Molecular Docking Study of Bio-Inhibitors Extracted From Marine Macro Alga Ulva Fasciata Against Hemolysin Protein of luminescence Disease Causing Vibrio Harveyi

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Abstract

Shrimp grow-out and hatchery systems are being affected by bacterial disease particularly *Vibrios*. The use of chemotherapeutic agents in aquaculture practices has to lead to the development of resistance among aquatic bacteria. Thus, health management becomes of major importance in aquaculture. Under this situation, progressing bio-inhibitors from marine resources are most appropriate to be considered against pathogenic bacteria. Molecular docking is an appropriate tool in structural biology and computer-assisted drug design to predict and neutralize a target protein of known diseases. In this study, marine macroalga *Ulva fasciata* was aimed at developing inhibitors against luminescence disease-causing pathogenic bacteria *Vibrio harveyi*. *U. fasciata* was collected from the intertidal zone of Thoothukudi, Tamil Nadu, India. Extract of *U. fasciata* was tested against growth and virulence factors of *V. harveyi* during *Penaeus monodon* larviculture. For molecular docking, the homology modeling of virulence of hemolysin protein of *V. harveyi* was designed and used for docking studies against the compounds of *U. fasciata* as identified by GC-MS analysis. Extract of *U. fasciata*@200 mg mL^{-1} had exhibited reductions on Cumulative Percentage of Mortality (32.40%) in postlarvae against the challenge of *V. harveyi* infection. In docking analysis, the bio-inhibitor Methyl dehydroabietate showed the highest binding affinity among compounds docked. Statistical analysis had revealed significant differences ($p<0.05$) in trials. Therefore, it was proved that the bio-inhibitors from *U. fasciata* will be a better option for controlling luminescence disease-causing *V. harveyi* in shrimp grow-out practices.

Introduction

The grow-out practices under the aquaculture sector are major sources in generating job opportunities and revenue to business sectors. The demand for seafood is being increased throughout the years. The shrimp grow-out practices have developed rapidly across the world due to the increased market demand (Harlioglu and Farhadi 2017). In shrimp grow-out system, disease problem that leads to severe economic losses worldwide. While considering bacterial diseases in shrimp culture *Vibrio* bacteria producing disease is known as Vibriosis a major contributor to other disease-causing agents (Krupesha-Sharma et al. 2016). Among other *Vibrio* bacteria, *V. harveyi* a bioluminescent bacterium, is a potent pathogen and resulting in major mortalities in grow-out and hatchery units. During its pathogenesis in host organisms, *V. harveyi* produces many extra-cellular virulence factors such as bioluminescence, proteases, phospholipases, lipases, siderophores, chitinases, hemolysins, etc (Soto-Rodriguez et al. 2012). Among other virulence factors, hemolysin is the most widely distributed exotoxin produced by the *V. harveyi* (Gavin et al. 2017). Further, hemolysins are capable to lyse erythrocytes and achieve two different modes of action, including cell pore-forming protein and phospholipase enzyme activity (Basso et al. 2017). Virulent isolates of *V. harveyi* were recognized for expressing *vhh* gene during infection of brine shrimp larvae whereas non-virulent isolates showed lesser expression of *vhh* gene (Ruwandeepika et al. 2012). *V. harveyi* virulent strain was found to contain two copies of the hemolysin gene (Zhang et al. 2001) which was infected salmon fish. Moreover, *V. harveyi* is associated with other luminescent *Vibrios* (Raissy et al. 2011) to spread diseases during the grow-out practices of shrimp *Penaeus monodon* and *Litopenaeus vannamei*. Most findings also suggested that larval and postlarval stages are more susceptible to bacterial infection than the juvenile and adult stages of shrimp (Rungrassamee et al. 2013). However, the mass mortalities resulting infections by *V. harveyi* were reported up to 80-100% in shrimp grow-out and larviculture system (Raissy et al. 2011).

