Antiviral activity of Turbinaria ornata against white spot syndrome virus in freshwater crab (Paratelphusa hydrodromous)

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Abstract

White spot disease in penaeid shrimp is caused by white spot syndrome virus and causing serious threat to shrimp farming industry. The present study was carried out to determine the anti-viral activity of *Turbinaria ornata* against WSSV in freshwater carbs *Paratelphusa hydrodromous*. The crabs were injected with acetone extract of *T. ornata* along with WSSV and the experimental groups were observed for more than 60 days post-infection. The efficacy of the *T. ornata* acetone extract was confirmed by bioassay, histopathology, and *in-silico*analysis. The results of the present study confirmed that acetone extract of *T. ornata* has significant anti-viral activity against WSSV and it can be used as a possible prophylactic in shrimp culture for prevention of WSSV infection.

1. Introduction

White spot syndrome virus (WSSV) is one of the most devastating and threatening viral pathogen, results in high mortality and huge economic losses in the crustacean aquaculture industry (Verbruggen et al. 2016). It causes 100% mortality within 3–10 days and it remains undetected until they produce a significant outbreak in shrimp farms. It consists of double-stranded circular DNA of 300 kbp in size and belongs to the family *Nimaviridae*, genus *Whispovirus* (Sundaram et al. 2016). WSSV infects a range of crustacean species including shrimp, crab, lobster, and crayfish. The mode of transmission of WSSV is both horizontal and vertical. The major physiological symptoms of WSSV are the occurrence of white spots inside the epidermis, appendages, and exoskeleton of shrimp (Sánchez-Paz 2010). WSSV infection progresses at various rates in different tissues with the stomach being the most preferred replication tissue in the early stages of infection. The skin, stomach, and gills are the most highly infected tissues causing symptoms such as skin colour changes, reduced food ingestion, and gathering at the water surface leading to hypoxia (Sun et al. 2013). Furthermore, WSSV causes apoptosis and oxidative stress in shrimp (Yuan et al. 2016). During viral infection, envelope proteins play a critical role in attachment to the host cellular receptor. In WSSV, VP28 is the major envelope protein that has been shown to exert systemic infection in shrimp and also interacts with several host proteins (Sivakumar et al. 2016).

To tackle the pathogens, the crustaceans depend on innate immunity since they lack an adaptive immune system. Although in the last 20 years substantial progress has been achieved in host anti-WSSV immune defense (Li et al. 2019) no effective approach has been established. There has been no successful production of commercial vaccine against WSSV, and safety issue has become a major concern for developing vaccines rather than their efficacy (Haq et al. 2012). Even though there are several drugs on the market with anti-WSSV activity, the problem remain unsolved.

Seaweeds are rich source of bioactive compounds and produce secondary metabolites with a varying range of biological functions such as antibacterial (Singh and Chaudhary 2010), antifungal (De Corato et al. 2017), antiviral (Immanuel et al. 2012), anti-inflammatory, nematocidal and anticoagulant (Caijiao et al. 2021). *Turbinaria ornata* is a brown seaweed found in the Indian coast. It has wide range of biological activities. However, there has never been any report on the anti-WSSV activity in *T. ornata*. Therefore, the aim of the study was to determine the antiviral properties of *T. ornata* against WSSV by *in-vivo* and i*n-silico* studies

2. Materials And Methods

2.1 Collection and maintenance of animal

Freshwater crabs *P. hydrodromous* (20–25 g body weight) were collected from the rice field located at Brahmapuram (12.9657° 79.1676°) Vellore, India. The animals were transported to the laboratory and kept at room temperature (25℃) in a 100L aquarium. The crabs were fed twice a day with boiled egg white (Karthikeyan et al. 2022). The crabs were maintained in the described condition for one week prior the experiment. Tissue samples including gills, muscle, and head-soft tissue were subjected to polymerase chain reaction to ensure the animals were WSSV-free before the experimental challenge (Sundaram et al. 2016).

2.2 Sample collection

Turbinaria ornata was collected from Thangchimadam (9.293434° 79.23951°) Rameswaram, Tamil Nadu, India. The samples were washed and cleaned with distilled water to remove impurities and salt present on the surface. The samples were then shade dried to avoid direct exposure to sunlight. The samples were then ground into fine powder using dry grinder and were sieved to get uniform particle size. They were then stored in air-tight container and was used for extraction (Roohi Fatima et al. 2016).

