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RESEARCH

In vivo and *in silico* preliminary evaluation of the cyanobacterial peptides portoamides A and B against the white spot syndrome virus in freshwater crabs (*Paratelphusa hydrodomous*)

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Abstract
White spot syndrome, caused by the white spot syndrome virus (WSSV), is a devastating viral disease responsible for significant economic losses in the shrimp farming industry. In the search for natural therapeutic alternatives against WSSV, this study explored the antiviral potential of portoamides, natural peptides produced by the cyanobacterium *Phormidium* sp. LEGE 05292. Given that the structural proteins of the viral envelope, specifically VP28, VP26, and VP24, are the primary mediators of host cell attachment, they serve as promising targets for antiviral drug development. Our approach combined *in vivo* post-infection histopathological analysis with *in silico* molecular docking to assess the antiviral efficacy of portoamides. In the *in vivo* study, crabs were injected with portoamides alongside WSSV and monitored for 30 days post-infection. The antiviral activity of portoamides was evaluated through survival rates and histopathological observations. The results revealed that crabs treated with portoamides showed improved survival and reduced signs of viral infection compared to the control group. In parallel, *in silico* molecular docking analysis was conducted to assess the binding affinity between portoamides and the viral envelope proteins VP28, VP26, and VP24. The docking results demonstrated that these proteins exhibited the highest binding energies with portoamides, indicating a strong interaction that could potentially inhibit viral attachment and replication. Our findings suggest that portoamides effectively inhibit WSSV replication by interacting with the viral envelope proteins, thereby preventing the virus from establishing infection in crabs. Moreover, it is hypothesized that portoamides may stimulate the immune system in crabs, further enhancing resistance to WSSV infection. However, additional studies are needed to fully understand the immunomodulatory mechanisms involved. These preliminary results highlight the potential of portoamides as natural antiviral agents for combating WSSV in aquaculture settings, paving the way for future research on their application in disease management strategies.

Keywords White spot syndrome virus · VP28 · VP26,VP24 · Portoamides · Cyanobacteria

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Extended author information available on the last page of the article

Introduction

Aquaculture is among the fastest-growing sectors in the food industry, substantially contributing to global food consumption. However, one of the major deterrents to its sustainable growth is viral infections (Dayana Senthamarai et al. 2023). Among these, white spot syndrome virus (WSSV), which causes white spot disease, is particularly notorious for posing a serious threat to the sustainable growth of the world's shrimp farming industry. This virus can inflict massive economic losses within 2 to 10 days of infection, often leading to 100% mortality in many cultured shrimp farms (Han-Ching Wang et al. 2010). The primary physiological signs of WSSV infection include the appearance of white spots on the inner surface of the carapace and the cuticle covering the abdominal segments. The common symptoms include decreased food intake, lethargy, and reddening of the appendages (Sánchez-Paz 2010).

WSSV is an enveloped non-occluded DNA virus of the genus *Wispovirus* belonging to *Nimaviride* family. It has a diverse host range, affecting cruatacean species such as lobsters, shrimp, crabs, and crayfish, and has been responsible for significant economic losses (Verbruggen et al. 2016). WSSV genome contains about 184 open reading frame (ORFs) with about 300 kb in size, encoding four types of genes: structural genes (envelope or nucleocapsid proteins), functional genes (associated with viral replication), latency-related genes, and temporal regulatory genes (actively involved in infection at particular phases) (Sánchez-Martínez et al. 2007). Despite the precise method of WSSV entry remaining unknown, several proteins and receptors have been implicated in the infection. In WSSV, VP28 is the primary envelope protein responsible for causing systemic infections in shrimp and interacts with various host proteins (Sivakumar et al. 2016). In contrast, VP26 (tegument protein) facilitates the adhesion of the virion to the crustacean cells though an actin-binding domain (Liao et al. 2021; Tsai et al. 2004). The multiprotein complex composed by the structural proteins VP28, VP26, VP24, and VP19 mediates the first response of infection in shrimp (Hasan et al. 2022). By disrupting the ligand-receptor binding interactions and eliminating the virus envelope, viral infection may be prevented.

The ineffectiveness of antibiotics in treating WSSV can be attributed to an insufficient understanding of proper dosage applications for chemotherapeutic agents, compounded by the negative impact of climate change on treatment efficacy (Li et al. 2016). The application of natural products to suppress viral infections in aquaculture systems shows great promise and has attracted increasing interest from researchers. Several medicinal plants have been evaluated for their anti-viral effectiveness against WSSV in shrimp, yielding positive outcomes (Rahimi et al. 2022).