Monitoring bacterial diseases in aquaculture practices, through the application of antimicrobial chemicals, has led to the emergence of more resistance among the bacterial pathogens (Chen et al. 2018). Under these circumstances, it is of vital importance to develop alternative agents which is sustainable and eco-friendly. While considering the modern drug designing industries, molecular docking has become a key tool in structural biology and computer-assisted drug design against the disease of our interest (de Ruyck et al. 2016). Generally, docking is done between a small molecule and a target macromolecule, which is often referred to as ligand-protein docking (Morris and Lim-Wilby 2008) aimed at predicting the predominant binding mode(s) of a ligand with a protein of known three-dimensional (3D) structure. Due to the lack of an experimentally established crystal structure of a given protein, homology modeling is the alternative to construct a reasonable (3D) model of the target (Bordoli et al. 2009). This model will be appropriate for applications like agonists or antagonists (Li and Wang 2007). Moreover, by generating binding energies in these docking studies, the position of the ligand in the enzyme binding site can be visualized (Wisastra et al. 2013).

Marine resources are an unmatchable reservoir of biologically active natural products, many of which exhibit structural features on par with the terrestrial organism (Saritha et al. 2013). Polysaccharides such as alginate, carrageenan produced by marine macroalgae were capable of improving the health status of marine fish (Peso-Echarri et al. 2012). *Ulva fasciata* is a more commonly available marine green macroalga (*Chlorophyceae*), which grows abundantly in both intertidal zone of the sea, and is recognized as an important source for antioxidant (Chakraborthy and Paulraj 2010). Also, the extracts from *U. reticulate* and *U. lactuca* were proved for antagonism against many human pathogens (Kolanjinathan and Stella 2011). The extract of *U. fasciata* shows inhibition against aquatic bacterial pathogens in laboratory trials (Priyadharshini et al. 2011). The efficacy of *U. fasciata* incorporated diet was also proved in controlling shrimp bacterial pathogens such as *V. fischeri, V. alginolyticus,* and *V. harveyi* during the infection on *P.monodon* postlarvae (Selvin et al. 2011). Several herbal plant diets were fed to *P. monodon* postlarvae and tested against *V. harveyi*, the results showed 75% improvement in survival (Velmurugan et al. 2010). Also, the reduced mortalities on *P. monodon* postlarvae tested against *V. harveyi* was observed during the oral administration of fucoidan compound isolated from brown algae *Undaria pinnatifida* through feed (Traifalgar et al. 2009). The extract from brown alga *Endarachne binghamiae* when administered to *L. vannamei* postlarvae (PL 10) through *Artemia* nauplii and observed enhanced survival against *V. alginolyticus* infection (Wong et al. 2008). When *V. harveyi* was tested against *P. monodon* postlarvae (PL 15), and fed with *Artemia* diet enriched by herbal products had found improved survival on postlarvae (Immanuel et al. 2007). Under this situation, it is crucial to know the pathogenic cum virulence mechanisms of *V. harveyi* and constitute different bio-inhibitors which would enable the beneficial approach through molecular docking analysis for controlling of *V. harveyi* in shrimp grow-out systems.

Materials And Methods

Isolation of *V. harveyi* strain

The *V. harveyi* strains were isolated from the infected *P. monodon* larviculture tanks. It was identified by using standard morphological and biochemical tests (Abraham and Palaniappan 2004). The strains were pre-enriched in alkaline peptone water (APW) and serially diluted with normal saline (0.85 % NaCl w/v), 0.1 ml of each sample was surface spread on Thiosulphate citrate bile salt sucrose agar medium (TCBS), Seawater complex agar (SWC) and *V. harveyi* selective agar medium (VHSA). In SWC agar, bio-luminous colonies were incubated at 30 °C/20 h and observed under darkroom. The isolates were compared with *V. harveyi* ATCC 25919 strain and then re-confirmed by PCR test (Maiti et al. 2009). *V. harveyi* bacteria isolates were named as (*Vh1* to *Vh20*).

Collection of marine macro alga and extraction of compounds

The marine green macro *U. fasciata* was collected from the intertidal zone of Thoothukudi coastal area (Latitude 8.7874°N; Longitude 78.1983°E) of Tamil Nadu, India. It was washed in freshwater and 10 mg L^{-1} (w/v) of KMnO$_4$ solution to remove epiphytes, sand, and other extraneous matters, later dried under shade condition. The algae were then weighed, pulverised by mechanical grinder and subjected to extraction using ethyl acetate solvent for obtaining compounds by cold extraction method (Das et al. 2005). The resultant extract was liquefied at 5 mg per mL of 30 % (v/v) DMSO (Dimethyl Sulfoxide).