2.3 Preparation of seaweed extract

The powder of *Turbinaria ornata* was extracted using acetone (Roohi Fatima et al. 2016) using a modified protocol. Ten grams of *Turbinaria ornata* was extracted with 100 mL of aqueous, acetone, and petroleum ether (1:10 w/v) and kept in a shaker at room temperature for 24 h. The extract was then filtered using Whatman No.1 filter paper. The filtrate was collected, and the solvent was removed by using rotary evaporator. The extract was lyophilized and stored in 4℃ for further studies.

2.3 Preparation of WSSV inoculum

Hemolymph was collected from shrimp through heart puncture using a 26G syringe. The collected hemolymph was centrifuged at 3000 rpm for 20 minutes at 4℃. The supernatant was collected and transferred to a fresh tube and it was centrifuged at 8000 rpm for 30 minutes at 4℃ the supernatant

was filtered with a 0.45µm filter and stored at -20°C for future studies. The presence of WSSV in hemolymph was confirmed by performing a PCR assay (Yoganandhan et al. 2003). The viral titter determined was 1.6×10^6 copies μL^{-1}.

2.4 Bio-assay analysis

The prepared *T. ornata* acetone extract was investigated for anti-viral activity efficacy against WSSV in freshwater crab, *Paratelphusa hydrodomous*. The experimental animal were divided into three experimental groups each group containing three crabs per group and the experiment was performed in triplicate (Sundaram et al. 2016). The viral inoculum mixture was incubated for 3 hours before injection. The viral inoculum mixture was injected onto the respective group after 3 hours of incubation. Briefly, healthy crabs received the following injection: in Group the crabs were injected with 100µL NTE buffer which served as a negative control, and in Group the crabs were injected with 95µl NTE buffer and 5µl viral inoculum which served as a positive control. In group the animals were injected with 85µl NTE buffer, 5µl viral inoculum, and 10 µl acetone extract. The crabs were fed twice a day and observed regularly for the gross sign of disease. The experiment was carried out up to 30 days post infection with WSSV and mortality was recorded to plot cumulative mortality graph.

2.5 GC-MS analysis

The analysis of compound in acetone extract of *T. ornata* was performed using Gas Chromatography (Perkin Elmer GC Clarus 680 system, United States) equipped with Elite-5MS column (30.0 m, 0.25 mm IID, 250µm df) and 70 eV of ionization energy was employed for GC-MS detection (Dinesh et al. 2017). The carrier gas was helium (99.99%) and was used at a constant flow rate of 1 mL/min and an injection volume of 1IL (split ratio of 10:1). The temperature of the injector was 260°C. The initial oven temperature was set to 60°C for 2 minutes followed by a increase of 10°C/min to 300°C, holding for 6 min. Mass spectra were collected at 70 eV, with a scanning interval of 0.5 s and fragments ranging from 50 to 600 Da. The total run time of GC was 32 minutes. Each component's relative percentage quantity was computed by comparing its average peak area to the overall area. TurboMass Version 5.4.2 was used to handle mass spectra and chromatograms. The retention periods of the matching compounds were compared to those of legitimate compounds, as well as the spectrum data collected from the library database, National Institute of Standards and Technology (NIST) (Eswaraiah et al. 2020).

2.5 *In-silico* analysis

2.5.1 Macromolecule and small molecule preparation

The 3D structure of the VP28 (2ED6) envelope protein of WSSV was obtained from Protein Data Bank (Berman et al. 2000). Using Auto dock 4.2 software the protein structure was prepared for docking by eliminating any non-amino acid moieties (Morris et al. 1998).The simplified Molecular-Input Line-Entry System (SMILES) is a line notation standard that represents the connectivity and chirality of a molecule. The canonical SMILES of the compound were obtained from PubChem and the PDB format of the compound was generated using the online SMILES Translator (https://cactus.nci.nih.gov/translate/) (Manimaran et al. 2018)

2.5.2 Molecular Docking

Molecular docking investigations were carried out using Autodock-4.2 to study the interaction between *T. oranta* compounds and envelope protein VP28 (Sudharsana et al. 2016) (Daniel and Devi 2019). Blind docking was used to allow the ligand to select their preferred binding sites. The partial charges for protein and ligand were calculated using Gasteiger-Marsili and Kollman charges. The grid box was set over the entire protein since blind docking performed and the grid space set to 0.375A. Using the Lamarckian genetic algorithm, the conformers of the ligand bound to the protein were generated. The number of docking runs was set to 10 leaving the other settings to default values. Results obtained from Autodock-4.2 were viewed using Discovery studio visualizer v20.1.0.19295, Dassault Systems Bio via corp (Thirumal Kumar et al. 2019).

2.6 Histopathology

Experimental crab injected with acetone extract and WSSV along with negative and positive control were dissected and organs such as gills and head soft tissue were rinsed in PBS buffer and preserved in 10% formalin for histopathological analysis. Then the tissues were stained with Haematoxylin and Eosin (H&E) and histological changes were then evaluated under a compound microscope (Carl Zeiss, India)(Lightner and Redman 1998).