Cyanobacteria are photosynthetic microbes that colonize a wide range of ecosystems, ranging from oceans to freshwater, soils, and extreme environments (Ali Shah et al. 2017). Apart from the well-known harmful effects of cyanotoxins, other natural products from cyanobacteria demonstrate a diverse range of bioactivities that may be valuable for various applications (Demay, et al. 2019; Pereira et al. 2024). The freshwater cyanobacterium *Phormidium sp*. LEGE 05292 is known to produce the cyclic depsipeptides portoamides A and B in a natural ratio of 3:1. This natural mixture has been shown to have allelopathic effects on other microalgae and cyanobacterial species (Dias et al. 2017; Leão et al. 2010), as well as promising antifouling effects by inhibiting the larval settlement of *Mytilus galloprovincialis* and biofilm formation of selected marine biofilm-forming bacteia (Antunes et al. 2019). Moreover, these natural compounds have also demonstrated anticancer activity (Ribeiro et al. 2017a; Sousa et al. 2020). Given the proven benefits of portoamides

A and B (hereafter referred to as portoamides) in addressing environmental and human health issues, we propose investigating their antiviral effects through *in vivo* and *in silico* approaches against WSSV, a disease for which no treatments are currently available.

Materials and methods

Collection and maintenance of experimental animals

Experiments were conducted as per the guidelines of The Committee for Control and Supervision of Experiments on Animals (CCSEA, India), in our counterpart laboratory, and also under ethical guidelines of the European Union Council (Directive, 2010/63/EU) and the Portuguese Ministry of Agriculture, Sea, Environment, and Spatial Planning (Decree-Law nr. 113/2013, 7 August) for the protection of animals used for experimental and other scientific purposes.

Freshwater crabs (*Paratelphusa hydrodromous)* weighing approximately 20–25 g were collected from the rice field at Brahmapuram (12.9657° N, 79.1676° E) Vellore, India. Their phenotypic traits were used to confirm species, and individuals were transported to the laboratory and kept at room temperature (27 ℃) in a 100-L aquarium. Crabs were fed twice a day (Raja and Radhakrishnan 2024). Crabs were carefully monitored them for any clinical symptoms and mortality due to any cause. Once their health status was confirmed, we specifically tested them for white spot syndrome virus (WSSV) using a molecular diagnostic procedure. Prior to the experiment, head-soft tissue, gills, and muscle samples were analyzed by polymerase chain reaction (PCR) to confirm absence of WSSV infection.

Preparation of viral inoculum

Initial WSSV stock was obtained from an OIE certified laboratory. Before use, concentration and purity are assessed using real-time PCR in the viral inoculum. As described in our earlier reports (Natarajan et al. 2017), haemoymph was drawn from WSSV infected shrimp using a 26-gauge syringe through heart puncture. The haemolymph was centrifuged at 3000 rpm for 20 min at 4 ℃. The supernatant was collected and it was centrifuged for 30 min at 4 ℃ and then it was filtered using 0.45 µm and stored at − 20 ℃ for further studies. The haemolymph was analyzed via PCR to comfirm the preseance of WSSV. To amplify the structural VP28 gene, a conventional PCR reaction was set up, which includes 10 µl of 2 × master mix, 5 pmol WSSV-VP28 primer, 3 µl PCR-grade water, and 1 µl template DNA (Sundaram et al. 2016; Yoganandhan et al. 2003). The viral titer quantified through real time PCR, was 1.6×10^6 copies μL^{-1} which served as the stock for this study.

Isolation of the cyanobacterial peptides portoamides

The cyanobacterium *Phormidium* sp. LEGE 05292, obtained from the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC, http://www.ciimar.up.pt/legecc/), was used to produce portoamides A and B. Biomass from scaled-up cultures was extracted in 100% methanol and subjected to flash chromatography on the Buchi Pure C-850 FlashPrep (Buchi Labortechnik AG, Flawil, Switzerland) until the compounds were isolated in their natural production ratio 3:1, as described (Ribeiro et al. 2017b; Singh et al. 2021). The

presence of portoamides and quality control were verified by LC–MS/MS analysis. This defined mixture of portoamides A and B (referred to as portoamides) was used in the following experiments according to previous experience (Antunes et al. 2019).

