mg/kg (T2), and 75 mg/kg (T3) of shrimp body weight, respectively. The animals under treatment groups were injected with 10 μl of viral suspension, 10 μl of plant extract, and 15 μl of PBS buffer solution to 20 shrimps. The experiment was carried out up to 15 days post-infection with WSSV. After intramuscular injection, three shrimp samples were randomly collected from each tank after 0 h, 6 h, 12 h, 18 h, 24 h, day 2, day 3, day 4 from PC and up to day 11 from NC and treatments post-inoculation (hpi). After removal from the tanks, the samples were preserved at − 80 °C before starting the pathogen confirmation test. Further, a representative sample of gill was subjected to PCR to identify the antiviral activity of the extract solution. At the end of the viral challenge test, the survival rate of *P. monodon* was calculated according to the following formula:

Survival rate (SR; %), $SR = \frac{\mathrm{NLA}}{\mathrm{NIA}} \times 100$, in which NLA = number of live animals and NIA = number of introduced or initial animals (Thirumurugan and Subramanian 2004).

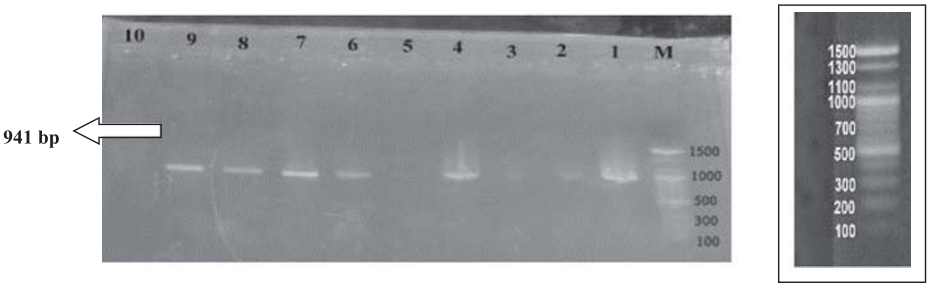

Fig. 1 UV visualization of PCR product electrophoresis in a 1.5% agarose gel. Lanes 1 to 5 are representative samples of Batiaghata, Khulna, and lanes 6 to 10 represent the samples of Rampal, Bagerhat. Lane M represents 100 bp ladder

Confirmation of WSSV using PCR

After sampling from each experimental group from the in vivo challenge test, DNA was extracted from the isolated shrimp sample. The DNA samples were diluted and subjected to PCR using primer 146F2/146R2 (941 bp) and IK 3–4 (298 bp) following the process described previously (in "Confirmation of WSSV using polymerase chain reaction"). Then, the bands of all groups were compared and settled upon the dose of *C. dactylon* that was combatant against WSSV.

Statistical analysis

Data generated on the survival of shrimp on administering the suspension of WSSV exposed to *C. dactylon* and the data generated on the survival when challenged with WSSV subsequent to intramuscular administration of the former were statistically analyzed employing chi-square test. The log-rank test was used to test whether there was a difference between survival times between the groups. Kruskal-Wallis test was performed to compare mean survival of shrimp between groups ($P < 0.01$). All statistical analyses were done using SPSS version 25.0.