3. Results

3.1 Bioassay

Freshwater Crabs *(P. hydrodromous)* were used as a model organism for the experiment to determine the anti-viral activity of *T. ornata* acetone extract. The result proved that *T. oranta* acetone extract has strong anti-viral activity against WSSV. In group crabs injected with *T. oranta* acetone extract and viral inoculum showed 100% survival until the end of the experiment (Fig. 1). In group the crabs injected with viral inoculum recorded 100% mortality within 7 days of post-infection. In group the crabs injected with NTE alone survived without any sign of WSSV infection and showed zero mortality till the end of the experiment. A decrease in feed consumption and slow movement were also observed in WSSV-infected crabs. The crabs in the treated groups survived more than 60 days post infection without any symptoms of WSSV as observed until completion of the experiment.

3.2 GC-MS analysis

GC-MS analysis was performed for *T. ornata* acetone extract; the chromatogram is shown in Fig. 2 The molecular weight (MW), molecular formula (MF), and the retention time (RT) for the bioactive compound present in the extract based on the search are given in Table 1 There were sixteen compounds identified in the extract. The prevailing compounds were butanoic acid, 3-amino-2-methyl-, n-[dimethylaminomethyl]aziridine, n-[3-methylaminopropyl] aziridine, Carbamic acid, 2-(dimethylamino)ethyl ester, n,n-diethylpropionamide, 2-butene-1,4-diamine, n,n'-dimethyl-,1,3-propanediamine, n,n-diethyl-, e-11,13-tetradecadien-1-ol acetate, n-chlorodimethylamine, 1-piperazinecarboxamide, n,n-diethyl-4-methyl-, 9-methyl-11-oxo-1,6-diazatricyclo [7.2.0.0(6,8)]undecane, 8-Azabicyclo[5.1.0]octane, Threo-4-hydroxy-l-lysine lactone,O-Methylcarbamoyl-3-tropanone oxime, 9-borabicyclo[3.3.1]nonane, 9-(3-aminopropyl)- and pregnane-3,20-dione, 17,21-[(methylborylene)bis(oxy)]-, (5.beta.).

Table 1
Compounds detected in Acetone extract of *Turbinaria ornata* through GC-MS analysis

S. No	RT (Retention Time)	Compound	Name	Pubchem ID	Molecular formula	Molecular weight	Area %	mg/g
1	3.679	butanoic acid, 3-amino-2-methyl-	butanoic acid, 3-amino-2-methyl-	541783	$C_5H_{11}NO_2$	117.15	7.121	71.21
2	9.511	n-[dimethylaminomethyl]aziridine	Aziridine, 1-((dimethylamino)methyl)	145718	$C_5H_{12}N_2$	100.16	6.413	60.13
3	12.392	n-[3-methylaminopropyl] aziridine	N-[3-Methylaminopropyl]aziridine	547048	$C_6H_{14}N_2$	114.19	13.635	136.35
4	14.943	Carbamic acid, 2-(dimethylamino)ethyl ester	dimethylaminoethyl carbamate	48131	$C_5H_{12}N_2O_2$	132.16	8.174	81.74
5	17.224	n,n-diethylpropionamide	Diethylpropionamide	66191	$C_7H_{15}NO$	129.20	2.285	22.85
6	17.615	2-butene-1,4-diamine, n,n'-dimethyl-	2-Butene-1,4-diamine, N1,N4-dimethyl-	5364149	$C_6H_{14}N_2$	114.19	1.126	11.26
7	19.290	1,3-propanediamine, n,n-diethyl-	Diethylaminotrimethylenamine	547048	$C_6H_{14}N_2$	114.19	1.059	10.59
8	19.660	e-11,13-tetradecadien-1-ol acetate	Ambrettolid	5365703	$C_{16}H_{28}O_2$	252.39	3.006	30.06
9	19.710	n-chlorodimethylamine	Dimethylchloramine	123122	C_2H_6ClN	79.53	1.339	13.39
10	20.366	1-piperazinecarboxamide, n,n-diethyl-4-methyl-	Carbamazine	3052	$C_{10}H_{21}N_3O$	199.29	6.799	67.99
11	26.253	9-methyl-11-oxo-1,6-diazatricyclo [7.2.0.0(6,8)]undecane	9-methyl-11-oxo-1,6-diazatricyclo [7.2.0.0(6,8)]undecane	565957	$C_{10}H_{16}N_2O$	180.25	17.944	179.44
12	26.458	8-Azabicyclo[5.1.0]octane	Cycloheptenimine	544549	$C_7H_{13}N$	111.18	2.114	21.14
13	27.198	Threo-4-hydroxy-l-lysine lactone	Threo-4-hydroxy-l-lysine lactone	91695432	$C_6H_{12}N_2O_2$	144.17	1.300	21.14
14	27.734	O-Methylcarbamoyl-3-tropanone oxime	O-Methylcarbamoyl-3-tropanone oxime	557471	$C_{10}H_{17}N_3O_2$	211.26	1.744	17.44
15	27.999	9-borabicyclo[3.3.1]nonane, 9-(3-aminopropyl)-	9-borabicyclo[3.3.1]nonane, 9-(3-aminopropyl)-	550741	$C_{11}H_{22}BN$	179	1.373	13.73
16	31.090	pregnane-3,20-dione, 17,21-[(methylborylene)bis(oxy)]-, (5.beta.)-	pregnane-3,20-dione, 17,21-[(methylborylene)bis(oxy)]-, (5.beta.)-	618762	$C_{22}H_{33}BO_4$	372	24.567	245.67