In vivo anti-viral bioassay

The anti-viral efficacy of portoamides against WSSV was assessed in the freshwater crabs previously acclimated to laboratory conditions. The crabs were divided into three experimental groups, each containing ten crabs, and the experiment was performed in triplicate (Karthikeyan et al. 2022; Sundaram et al. 2016, 2014). The viral inoculum mixture was injected into the respective group after 3 h of incubation. All the crabs were injected intramuscularly at the second abdominal segment. Briefly, healthy crabs received the following injections: Group I: 100 µL NTE buffer (negative control); Group II: 95 µL NTE buffer + 5 µL of WSSV (positive control); Group III: 85 µL NTE buffer + 5 µL viral inoculum + 10 µL of portoamides (1 mg per individual crab as a dosage) (treatment). The experiment was carried out up to 30 days post-infection with WSSV and mortality was recorded to plot a cumulative mortality graph. During the experiment, crabs were fed twice a day and observed regularly for gross signs of disease.

Haematoxylin and eosin stain

At the end of the bioassay, all the crabs from the three experimental groups were dissected and tissue (gills and head soft-tissue) were rinsed in PBS buffer and fixed with 10% formalin. Samples were dehydrated with sucessive concentrations of alcohol, washed and embedded with paraffin wax. Samples were sectioned using microtome (5 µm), and the sections fixed with glass slide containing BSA (bovine serum albumin). Rehydratation and stained with H&E (Haematoxylin and Eosin) was performed, and histological patterns were evaluated microscopically (Carl Zeiss, India) (Lightner and Redman 1998; Sundaram et al. 2016).

Preparation of simplified molecule

SMILES (Simplified Molecular Input Line Entry System) of the portoamides was obtained from PubChem and the PDB format of the compound was generated using the online SMILES Translator (https://cactus.nci.nih.gov/translate/). The structure was optimized and energy minimization was done in Swiss PDB Viewer 4.1.0 software (Manimaran et al. 2018).

Preparation of macromolecule

The Protein database (PDB, http://www.rcsb.org) was accessed to obtain the 3D structure of WSSV envelope protein VP28 (PDB ID: 2ED6) VP26 (PDB ID: 2EDM) and VP24 (PDB ID: 5HLJ) (Dinesh et al. 2017; Sudharsana et al. 2016; Yadav et al. 2021). Using AutoDock v4.2 software, the protein structure was prepared for docking by eliminating any non-amino acid moieties.

Molecular docking

VP28, VP26, and VP24 were used as target proteins. The docking of portoamides A and B with the envelop proteins was carried out using AutoDock v4.2 (Dinesh et al. 2017). Blind docking was employed to enable the ligand to select autonomously its preferred binding sites. The Gasteiger-Marsili and Kollman charge methods were utilized to compute the partial charges for both the receptor and ligand. Given that blind docking was utilized the entire receptor was encompassed and a grid space of 0.375 Å was assigned accordingly. The Lamarckian genetic algorithm was applied to produce various conformations of the ligand interacting with the receptor, facilitating a comprehensive exploration of binding configuration A total of ten docking runs were executed, maintaining the default parameters for all other settings. In post-docking, the results obtained were visualized using Discovery Studio Visualizer v20.1.0.19295, developed by Dassault Systèmes Biovia Corp (Raja et al. 2023; Sudharsana et al. 2016).

Results

Bioassay

Experimental crabs from group I, injected with NTE alone (negative control) exhibited 0% mortality throughout the bioassays. Crabs in group II, injected with the viral inoculum (positive control), showed 100% mortality 5 days post-infection. Crabs from group III, inoculated with viral inoculum and portoamides, recorded 100% survival throughout the exposure period (Fig. 1), showing the same pattern as the negative control crabs without WSSV inoculum. Also, the WSSV-infected crabs from group II showed decreased feed consumption and sluggish movement. The crabs in the treated group III survived more than 30 days post-infection without exhibiting any symptoms of WSSV until the end of the experiment. These results showed that portoamides acted to inhibit the development of WSSV infection and white spot syndrome.