Preparation of inhibitors data

The compounds were identified through Gas Chromatography and Mass Spectrometry (GC-MS) analysis from the extract of *U. fasciata* which was used as ligand molecules in the present docking studies. PUBCHEM (http://pubchem.ncbi.nlm.nih.gov) – PubChem is a 3D structural database, organized as three linked databases within the NCBI's Entrez information retrieval system. The 3D structure of compounds was downloaded from this database based on GC-MS results. These 3D structures were further converted to PDB (Protein Data Bank) format using PyMOL (0.99rc6) software (Saeed et.al. 2011). These PDB files were used for the docking study.

Homology modeling of hemolysin protein

The 3D structure of the hemolysin protein of *V. harveyi* (as of June 2014) was not available in the Protein Data Bank (PDB; http://www.pdb.org). The primary sequence of hemolysin protein of *V. harveyi* was obtained from NCBI through Protein sequence ID AAG25957.1. However, there was no suitable template/model available for hemolysin of *V. harveyi*. Therefore, the sequence was used to build the 3D model of protein structure using Phyre[2] (Protein Homology/AnalogY Recognition Engine, Version 2.0) (Rounak et al. 2014). Hence, the modeled structure was validated in the Phyre server for structural similarity analysis against the PDB database (Kelley and Sternberg 2009). Phi/Psi dihedral angle for the predicted model was validated using Ramachandran plot from PROCHECK (Laskowski et al. 1993).

Hemolysin protein preparation and active site prediction

The structure modification was performed in hemolysin protein for docking studies like the addition of hydrogen atoms, assigning correct bond orders, fixing of the charges, and orientation of groups. Following this, optimization of the amino acid orientation of hydroxyl groups, amide groups of ASN, GLN, and HIS was carried out. All amino acid flips were assigned and H-bonds were optimized. Non-hydrogen atoms were minimized until the average root mean square deviation reached the default value of 0.3 Å (Parasuraman et al. 2012). For docking studies, the active site/binding site of hemolysin protein was recognized by superimposing with esterase EstA protein from *Pseudomonas aeruginosa*. The active-site residues SER153, HIS393, and ASP390 were considered as the catalytic reaction mechanism of the hemolysin protein of *V. harveyi* (Van den Berg 2010).

Docking protocol

In the current study, the AutoDock 4.0 program was used to investigate the affinity of marine algal inhibitors at the binding pocket of hemolysin protein of *V. harveyi* through the implemented empirical free energy function and the Lamarckian Genetic Algorithm (LGA). During docking, an extended PDBQT format of PDB file was used for coordinate files, which include atomic partial charges. All the hydrogen atoms of the macromolecule (protein) were added using the AutoDockTools software (Version 1.5.2 revision 2) for preparation of the target protein hemolysin (unbound target), which is an essential step for the correct calculation of partial atomic charges. Kollman charges were calculated for each atom of the macromolecule in AutoDock 4.0. In docking, the grid dimensions were 60´60´60 Å with points separated by 0.375 Å, and the grid center was set to 1.778, 36.282, and 81. 809 for X, Y, and Z respectively, which covered all the active site amino acids include important 3 amino acid residues (SER153, HIS393, and ASP390) in the considered active pockets. LGA was employed as the docking algorithm with 10 runs, 150 population sizes, 2,500,000 maximum numbers of energy evaluations, and 27,000 maximum numbers of generations. The best-performing compounds were ascertained by computation of drug-likeness properties. The drug-likeness scores of the compounds were evaluated with the help of Lipinski's rule. During the docking process, a maximum of 10 conformers or poses were considered for each compound. The best interaction(s) pose was showed by protein and ligand molecule along with least binding energy exhibited (kcal mol^{-1}) that was considered as the highest inhibiting activity of the respective compound and compared to the standard drug Oxytetracycline dihydrate (OTC) (Hi-Media, India). The binding energy of the individual compound was decided based on the amount of free energy required, for the interaction(s) of ligand molecule with active site amino acid residues of target protein either by H-bond or hydrophobic interactions. Besides, other docking parameter results such as inhibition constant (mM) and intermolecular efficiency (kcal mol^{-1}) were also measured along with binding energy exhibited, since they are directly proportional to binding energy (Madeswaran et al. 2012; Tomi et al. 2016). PyMOL and UCSF Chimera (Version 1.8.1) software (Pathak et al. 2014) were used for graphical visualization, analyzing hydrogen bond interactions, and producing quality images. Hydrophobic interactions were observed between protein and ligand using Chimera software. All the docking calculations were performed in Intel(R) Core™ i3-2310M CPU@2.10GHz of 32-bit Operating system of Lenovo, with 2.00 GB RAM. AutoDock 4.0 was compiled and run under Microsoft Windows 7 operating system.