Table 2
In silico docking investigation of *Turbinaria ornata* compounds interactions with WSSV target proteins VP28.

S. No	Compound	VP28 (Binding Affinity)	No of H bond
1	butanoic acid, 3-amino-2-methyl-	-4.8	3
2	n-[dimethylaminomethyl]aziridine	-3.2	3
3	n-[3-methylaminopropyl] aziridine	-3.5	2
4	Carbamic acid, 2-(dimethylamino)ethyl ester	-3.2	3
5	n,n-diethylpropionamide	-3.2	3
6	2-butene-1,4-diamine, n,n'-dimethyl-	-3.7	2
7	1,3-propanediamine, n,n-diethyl-	-3.5	2
8	e-11,13-tetradecadien-1-ol acetate	-3.4	3
9	n-chlorodimethylamine	-2.6	3
10	1-piperazinecarboxamide, n,n-diethyl-4-methyl-	-5.5	2
11	9-methyl-11-oxo-1,6-diazatricyclo [7.2.0.0(6,8)] undecane	-6.0	2
12	8-Azabicyclo[5.1.0]octane	-3.7	2
13	Threo-4-hydroxy-l-lysine lactone	-3.7	2
14	O-Methylcarbamoyl-3-tropanone oxime	-3.7	2
15	9-borabicyclo[3.3.1]nonane, 9-(3-aminopropyl)-	-3.2	1
16	pregnane-3,20-dione, 17,21-[(methylborylene)bis(oxy)]-, (5.beta.)-	-7.1	1

3.2 Molecular docking studies

Docking of the different compounds identified from *T. ornata* acetone extract with the target protein of WSSV VP28 showed the least binding energy of -7.1kcal/mol respectively. The amino acid residues involved in the interaction with VP28 envelope protein were visualized using the Discovery Studio visualizer. The lead compound pregnane-3, 20-dione, 17, 21-[(methylborylene)bis(oxy)]-, (5.beta.)- formed 2 hydrogen bonds(PRO145 & LYS147) with VP28 during interaction (Fig. 3). The strongest interactions responsible for the binding are hydrogen bonds. The amino acid residues involved in the interaction were methylborylene) bis(oxy)]-, (5.beta)- interacts with PROD:145 LYSD:147,ALAE:107 and VALE:172. The interaction involved includes van der Walls, hydrogen bonds, and alkyl interactions which are depicted in Fig. 3. The possible ligand binding site of VP28 contains all of the amino acids involved in the interaction.

3.3 Histopathology

The negative control crabs (NTE buffer alone) showed no histological changes (Fig. 4a & b). In WSSV infected group the H&E-stained sections of gills and head soft tissue showed histopathological changes which includes condensed nucleus and cells with hypertrophied nuclei with intracellular which are typical of WSSV infection (Fig. 4c & d). Whereas in treated group no histological alteration was observed (Fig. 4e & f).

4. Discussion

WSSV infection in shrimp is still a major problem for shrimp farmers. There is no proper treatment to control the WSSV infection in shrimp. It is important to develop anti-viral drugs against WSSV. Several investigations have been carried out in search of seaweed extract with a substantial anti-WSSV activity that can protect cultured shrimp against this virus. Seaweed extracts possess antiviral as well as immunostimulant characteristics (Güroy et al. 2022). Fucoidans extracted from different seaweeds reported to inhibit the replication of several enveloped virus which includes HIV, HSV, and human cytomegalovirus(Thuy et al. 2015) (Garrido et al. 2017). *Turbinaria ornata* illustrated biological activity such as antibacterial, antioxidant anti-inflammatory activity, and wound healing properties (Shaibi et al., 2021).