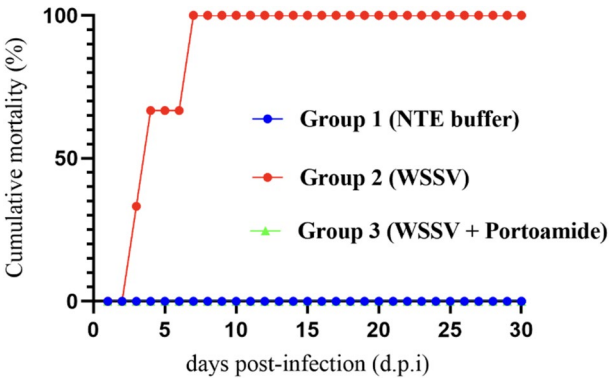

Fig. 1 *In vivo* antiviral assessment of portoamides against WSSV. Cumulative mortality of freshwater crabs over 30 days post-infection with WSSV is presented. Group I (blue circles) served as the negative control (NTE buffer). Group II (red circles) was infected with WSSV (in NTE buffer) and represents the infection control. Group III (green triangles) was the treatment group, receiving viral inoculum and portoamides. Mortality was recorded daily. $n = 10$ (per group)

Histopathological analysis

The crabs in group I and group III exhibited no histological alteration. The head-soft tissue of group II (positive control) (Fig. 2e) showed histopathological alterations such as shrunken nuclei and cells with intercellular hypertrophied nuclei compared with groups I (Fig. 2b) and III (Fig. 2f). The H&E section of gills from group II (Fig. 2b) displayed histological changes such as cell bursting and cell gaps when compared with groups I (Fig. 2a) and III (Fig. 2c).

Molecular docking studies

Docking studies of portoamides A and B with the WSSV target proteins VP28, VP26, and VP24 revealed molecular interactions that may account for the observed inhibitory activity. Portoamides A and B are dodecapeptides with nearly identical amino acid sequences, differing at a single position: portoamide A contains O-methoxy-homotyrosine, while portoamide B contains homophenylalanine. Despite this structural similarity, their binding energies toward the envelope proteins of WSSV differ (Table 1), indicating distinct affinities between the two compounds. For VP28, portoamide A displayed a binding energy of − 8.0 kcal/mol, while portoamide B showed a strongeraffinity with a binding energy of − 9.0 kcal/mol. An analysis of the binding patterns showed that portoamide A formed 5 hydrogen bonds and 3 hydrophobic bonds with VP28 (Fig. 3a), whereas, portoamide B formed 9 hydrogen bonds and 6 hydrophobic interactions with VP28 (Fig. 3b).

Similarly, the interactions of portoamides with envelope protein VP26 were analyzed, with portoamide A exhibiting a binding energy of − 5.6 kcal/mol and portoamide B of − 5.3

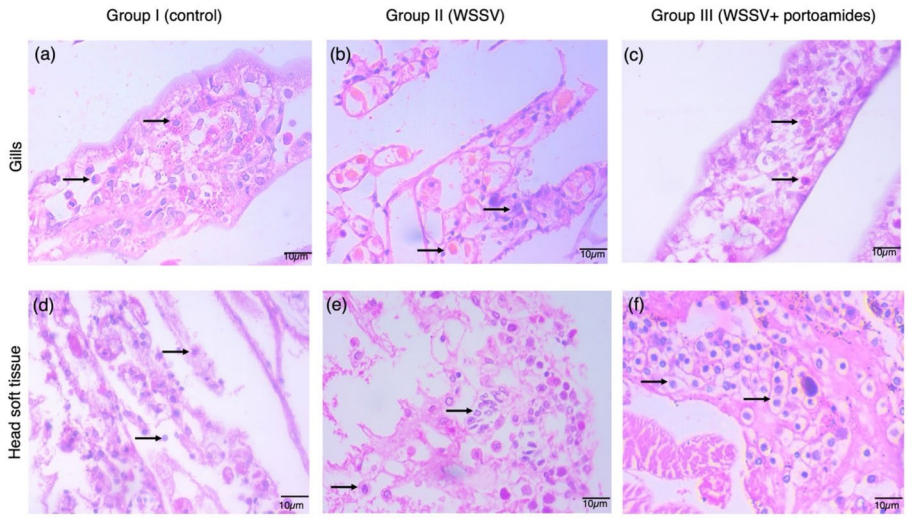

Fig. 2 Photomicrographs of hematoxylin and eosin (H&E) stained tissue sections: gills (**a**–**c**) and head soft tissue (**d**–**f**) from crabs in the control group (Group I: **a** and **d**), WSSV-infected group (Group II: **b** and **e**), and treatment group (Group III WSSV + portoamides: **c** and **f**). Original magnification is 1000×. Black arrows in photomicrographs **a**, **c**, **d**, and **f** indicate normal nuclei, while black arrows in photomicrographs **b** and **e** illustrate infected nuclei