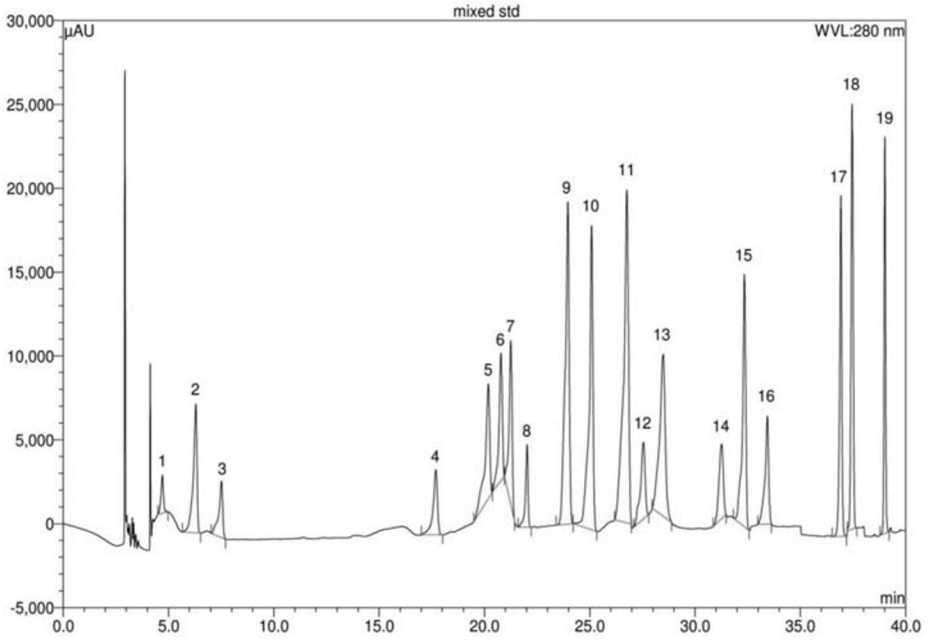

Fig. 2 HPLC chromatogram of a standard mixture of polyphenolic compounds. Peaks: 1, arbutin; 2, gallic acid; 3, hydroquinone; 4, (+)-catechin; 5, vanillic acid; 6, caffeic acid; 7, syringic acid; 8, (−)-epicatechin; 9, vanillin; 10, *p*-coumaric acid; 11, *trans*-ferulic acid; 12, rutin hydrate; 13, ellagic acid; 14, benzoic acid; 15, rosmarinic acid; 16, myricetin; 17, quercetin; 18, *trans*-cinnamic acid; 19, kaempferol

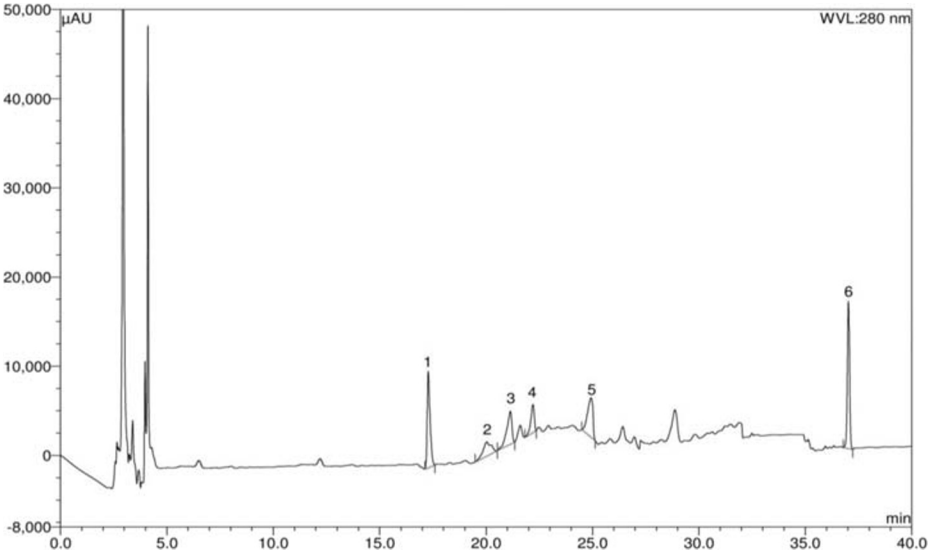

Fig. 3 HPLC chromatogram of ethanolic extract of *C. dactylon*. Peaks: 1 ((+)-catechin), 2 (vanillic acid), 3 (syringic acid), 4 ((−)-epicatechin), 5 (*p*-coumaric acid), 6 (quercetin)

Results

Detection and comparison of WSSV from study area

Most of the randomly selected samples produced a DNA band with varying degree of intensity and smearing pattern. The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of DNA (Table 1).

Detection of WSSV through PCR

Depending on the DNA concentration, 10 samples were selected for further PCR testing using 146F2/146R2 (941 bp) primer. The results of PCR product visualization revealed that out of 10 samples collected from two different places viz. Batiaghata and Rampal Upazillas, four samples (lanes 6, 7, 8, 9) collected from Rampal and two samples (lanes 1 and 4) collected from Batiaghata exhibited positive results for WSSV infection. Presence of WSSV in shrimp

Table 2 Contents of polyphenolic compounds in the ethanolic extract of *C. dactylon* ($n = 5$)