Earlier, several attempts were done to find anti-WSSV properties from seaweeds and plants. In this present study, *T. ornata* acetone extract was tested for antiviral activity against WSSV in freshwater crab *P. hydrodomous*. In this experiment, we found that the acetone extract of *T. ornata* showed strong activity against WSSV with 100% survival. In the positive control showed 100% mortality. A similar study was done with methanolic extract of *Hypane spinella* that showed antiviral activity against WSSV in freshwater crabs (Sundaram et al. 2014) The WSSV inoculum incubated with *T. ornata* extract effectively inactivated the virus replication by the interaction between the extract and the envelope proteins of the WSSV virus. Furthermore, the effect of the extract on WSSV replication prevents virus multiplication in host organisms. It is assumed that the immune mechanism is triggered by *T. ornata* extract which tries to combat against WSSV in the crab.

Sudheer et al.(2011) reported that aqueous extract of *Ceriops tagal* when administered as feed to *P. monodon* showed 74% survival rate against WSSV. The shrimp feed with ethanolic leaf extract of *Pongamia pinnata* showed 80% survival rate against WSSV (Rameshthangam and Ramasamy 2007). Fucoidan isolated from *Sargassum wightii* z fed to *P. monodon* enhanced the immunity and resistance against WSSV(Immanuel et al. 2012).Similarly sulfated galactans isolated from *Gracilaria fisheri* exhibited antiviral activity against WSSV in *P.monodon* and the mortality rate was lower (Wongprasert et al. 2014). It was reported that the hot water extract of *Ulva instestinalis* when supplemented as feed to *Litopenaeus vannamei* upregulated the immune response and inhibited WSSV (Klongklaew et al. 2021).

Dupuy et al., (2004) reported that pre incubation of WSSV with Mytillin for 3hours at room temperature showed anti-WSSV activity by decreasing the mortality in shrimp. In this study, the acetone extract of *T. ornata* administered along with WSSV was incubated for 3hours at room temperature showed virucidal activity suggesting the presence of molecules in extract that could render the virus inactive.

GC-MS analysis revealed that *T. ornata* acetone extract contains bioactive compounds that are considered to have anti-WSSV activity in *P. hydrodomous*. In this experiment pregnane-3,20-dione, 17,21-[(methylborylene)bis(oxy)]-, (5.beta)- was recorded highest in the acetone extract of *T. ornata* and this compound may be responsible for the suppression of WSSV. It has been reported that pregnane-3,20-dione, 17,21-[(methylborylene)bis(oxy)]-, (5.beta)- has anticancer activity (Parthasarathi et al. 2021).

A chemically synthesised compound methyl 1-chloro-7-methyl-2-propyl-1h-benzo[d] imidazole-5-carboxylate was tested against WSSV target protein VP28 by *in-silico* docking analysis (Karthikeyan et al. 2022). They also reported that the compound interacted with METB139 of VP28 envelope protein. According to pervious reports VP28 binds to shrimp cells as an attachment protein, allowing the virus to enter the cytoplasm (Yi et al. 2004).As a result blocking of this protein will prevent the entry of WSSV in host. Similarly docking of VP28 with *A. marina* derived phytochemicals exhibited the potential to block VP28 protein (Sahu et al. 2012). In this study *in-silico* analysis revealed that pregnane-3,20-dione, 17,21-[(methylborylene)bis(oxy)]-, (5.beta)- interacts with PROD:145 LYSD:147,ALAE:107 and VALE:172. of VP28 protein and it also showed highest binding affinity to the protein VP28 which can inhibit the replication of WSSV. It is proposed that the interaction between pregnane-3,20-dione, 17,21-[(methylborylene)bis(oxy)]-, (5. beta)- with VP28 can prevent the formation of PmRab-VP28 complex. There have been very few efforts to purify components responsible for anti-WSSV activity and all these studies focused primarily on crude extract from single seaweed or a combination of both. Hence, pregnane-3,20-dione, 17,21-[(methylborylene)bis(oxy)]-, (5. beta)- showed minimum docking score and could be considered for further *in-vitro* and *in-vivo* studies. The *in-silico* results further support the anti-WSSV activity of *T. ornata* acetone extract.

5. Conclusion

It is concluded from the present study that acetone extract of *T. ornata* showed significant antiviral activity against WSSV. The presence of antiviral compound in *T. ornata* was also confirmed from this study. The acetone extract of *T. ornata* could be effectively used as prophylactic and both *in-silico* and *in-vivo* approaches reveal potential activity of *T. ornata* to control WSSV infection shrimp farms. The computational analysis reveals that the phytocompound pregnane-3,20-dione, 17,21-[(methylborylene)bis(oxy)]-, (5. beta)- from *T. ornata* could be the potential molecule for the treatment of WSSV in shrimp culture.