Larviculture trial of *U. fasciata* extract against *V. harveyi*

The experimental tubs were washed with 10 mg L^{-1} $KMNO_4$ solution (w/v) and filled with 20 L of saline water at 20 ppt (Parts per thousand). *P. monodon* disease-free postlarvae (PL 15) (Ananda Raja et al. 2017) (after checking OIE listed diseases) were procured from the commercial Shrimp Hatchery located at Chennai, India. These postlarvae were acclimatized at 20 ppt for 5 days at 29±1 °C with sufficient aeration. In each tub the average weight of postlarvae of 17 to 18 mg was stocked at 1000 numbers. The control tub was inoculated with *V. harveyi* (10 mL of 1.80 OD) and PL. In treatment tub the PL was inoculated with *V. harveyi* and 200 mg of extract of *U. fasciata* per mL of saline water (2g/10L). Another control as reference tub was added extract @200 mg mL^{-1} alone and PL. The 4th tub was an additional reference control for PL alone without *U. fasciata* extract and *V. harveyi*. The fifth tub was known as a reference control for extract, it was added with *V. harveyi* and OTC (200 mg mL^{-1}). The sufficient level of dissolved oxygen was maintained by aeration in each tub. The feed was provided to PL daily two times (morning and evening) @15 % body weight. The water quality parameters were examined daily such as temperature, salinity, and pH. The mortality of PL was noted every day. The water exchange was not given for all the tubs for 30 days experimental period, but the evaporated water was filled with sterile saline water. The sample water from the each experimental tub was collected once every 5 days using sterile water bottles. The total *V. harveyi* counts and heterotrophic bacteria were enumerated with VHSA and SWC medium correspondingly by the spread plate technique. The experimental tubs were provided with shelters on top to avoid the possibility of external contamination. Triplicate was performed for each experiment. The mean values were represented in standard deviation. The value of cumulative percentage of mortality (CPM) in *P. monodon* larviculture was noted at end of 30 days trial (Kannappan et al. 2013).

Effect of *U. fasciata* extract against growth and virulence factors of *V. harveyi* during *P. monodon* larviculture trial

The effect of *U. fasciata* extract on virulence factors produced by *V. harveyi* was observed during the experiment for every 5 days. The luminous *V. harveyi* colonies were examined from *U. fasciata* extract treatments by VHSA medium, which was later inoculated into LB broth and incubated at 28 °C/100 rpm/24 h. The spent culture of *V. harveyi* was taken and various virulence factors were produced such as hemolytic activity, phospholipase activity, extracellular crude protein (Bacteriocin), luciferase, and luminescence production, protease enzyme production, and growth of *V. harveyi* were measured. Also, Cell surface hydrophobicity was examined by Salt aggregations test (Soto-Rodriguez et al. 2012; MadhusudanaRao et al. 2013). The results of the agar plate assay were evaluated and graded based on the hydrolysis of the medium around the inoculated colonies. Thus, the activity was coded by qualitative parameters like non−existent (−), weak (+), moderate (++), high (+++), and very high (++++) (Soto-Rodriguez et al. 2003). Each test was performed in triplicates and the mean value was expressed with standard deviation. One factor analysis of variance was used for statistical evaluation using SPSS ver. 16.0 software to assess the significance in the present study.