Table 1 Binding energy of portoamides A and B with WSSV envelope proteins VP28, VP26, and VP24

Protein	Binding energy (kcal/mol)	
	Portoamide A	**Portoamide B**
VP28	−8.0	−9.0
VP26	−5.6	−5.3
VP24	−5.7	−6.2

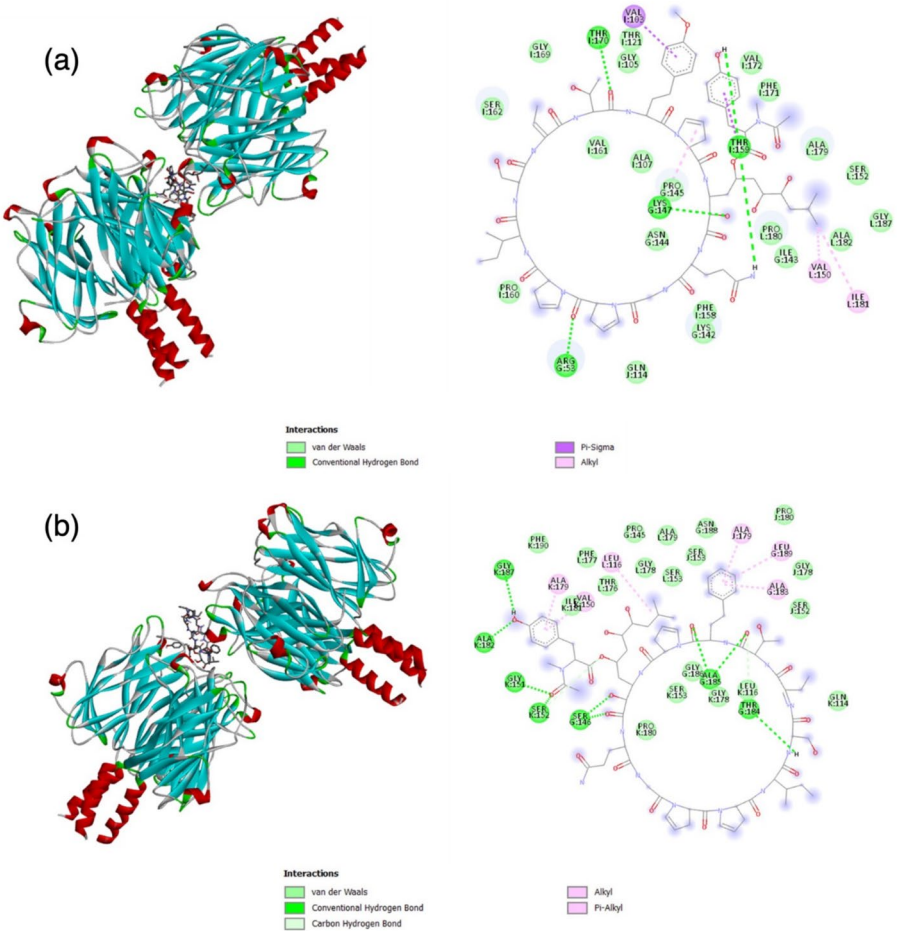

Fig. 3 Schematic representation of the binding of portoamides A (**a**) and B (**b**) with WSSV protein VP28. The interations involved between the ligands and the amino acid residues of VP28 are presented. Binding energy of portoamide A with VP28 − 8.0 kcal/mol; Binding energy of portoamide B with VP28 − 9.0 kcal/mol

kcal/mol. Both molecules formed interactions with VP26, with portoamide A forming forming 4 hydrogen bonds and 4 hydrophobic bonds (Fig. 4a), while portoamide B formed 4 hydrogen bonds and 2 hydrophobic interactions (Fig. 4b).