Polyphenolic compound	Ethanolic extract of *Cynodon dactylon*	
	Content (mg/100 g of dry extract)	% RSD
Catechin	89.02	1.71
Vanillic acid	16.01	0.51
Syringic acid	27.11	0.69
Epicatechin	45.09	1.18
p-Coumaric acid	7.18	0.16
Quercetin	94.05	1.88

RSD relative standard deviation

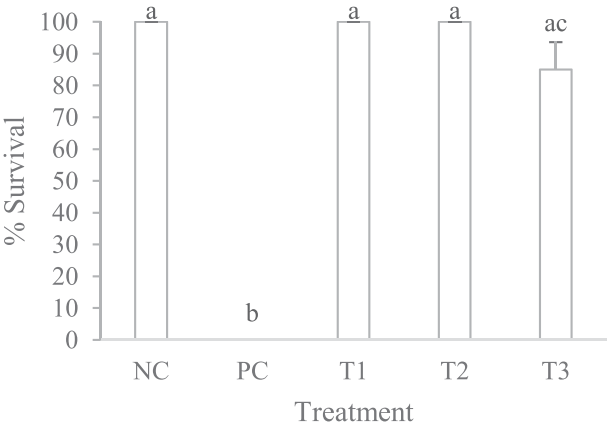

Fig. 4 Percentage survival (mean ± standard deviation) of *P. monodon* juveniles in the in vivo challenge test with WSSV and three different concentrations (75, 100, and 150 mg/kg) of *C. dactylon* ethanolic extract. NC is the negative control without any viral injection as well as ethanolic extract of *C. dactylon* given to the animal. *P. monodon* under negative control was only injected with 35-μl PBS buffer solution. Positive control was indicated as PC and treated with viral suspension. The treatments were T1 = 150 mg/kg, T2 = 100 mg/kg, and T3 = 50 mg/kg. The error bars represent the standard deviations of three replicates. Different letters indicate significant difference among the treatments (Kruskal-Wallis test, $P < 0.01$)

of Rampal was higher than the shrimp in Batiaghata. Among 10 samples, 60% were WSSV positive (Fig. 1).

Analysis of ethanolic extract of *C. dactylon* by HPLC-DAD

Identification and quantification of individual phenolic compounds in the ethanol extract of *C. dactylon* were analyzed by HPLC-DAD (Figs. 2 and 3). The content of each phenolic compound was calculated from the corresponding calibration curve and presented as the mean of five determinations (Table 2). The experimental results indicated that ethanolic extract of

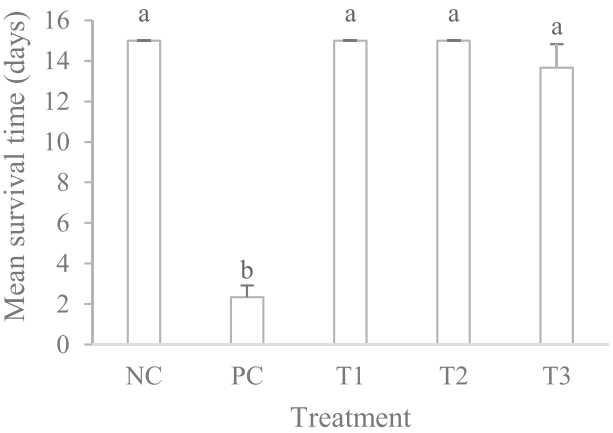

Fig. 5 Mean survival time (days) of different groups (NC = negative control, without any viral injection as well as ethanolic extract of *C. dactylon* given to the animal; PC = positive control, treated with viral suspension and no ethanolic extract dose was given; T1 = 150 mg/kg; T2 = 100 mg/kg; and T3 = 50 mg/kg). The error bars represent the standard deviations of three replicates. Different letters indicate significant differences among the treatments (Log-ranked test, $P < 0.001$)