Results

Identification and characterization *V. harveyi*

All the *Vibrio* bacteria isolates were known as *V. harveyi*, since they were Gram-negative rods, motile, oxidase-positive, fermented glucose and were sensitive to the vibriostatic agent O/129 and used D-mannitol as a sole source of carbon and made bioluminescence as observed in the darkroom. The isolates of *V. harveyi* obtained (negative for Voges−Proskauer, citrate positive, positive growth with 8% NaCl and negative with 0% NaCl) were stored in Luria−Bertani (LB) broth containing sterile glycerol (15 % v/v). Further, the PCR assay was performed for the expected fragment size of 235 bp of *vhh* gene indicating the presence of hemolysin among the isolates were tested. The *V. harveyi* bacteria isolates confirmed were named as (*Vh*1 to *Vh*20). The pathogenicity of *V. harveyi* was ascertained by spotting in 3% blood agar and the *V. harveyi* strain *Vh1* was used in the present study.

Effect of bio-inhibitors from *U. fasciata* extract against hemolysin protein of *V. harveyi*

The different structure/model of hemolysin protein of *V. harveyi* and its best interaction pose formed with OTC as depicted (Fig. 1). Based on the results observed from the molecular docking (Table1), the best leading three compounds were selected (Fig. 2a, b, and c) from the inhibitors of *U. fasciata*. Totally 36 compounds from *U. fasciata* were subjected to the docking analysis and found interactions only with 18 compounds by exhibiting H-bonding or hydrophobic interactions or both with 3 important active site residues (SER153, HIS393, and ASP390) including other residues of hemolysin protein of *V. harveyi*. The remaining 18 compounds did not show such interaction with active site residues of *V. harveyi*. Among the inhibitors docked from *U. fasciata*, the inhibitor Methyl dehydroabietate showed highest binding activity by exhibiting docking scores like lowest binding energy (-6.46 kcal mol^{-1}), inhibition constant (0.018 mM), and intermolecular efficiency (-0.28 kcal mol^{-1}) followed by inhibitor Bis(2-ethylhexyl) phthalate of binding energy (-5.98 kcal mol^{-1}), inhibition constant (0.041mM) and intermolecular efficiency (-0.21 kcal mol^{-1}), and inhibitor 1,2-Benzenedicarboxylic acid, butyl 2-ethylhexyl ester of binding energy (-5.77 kcal mol^{-1}), inhibition constant (0.058 mM) and intermolecular efficiency (-0.24 kcal mol^{-1}) respectively, when compared to standard drug OTC of binding energy (-5.66 kcal mol^{-1}), inhibition constant (0.071 mM) and intermolecular efficiency (-0.17 kcal mol^{-1}).

Evaluating of *U. fasciata* extract against *V. harveyi* in *P. monodon* larviculture

In *P. monodon* larviculture trial found decline of CPM at 32.40 % in PL as compared to the control (76.30 %) by *U. fasciata* extract treatment with *V. harveyi*. Other reference controls, the results on CPM in PL for extract was 29.56 %, with PL alone was 28.39 %, whereas, in OTC trial, it was 46.80 %. In PL average body weight, there were not much of difference observed among treatment and control and it had been recorded as follows of 269.3 mg and 266.5 mg for control and treatment respectively in end of the trial. The maximum decline on *V. harveyi* counts was observed on the 5th, 10th, 15th, and 20th days and the mean values for treatment were 2.38´10^4, 1.56´10^4, 4.30´10^3, and 3.40´10^3 CFU mL^{-1} as compared to control which is 3.40´10^5, 1.44´10^5, 1.45´$10^{5,}$ and 2.49´10^4 CFU mL^{-1} correspondingly. The recorded water quality parameters like temperature, salinity, and pH were in every sampling shown in Table 2 and it was not much of variations noted among treatment and control. Although, the light greenish color was observed in the treatment and extract alone tubs when compared to control due to the nature of extract color.

Effect of *U. fasciata* extracts against growth and virulence factors of *V. harveyi* during *P. monodon* larviculture

During the trial period, the growth of *V. harveyi* (OD) was decreased as compared to control for all the sampling days. Further, the virulence factors of *V. harveyi* had also shown decreases by *U. fasciata* extract for all the sampling days as compared to the control (Table 3).

Statistical analysis

The results of growth and virulence factors of *V. harveyi* and values of CPM in *P. monodon* larviculture among *U. fasciata* extract treatment and control showed significances at $p<0.05$ level by interpreting with statistical analysis.