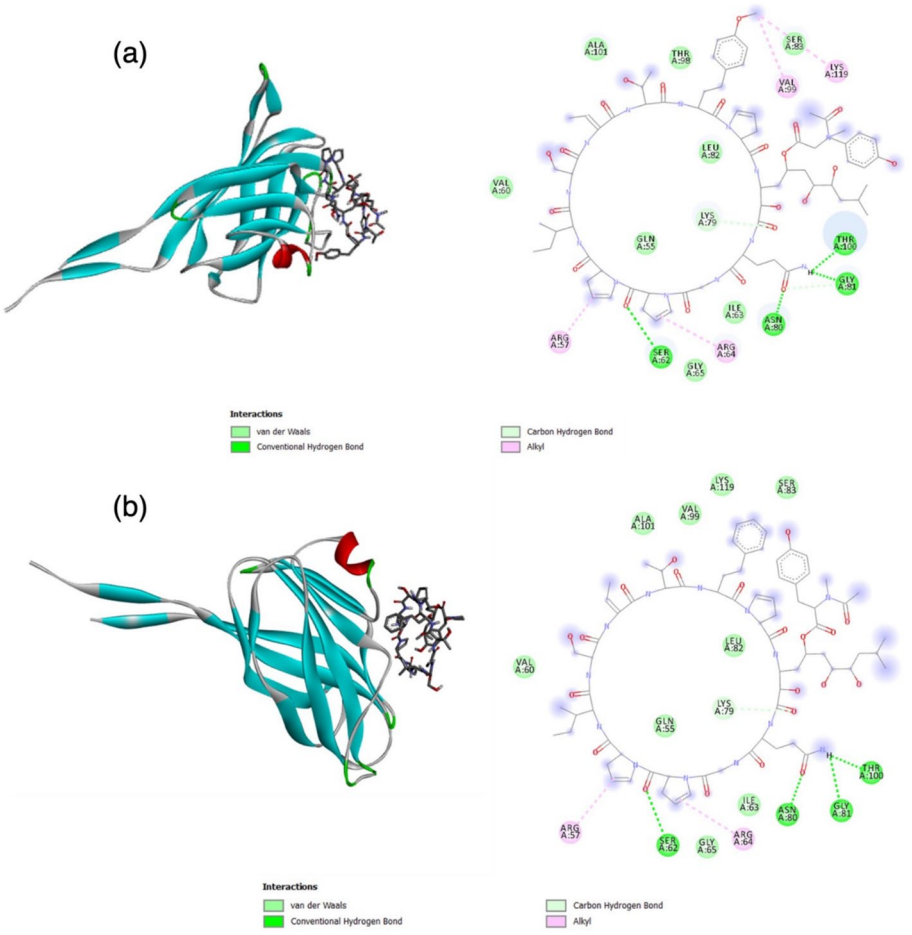

Fig. 4 Schematic representation of the binding of portoamides A (**a**) and B (**b**) with WSSV protein VP26. The interations involved between the ligands and the amino acid residues of VP26 are presented. Binding energy of portoamide A with VP26 – 5.6 kcal/mol; binding energy of portoamide B with VP26 – 5.3 kcal/mol

For the envelope protein VP24, portoamide A exhibited a binding energy of – 5.7 kcal/mol, and portoamide B exhibited a binding energy of – 6.2 kcal/mol. Analysis of the binding patterns revealed that portoamide A interaction with VP24 formed 3 hydrogen bonds and 4 hydrophobic bonds (Fig. 5a), while portoamide B interaction with VP24 formed 8 hydrogen bonds, 3 hydrophobic and one electrostatic interactions (pi-cation) (Fig. 5b). The amino acid residues involved in these interactions were visualized using the Discovery Studio visualizer, highlighting hydrogen bonds as the primary contributors to binding strength. The results highlight portoamides as a highly promising candidate for the development of an effective anti-WSSV treatment.