Declarations

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Conflict of interest

The authors declare that there were no competing interests either financial or non-financial.

Author contribution

Authors of the manuscript have contributed in various sections such as designing and executing experiments, resulting in analysis, manuscript writing, and reviewing.

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Figures

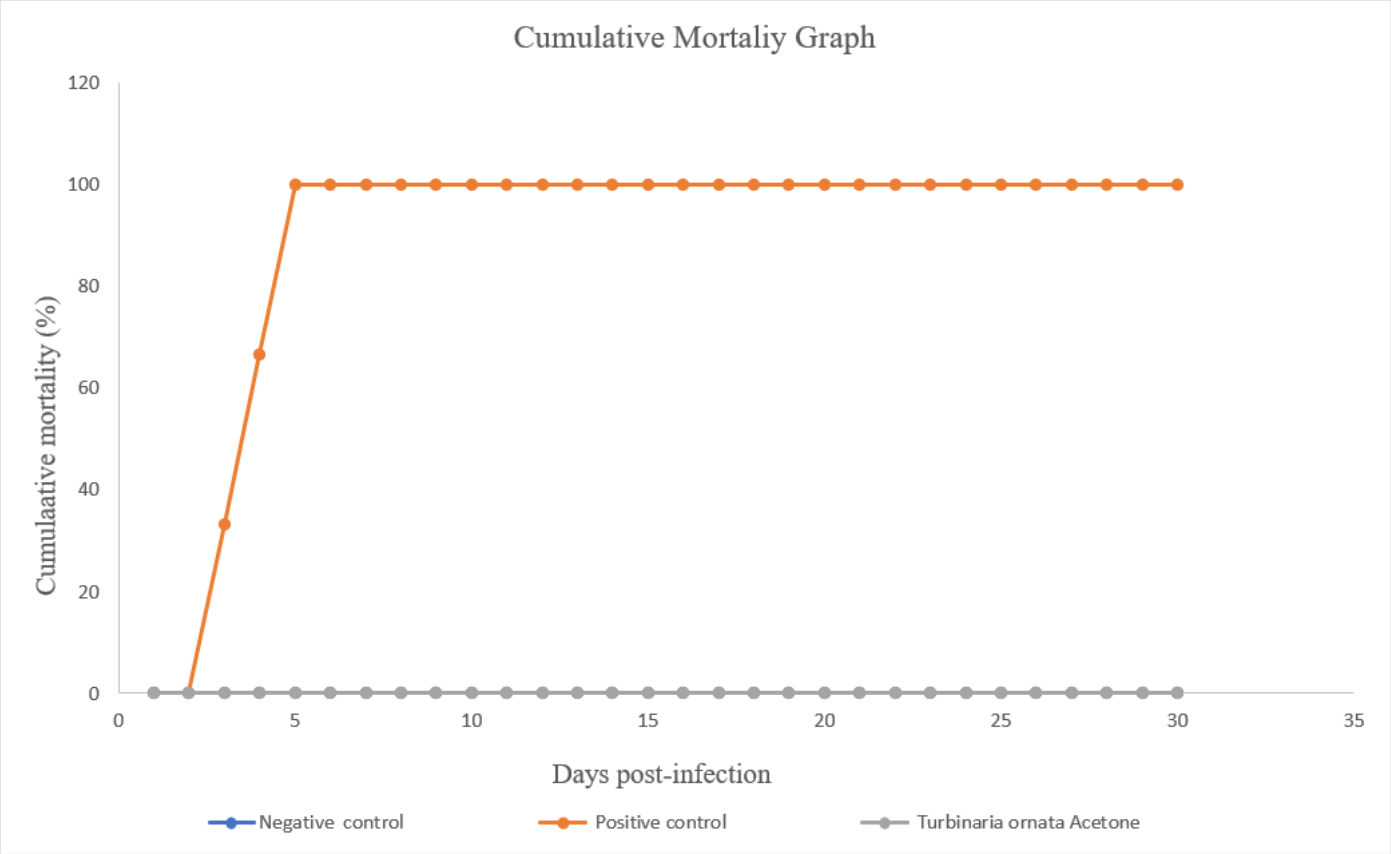

Figure 1

Cumulative mortality of crabs experimentally injected WSSV along with acetone extract of *Turbinaria ornata*