Table1 Molecular docking parameters of bio-inhibitors from *U. fasciata* against hemolysin protein of *V. harveyi* in comparison with standard drug Oxytetracycline dihydrate

Compounds	Binding energy (kcal mol^{-1})	Intermolecular efficiency (kcal mol^{-1})	Inhibition constant (mM)	H-bond interaction	Inhibitor Atom	Amino acid residue	Distance of D….A (Å)	Hydrophobic interaction
Oxytetracycline dihydrate – Standard drug	-5.66	-0.17	0.071	N-H….O O-H….O	O O	GLN210 NE2 ASP 390 OD2	3.013 3.095	LEU176; SER164; GLN165; ALA162; ASN162; TRP166; ASP390; TRP389; VAL391; ARG167; GLN210; GLY204; SER153; HIS393
1,2-Benzenedicarboxylic acid, butyl	-4.31	-0.18	0.697	NH….O	O	GLN210 NE2	2.689	TRP166; TRP389; ASP390; VAL391; HIS393; ARG167; SER153; GLN210; ILE160
1,2-Benzenedicarboxylic acid,butyl 2-ethylhexyl ester	-5.77	-0.24	0.058	N-H….O	O	GLN210 NE2	2.971	GLN210; ASP156; ALA163; TRP166; SER153; GLY204; ASN248; HIS393; ARG167
1-Decene	-	-	-	-	-	-	-	No interaction
1-Octadecene	-3.95	-0.22	1.270	-	-	-	-	LEU176; TRP166; TRP389; ARG167; ASP390; HIS393; SER153; ILE160; GLN210
1-Tetradecene	-	-	-	-	-	-	-	No interaction
2-Undecanone, 6,10-dimethyl-	-	-	-	-	-	-	-	No interaction
5-Eicosene, (E)-	-4.38	-0.22	0.618	-	-	-	-	ALA163; TRP166; TRP389; ILE160; GLN165; GLN210; SER164; SER153; HIS393; ARG167
5-Tetradecene, (E)-	-	-	-	-	-	-	-	No interaction
8-Heptadecene	-4.29	-0.25	0.716	-	-	-	-	ARG167; TRP166; SER153; GLN210; ILE160
Anisole	-	-	-	-	-	-	-	No interaction
Behenic alcohol	-3.89	-0.17	1.41	N-H….O	O	HIS393 NE2	2.783	ALA163; TRP166; ILE160; GLN210; ASP152; GLY204; ASN248; LEU247; HIS393; LEU154; SER153
Benzo[h]quinoline, 2,4-dimethyl-	-	-	-	-	-	-	-	No interaction
Benzothiazole	-	-	-	-	-	-	-	No interaction
Bicyclo[3.1.1]heptanes, 2,6,6-trimethyl-, (1.alpha.,2.beta.,5.alpha.)	-	-	-	-	-	-	-	No interaction
Bis(2-ethylhexyl) phthalate	-5.98	-0.21	0.041	O-H….O N-H….O	O O	SER153 OG GLN210 NE2	3.232 2.959	ALA163; SER164; GLN165; TRP166; ILE160; GLN210; TRP389; SER364; SER365; ASP390; VAL391; HIS393; ARG167; SER153
Cetene	-	-	-	-	-	-	-	No interaction
Dibutyl phthalate	-4.43	-0.22	0.563	N-H….O	O	GLN210 NE2	2.789	ILE160; TRP166; GLN210; ARG167; TRP389; HIS393; SER153
Dodecane	-	-	-	-	-	-	-	No interaction
Dodecane, 1,1'-oxybis-	-2.91	-0.12	7.41	-	-	-	-	ALA163; TRP166; ILE160; GLN210; SER153; GLY204; ASN248; HIS393; ASP390
Hentriacontane	-3.48	-0.11	2.82	-	-	-	-	ALA163; ALA213; GLU208; TRP166; ILE160; GLN210; SER153; HIS393; ASP390; VAL391; GLN292; TYR368; SER364; SER365; TRP389
Heptadecane	-4.18	-0.25	0.867	-	-	-	-	LEU176; GLN165; SER164; ALA163; ASN162; TRP166; GLN210; ILE160; SER153; HIS393; ARG167; TRP389
Methyl dehydroabietate	-6.46	-0.28	0.018	N-H….O	O	ALA163 N	2.989	ALA163; SER164; TRP166; ILE160; GLN210; TRP389;