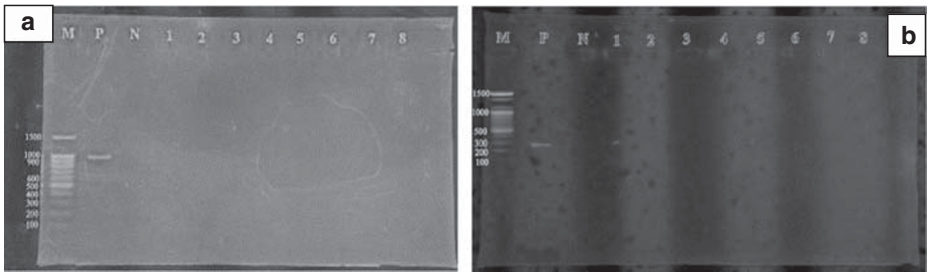

Fig. 6 **a** UV visualization of PCR product electrophoresis in a 1.5% agarose gel using primer 146F2/146R2 (941 bp). **b** Visualization of PCR product in a 1.5% agarose gel using primer IK 3–4 (298 bp). In both figures, lane M represents 100 bp ladder or marker, lanes P and N represent positive and negative control, respectively. Lanes 1 to 8, respectively, represent samples at 0 h to 11 days which were injected with PBS solution. Here, 1 = 0 h, 2 = 6 h, 3 = 12 h, 4 = 24 h, 5 = 3 days, 6 = 5 days, 7 = 7 days, 8 = 11 days

C. dactylon contained a high concentration of catechin, epicatechin, and quercetin. Vanillic acid, syringic acid, and p-coumaric acid were also detected at moderate to low concentration.

Investigation of anti-WSSV activity of *C. dactylon*

Anti-WSSV activity of *C. dactylon* was determined by observing the survival of shrimp through an in vivo challenge test. Complete mortality was observed in PC within 4 days of infection. After 15 days, the survival was found significantly higher in NC (100%) as well as in three different treatments (100%, 100%, and 85% respectively; $P < 0.008$; Fig. 4). In addition, a lower mean survival time was found in PC compared to other groups ($P < 0.001$; Fig. 5).

Detection of WSSV by PCR in the experimental groups

The samples from PC were subjected to PCR test using two primers (146F2/146R2 (941 bp) and IK 3–4 (298 bp). In both primers, WSSV band was found under UV visualization after electrophoresis in 1.5% agarose gel (Figs. 6a and b). In addition, in PC group, 100% mortality occurred within 4 days of WSSV infection. First, PCR ran with 146 F2/146R2 (941 bp) primer and found WSSV-specific band at day 2, day 3, and day 4 (Fig. 7a) after viral injection to the shrimp muscle. The PCR product of remaining samples after confirmation of WSSV positive

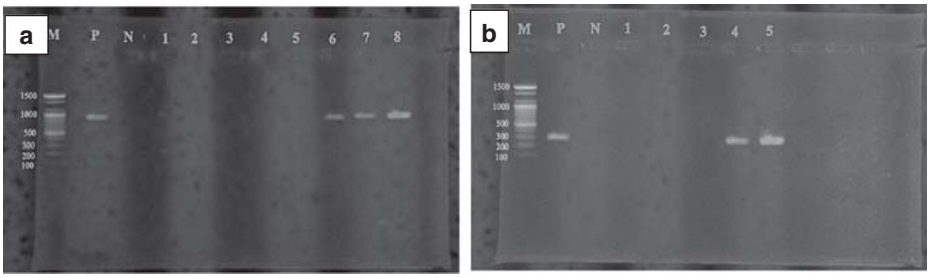

Fig. 7 **a** UV visualization of PCR product electrophoresis in a 1.2% agarose gel using primer 146F2/146R2 (941 bp) and **b** using primer IK 3–4 (298 bp). Lane M represents 100 bp ladder or marker; lanes P and N represent positive and negative control, respectively. Lanes 1 to 8, respectively, represent samples at which were injected with WSSV viral solution. Here, 1 = 0 h, 2 = 6 h, 3 = 12 h, 4 = 18 h, 5 = 24 h, 6 = 2 d, 7 = 3 d, 8 = 4 d. N.B. h, hour; d, day