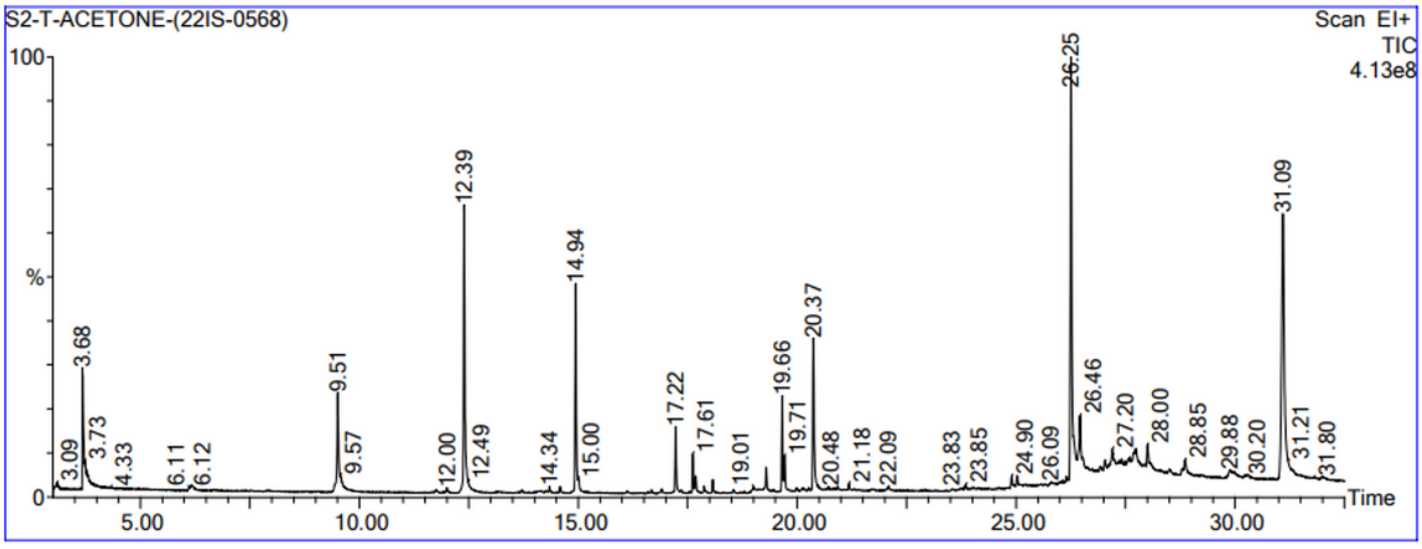

Figure 2

Chromatogram obtained from GC-MS analysis of *T. ornata*acetone extract

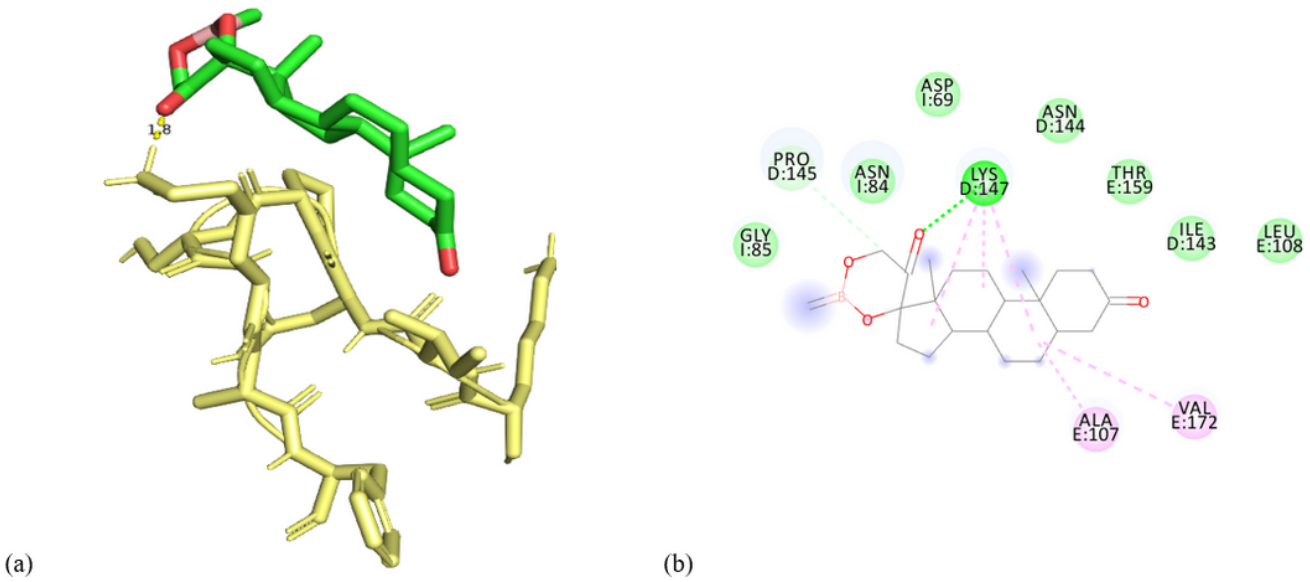

Figure 3

(a) PyMol was used to view the interaction of pregnane-3,20-dione, 17,21-[(methylborylene)bis(oxy)]-, (5.beta.), with WSSV protein VP28.