Compound								
Naphthalene, 6-chloro-1-nitro-	-	-	-	-	-	-	-	No interaction
Octacosanol	-3.58	-0.12	2.38	N-H....O	O	SER164 N	2.834	ALA163; GLN165; SER164; LEU176; GLN210; ALA213; GLU208; GLY204; ASN248; SER153; ARG167; HIS393; ASP390; TRP389
				O-H....O	O	GLN165 O	2.989	
Octadecane	-4.12	-0.23	0.961	-	-	-	-	ILE160; GLN210; SER153; ARG167; ASP390; TRP389; TRP166; ASN162; ALA163
Phenol 2,4-bis(1,1-dimethylethyl)	-	-	-	-	-	-	-	No interaction
Phenol, 2-(1-phenylethyl)-	-	-	-	-	-	-	-	No interaction
Phenol, 2,4-bis(1-phenylethyl)-	-	-	-	-	-	-	-	No interaction
Phthalic acid, 2-ethylhexyl pentyl ester	-5.68	-0.23	0.069	-	-	-	-	ALA163; TRP166; ILE160; GLN210; ASP156; SER153; ARG167; HIS393; ASP390; SER364; TRP389
Phthalic acid, butyl isohexyl ester	-5.23	-0.24	0.146	N-H....O	O	GLN210 NE2	2.969	ALA163; ASN162; ILE160; GLN210; SER153; TRP166; ARG167
Phytol, acetate	-4.61	-0.19	0.416	N-H....O	O	ASN248 ND2	2.926	ILE160; TRP166; ARG167; TRP389; HIS393; SER153; GLY204; GLU208; ASN248; ASN252; GLN210
Silanetriamine, 1-azido-N,N,N',N', N'',N''-hexamethyl-	-3.23	-0.25	4.27	-	-	-	-	TRP166; TRP389; ASP390; HIS393; SER153; ARG167; GLN210
Tetradecane	-	-	-	-	-	-	-	No interaction

O – Oxygen, N – Nitrogen, H – Hydrogen, D – Donor and A – Acceptor of H ion; SER – Serine, ALA – Alanine, ARG –Arginine,ASN– Asparagine, ASP – Aspartic acid, GLN – Glutamine, GLY – Glycine, HIS –Histidine, ILE – Isoleucine, LEU –Leucine, PHE – Phenylalanine, TRP – Tryptophan, VAL –Valine

Table 2 Evaluating of *U. fasciata* extract against *V. harveyi* in *P. monodon* postlarvae

Parameters	Days						
	0	5th	10th	15th	20th	25th	30th
Cumulative percentage of mortality (CPM)							
Control (PL and *V. harveyi*)	0	13.66 ± 0.3	26.05 ± 0.9	35.63 ± 1.1	47.33 ± 1.5	62.13 ± 2.3	76.30 ± 2.9
Treatment (PL, extract and *V. harveyi*)	0	6.96 ± 0.2	14.36 ± 0.3	21.33 ± 0.6	27.81 ± 1.1	36.63 ± 1.3	43.90 ± 1.3
Reference Control (PL and extract only)	0	2.39 ± 0.1	6.19 ± 0.2	12.05 ± 0.5	18.13 ± 0.6	24.69 ± 0.9	29.56 ± 1.0
Reference Control (PL only)	0	3.23 ± 0.1	6.03 ± 0.2	13.33 ± 0.5	17.43 ± 0.5	23.86 ± 1.0	28.39 ± 1.0
Reference Control (PL, OTC and *V. harveyi*)	0	7.91 ± 0.1	16.10 ± 0.3	23.66 ± 0.5	30.33 ± 1.33	39.11 ± 1.1	46.80 ± 1.1
Treatment (CFU mL-1)							
Total plate count	1.24×10^6	2.42×10^4	2.15×10^4	7.40×10^4	1.29×10^4	9.40×10^4	8.20×10^4
V. harveyi count	1.15×10^6	2.38×10^4	1.56×10^4	4.30×10^3	3.40×10^3	5.40×10^3	4.10×10^3
Control (CFU mL-1)							
Total plate count	1.18×10^6	3.18×10^5	2.94×10^5	1.51×10^5	2.66×10^4	1.80×10^4	1.74×10^4
V. harveyi count	2.41×10^6	3.40×10^5	1.44×10^5	1.45×10^5	2.49×10^4	1.74×10^4	1.51×10^4
Average body weight of PL (mg)							
Treatment	17.7 ± 3	60.9 ± 4	121.1 ± 4	156.3 ± 5	201.5 ± 9	236.9 ± 8	269.3 ± 9
Control	18.1 ± 2	63.6 ± 3	127.5 ± 5	157.5 ± 5	197.9 ± 7	240.1 ± 9	266.5 ± 8
Water quality parameters (Treatment and Control)							
Temperature (°C)	29.0 ± 1.0	29.5 ± 1.0	29.0 ± 1.0	30.0 ± 1.0	30.0± 1.0	31.0± 1.0	30.0 ± 1.0
Salinity (ppt)	20 ± 0.5	20 ± 0.5	20 ± 0.5	20 ± 0.5	21 ± 0.5	21 ± 0.5	21 ± 0.5
pH - Control	8.40 ± 0.2	8.50 ± 0.2	8.20 ± 0.2	8.40 ± 0.2	8.10 ± 0.2	8.40 ± 0.2	8.10 ± 0.2
pH - Treatment	8.30 ± 0.2	8.40 ± 0.2	8.30 ± 0.2	8.50 ± 0.2	8.30 ± 0.2	8.20 ± 0.2	8.00 ± 0.2