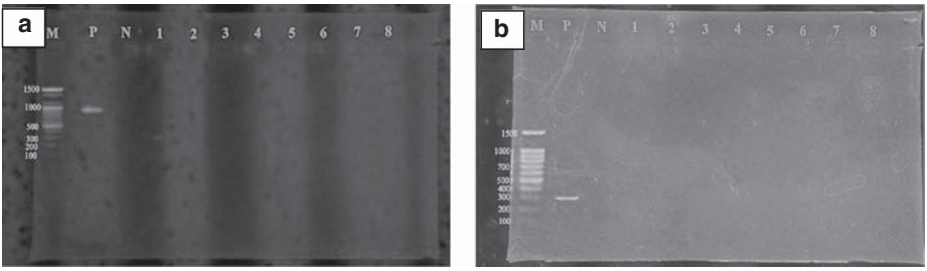

Fig. 8 **a** UV visualization of PCR product following electrophoresis in a 1.2% agarose gel using primer 146F2/146R2 (941 bp) and **b** using primer IK 3–4 (298 bp). Lane M represents 100 bp ladder or marker; lanes P and N represent positive and negative control, respectively. Lanes 1 to 8, respectively, represent samples at 0 h to 11 days which were injected with *C. dactylon* extracts (150, 100, and 75 mg/kg of BW) mixed with WSSV viral solution. Here, 1 = 0 h, 2 = 6 h, 3 = 12 h, 4 = 24 h, 5 = 3 days, 6 = 5 days, 7 = 7 days, 8 = 11 days

with 146 F2/146 R2 (941 bp) was subjected to nested PCR using primer IK 3–4 (298). UV visualization following electrophoresis results exhibited (Fig. 7b) nested PCR positive at 18 h and 1 day after inoculation of viral solution intramuscularly using injection. It could be mentioned that WSSV was very venturesome and quickly affected the shrimp.

In the ethanolic extract treatment groups, all the shrimps survived throughout the study period and there were no visible signs of WSSV infection in shrimp body where the concentration was 150 mg/kg of shrimp body weight (T1) and 100 mg/kg of shrimp body weight (T2). Furthermore, it was also observed that there were no WSSV-specific DNA bands found in PCR testing method using 146F2/146R2 (941 bp) primer. No WSSV-specific bands were found in a nested PCR using IK 3–4 (298 bp) primers (Fig. 8a and b).

In the case of treatment 3 (75 mg/kg of BW), 85% of survival rate ($P < 0.008$) was found during the experiment with no WSSV-specific band (Fig. 7a) after PCR analysis using primer 146F2/146R2 (941 bp). However, on the day 08 of the challenge test, WSSV-specific bands were found in nested PCR using IK 3–4 (298 bp) primer (Figs. 9).

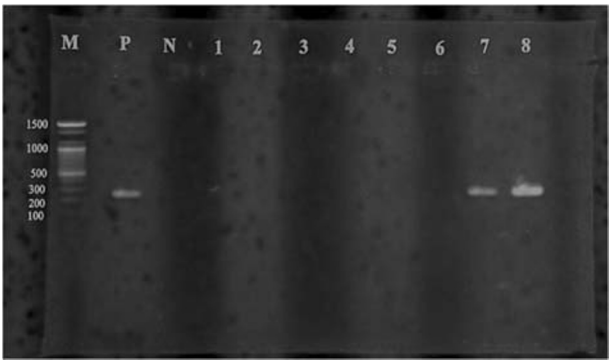

Fig. 9 UV visualization of PCR product through electrophoresis in a 1.3% agarose gel. Lane M represents 100 bp ladder or marker; lanes P and N represent positive and negative control, respectively. Lanes 1 to 8, respectively, represent nested PCR products at 0 h to 9 days which were injected with *C. dactylon* extracts 75 mg/kg of BW mixed with WSSV viral solution, where IK 3–4 (298) primer was used during PCR. Here, 1 = 0 h, 2 = 12 h, 3 = 24 h, 4 = 3 days, 5 = 5 days, 6 = 7 days, 7 = 8 days, 8 = 9 days

Discussion

Like all other crustaceans, tiger shrimp has a non-specific innate immune response which is not able to protect the organism from WSSV infection (Sarathi et al. 2007). Several studies have been conducted in search of plant extracts with significant anti-WSSV properties to protect cultured shrimp from this virus (Citarasu et al. 2006). In this regard, Balasubramanian et al. (2006) explained three possible mechanisms of antiviral activity of the plant extracts; for instance, (i) viral inactivation through reaction between the extract and the envelop proteins of the virus in which viruses may lose their entry capacity into the host body, (ii) interfering capability of plant extracts in the replication mechanism of viruses, which prevents the multiplication activity of viruses into the host body, and (iii) plant extracts might act as immunostimulant which enhances the innate immunity, e.g., pro-phenoloxidase enzyme activity, superoxide dismutase, as well as reactive oxygen or nitric oxide activity of shrimp against WSSV. Plant extracts also have antioxidant property which can protect the cells from the free radicals resulted from WSSV infection (Balasubramanian et al. 2006).