(b) The interaction of pregnane-3,20-dione, 17,21-[(methylborylene)bis(oxy)]-, (5.beta.)-, with WSSV protein VP28 was viewed in Discovery studio, displaying hydrogen bonds and the amino acid involved

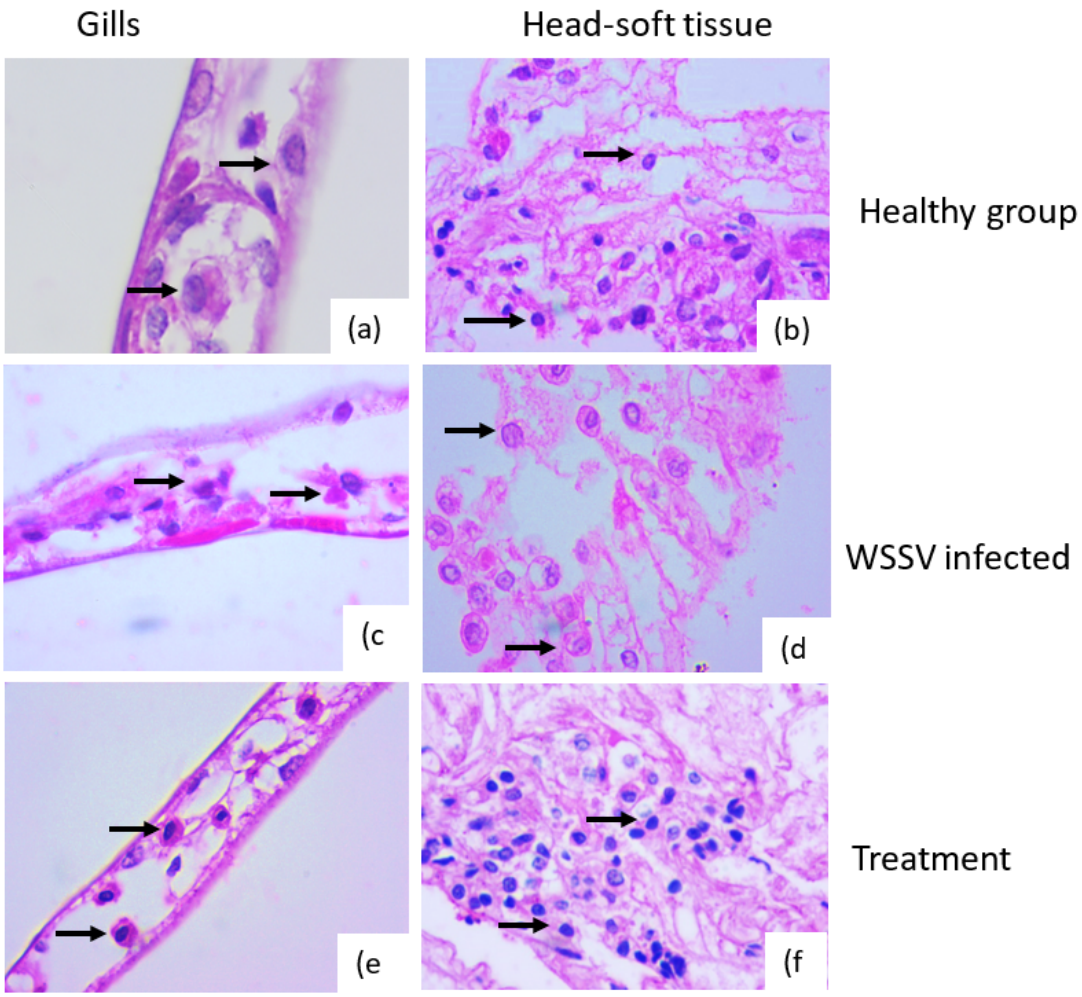

Figure 4

Haematoxylin and Eosin (H&E) stained section of gills and head soft tissue healthy, WSSV infected and acetone extract treated group. Original magnification 1000× (Arrow marks in figures (a, b, e and f) shows normal nuclei and arrow mark in figures (c, d) shows infected nuclei).