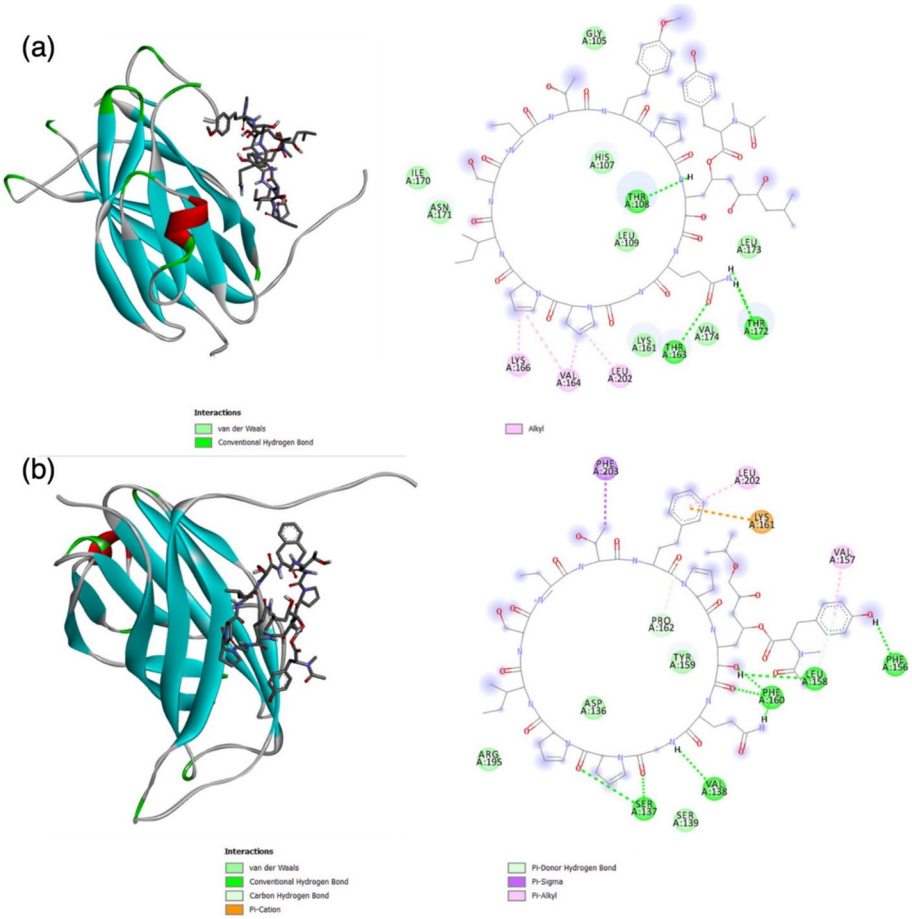

Fig. 5 Schematic representation of the binding of portoamides A (**a**) and B (**b**) with WSSV protein VP24. The interations involved between the ligands and the amino acid residues of VP24 are presented. Binding energy of portoamide A with VP24 – 5.7 kcal/mol; binding energy of portoamide B with VP24 – 6.2 kcal/mol

Discussion

WSSV infection in shrimp continues to pose a significant challenge for shrimp farmers. To date, there is no effective treatment to prevent the spread of WSSV infection in shrimp farms due to a combination of known and unknown vectors, and anti-viral drugs against WSSV are needed. Research efforts have been focused on finding effective anti-WSSV agents that can prevent WSSV infection in shrimp (Citarasu et al. 2006; Güroy et al. 2022; Peraza-Gómez et al. 2014). For example, the chemically synthesized compound methyl 1-chloro-7-methyl-2-propyl-1h-benzo[d] imidazole-5-carboxylate showed anti-viral activity against WSSV in freshwater crabs (Karthikeyan et al. 2022). Similarly, the synthesized coumarin derivatives have shown strong antiviral activity against WSSV infection in *Litopenaeus vannamei* larvae, offering significant environmental stability and preventing horizontal transmission among the larvae (Liu et al. 2021).

Natural compounds obtained from cyanobacteria have been identified as promising for several pharmaceutical applications (Demay e al., 2019). Portoamides produced by the cyanobacterium *Phormidium* sp. LEGE 05292 have demonstrated several bioactivities such as allelopathic, anticancer, and antifouling (Antunes et al. 2019; Leão et al. 2010; Ribeiro et al., 2017c) but have not yet been studied in the context of viral infections of aquatic animals. In the present study, a natural mixture of these cyclic dodecapeptides was studied for its antiviral activity against WSSV. This is the first report on antiviral activity of portoamides. Our results showed that portoamides present strong activity against WSSV in infected *Paratelphusa hydrodomous* crabs, with 100% survival in the treatment group, preventing the development of the white spot syndrome, when compared to the infected control group. Further studies on the dose–response activity of the compounds with WSSV should be performed in future work to fully understand their interaction.