Our experiment demonstrates that *C. dactylon* ethanolic extract has some bioactive compounds considered to have anti-WSSV activity in tiger shrimp culture (Fig. 3). We found six compounds in considerable concentrations that are a rich source of alkaloids, polyphenols, and flavonoids. Phenolic acids are known to possess antiviral, anticancer, and antioxidative bioactivities (Bahorun et al. 2004; Inoue et al. 1995; Scalbert et al. 2005). In this experiment, quercetin, a flavonoid from the flavonol subgroup, was recorded highest in the ethanolic extract of *C. dactylon*. Quercetin exhibits antiviral activities against some viruses, including influenza virus, some herpes viruses, porcine epidemic diarrhea virus, and some types of human adenoviruses (Choi et al. 2009; Evers et al. 2005; Kim et al. 2010; Lyu et al. 2005; Xu et al. 2010).

Quercetin contains properties to initiate and/or inhibit the activities of several pathways of cell proliferation and induce autophagy as well as cancer cell death (Feitelson et al. 2015). It also has an inhibitory effect against the in vitro replication of dengue virus (Chiang et al. 2003). The water-soluble phenolic compound (p-coumaric and some others) found in the ethanolic extract in this experiment exhibits anti-herpes virus and anti-adenovirus properties (Chiang et al. 2002). Catechin is a flavan-3-ol, a type of natural phenol and antioxidant, which has an infection-inhibiting factor in strawberry leaves (Yamamoto et al. 2000). Catechins and their metabolites have binding capability to red blood cells that might induce release of auto antibodies (Martinez et al. 2013). Epicatechin and catechin may prevent coffee berry disease by inhibiting appressorial melanization of *Colletotrichum kahawae* (Chen et al. 2006).

We observed that ethanolic extract of *C. dactylon* provides 100% protection of *P. monodon* against WSSV infection at the concentration 100–150 mg/kg body weight. During the challenge test period, no WSSV-specific bands were found by PCR analysis using primer 146F2/146R2 (941 bp) and nested PCR using primer IK 3–4 (298 bp). The results indicate that the extracted chemicals of *C. dactylon* might contain anti-WSSV properties either independently or combinedly and those chemicals can provide complete protection of the animals against the WSSV. However, the concentration of 75 mg/kg might protect the shrimp by up to 85% during the challenge test period and we got WSSV-specific band in nested PCR on day 08. That means that those chemicals need a minimal inhibitory concentration to combat WSSV for complete protection of the tiger shrimp and it should be 100 mg/kg or more. Oral administration of methanolic extracts of five different medicinal plants, including *C. dactylon*, protects 74% of *P. monodon* at 800 mg/kg concentration of animal body weight

(Citarasu et al. 2006). In addition, 2% aqueous extract of *C. dactylon*-coated feed can protect *P. monodon* completely without presence of any DNA bands in PCR analysis (Balasubramanian et al. 2008). In an earlier study, Balasubramanian et al. (2007) reported that aqueous extracts of *C. dactylon* exhibited protective effects against WSSV in *P. monodon* and the strongest antiviral activity is found at 100 mg/kg of animal body weight.

The results of this study show that *C. dactylon* contain strong antiviral properties with many phenolic compounds. The in vivo challenge test showed that *C. dactylon* also has anti-WSSV properties which protect tiger shrimp from WSSV infections. Since *C. dactylon* has a strong activity against WSSV, further works on isolation, characterization, and purification of active compounds of this plant should be carried out to apply them in shrimp culture industry. As it was beyond the scope in this study, we would suggest doing this experiment using qRT-PCR for understanding the real status of WSSV infectivity range in shrimp culture.

Conclusion

This study confirmed the presence of some antiviral compounds in *C. dactylon* using HPLC-DAD analysis. The in vivo challenge test showed that ethanolic extracts of *C. dactylon* can protect *P. monodon* juveniles from WSSV infection at 100 to 150 mg/kg concentration. The absence of WSSV-specific bands further confirmed the anti-WSSV activity of *C. dactylon* in tiger shrimp culture. Further experiments with ethanolic or other solvent extracts of *C. dactylon* could help us to investigate the anti-WSSV activity of this grass more clearly.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

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