In our study, the interaction between the portoamides and WSSV sustainably inhibited the virus replication by interacting with the envelope proteins of the virus (VP28, VP26, and VP24), thus preventing the viral infection in crabs. It is hypothesized that portoamides may stimulate the immune system in crabs to combat WSSV infection; however, further studies need to be conducted to clarify this question. Dupuy et al. (2004) observed that pre-incubating WSSV with the *Mytilus galloprovincialis* peptide mytillin for 3 h at room temperature, induced anti-WSSV activity by reducing mortality in shrimp. In this study, portoamides were incubated along with WSSV for 3h and virucidal activity was observed by rendering the virus inactive. The results obtained from the bioassay and histopathological examinations provide evidence in favor of the potential antiviral effects of portoamides against WSSV.

Structural proteins of WSSV, such as VP28 and VP24, play a major role in viral attachment to the host cell membrane and initiation of viral replication. As a result, blocking of these proteins will prevent the entry of WSSV in the host. These viral proteins have become a suitable molecular target for drug development. Inhibiting these proteins can block the entry of WSSV in the host cells, providing a strategy for preventing the virus (Xiao et al., 2020). *In silico* docking analysis of the target protein VP28 has previously been used to evaluate the synthethic compound methyl 1-chloro-7-methyl-2-propyl-1h-benzo[d] imidazole-5-carboxylate against WSSV (Karthikeyan et al. 2022). The authors reported a binding energy of − 6.61 kcal/mol for this compound, which falls within a similar binding affinity range as that of portoamides A and B. Similarly, docking studies of VP28 with phytochemicals derived from *Avicenna marina* exhibited potential to block VP28 protein (Sahu et al. 2012). According to previous studies, VP28 acts as an attachment protein in shrimp cells, permitting viral entrance in the cytoplasm (Yi et al. 2004). VP26 acts as a linker protein that interacts directly with other proteins to bridge the envelope to the nucleocapsid of WSSV, implying that VP26 plays a significant role in WSSV virion envelopment (Liao et al. 2021). Consequently, targeting these proteins could prevent the entry of WSSV in the host. Similar to our results, the plant-derived flavonoid quercetin exhibited strong interaction and binding affinity with the viral envelope protein VP26 (Kumar et al. 2023). Molecular docking studies have shown that hydrogen and hydrophobic bonds are the key factors driving the binding of compounds to WSSV proteins, as demonstrated by the strong binding affinities (Bulusu and Desiraju 2020).

Sudharsana et al. (2016) investigated the antiviral activity of 3-(1-chloropiperidin-4-yl)−6-fluoro benzisoxazole against WSSV envelope proteins VP28 and VP26. Additionally, another *in silico* docking simulation study targeted VP28 to identify potential marined-derived compounds against WSSV (Sivakumar et al. 2016). Various phytocompounds identified from *Phyllanthus amarus* extract were examined in a docking simulation study, with the compound

2H-1-benzopyran-6-ol,3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-acetate exhibiting the highest binding energies of − 8.94 kcal/mol for VP28 and − 6.71 kcal/mol for VP26 (Sundaram et al. 2016). Our findings emphasize the role of envelope structural proteins as promising targets for antiviral treatment development, particularly antiviral therapies against WSSV. They also highlight the potential of targeting structural proteins like VP28, VP26, and VP24. In our study, portoamides A and B demonstrated significant binding energy to WSSV envelope proteins, strongly supporting our in vivo results that indicate these compounds to have a promissing anti-viral activity against WSSV.

Conclusion

This is the first report on the antiviral activity of portoamides isolated from the cyanobacterium *Phormidium* sp. LEGE 05292 against WSSV. The results of this preliminary study indicate that the compounds seem to show significant antiviral activity against WSSV, both *in silico* and *in vivo*, revealing the potential activity of portoamides to control WSSV in crustacean culturable species. Further insights on ecotoxicological effects (dose–response study) immunomodular and molecular biology traits are needed to fully understand the viral interaction with the compounds. The use of portoamides for controlling pathogens in shrimp and fish could provide new opportunities for aquatic animal health management.

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Author contribution Bharath Raja: formal analysis, investigation, methodology, writing—original draft. Vidya Radhakrishnan: investigation, supervision, validation, writing—review and editing. Sudhakaran Raja: supervision, validation, writing—review and editing. Sandra Pereira: formal analysis, investigation, methodology writing—review and editing; Catarina Gonçalves: formal analysis, investigation, methodology writing—review and editing. Vitor Vasconcelos: supervision, resources, writing—review and editing. Mariana Reis: conceptualization, validation, writing—review and editing. Joana R. Almeida: conceptualization, funding acquisition, methodology, project administration, validation, resources, supervision, writing—review and editing.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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