Values of average of three determinations with standard deviation

Table 3 Testing of *U. fasciata* extract against the growth and virulence factors produced by *V. harveyi* during *P. monodon* larviculture

Day	Growth and Virulence factors production													
	Growth (OD 600nm)		Phospholipase activity		Hemolytic activity		Cell surface hydrophobicity SAT (M)		Luminescence production (CPS)		Luciferase production (CPS)		Crude bacteriocin (OD 660nm)	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated
5th	1.033 ± 0.03	0.778 ± 0.02	++++	−	++++	−	0.75 ± 0.03	1.25 ± 0.04	136 ± 3.31	121 ± 4.33	137 ± 4.16	106 ± 2.93	2.119 ± 0.09	2.022 ± 0.07
10th	1.482 ± 0.05	1.313 ± 0.04	++++	−	++++	−	0.50 ± 0.02	1.50 ± 0.05	110 ± 4.01	099 ± 3.74	123 ± 4.56	117 ± 1.96	1.985 ± 0.06	1.929 ± 0.05
15th	1.647 ± 0.05	1.393 ± 0.01	++++	−	++++	−	0.50 ± 0.01	1.75 ± 0.06	118 ± 3.56	105 ± 4.91	130 ± 5.63	119 ± 3.71	2.161 ± 0.05	1.618 ± 0.07
20th	1.357 ± 0.04	1.235 ± 0.03	++++	−	++++	−	0.75 ± 0.03	1.75 ± 0.04	150 ± 5.06	116 ± 1.93	148 ± 6.33	116 ± 4.61	1.950 ± 0.01	1.736 ± 0.05
25th	0.771 ± 0.01	0.615 ± 0.03	++++	−	++++	−	0.50 ± 0.02	2.00 ± 0.09	154 ± 1.91	125 ± 3.33	135 ± 3.69	115 ± 5.05	1.956 ± 0.07	1.493 ± 0.05
30th	1.152 ± 0.05	1.056 ± 0.04	++++	−	++++	−	0.50 ± 0.01	1.75 ± 0.04	128 ± 2.63	096 ± 1.96	129 ± 2.93	103 ± 1.11	2.373 ± 0.11	2.188 ± 0.09

Phospholipase and Hemolytic activity (− = non-existent; + = weak; ++ = moderate; +++ = high; ++++ = very high); SAT test (0.0 to 1.0 Molarity (M) = strongly hydrophobic, 1.0 to 2.0 M = moderately hydrophobic; 2.0 to 4.0 M = weakly hydrophobic, and >4.0 M = not hydrophobic)

Discussion

Molecular docking tools have played an essential role in the development of therapeutically vital small molecules, which follow either structure-based or ligand-based methods (Sliwoski et al. 2014). Due to the lack of an experimentally established crystal structure of a given protein, homology modeling has been applied to various areas of drug discovery including structure related aspects of target validation such as site-directed mutagenesis and drug ability assessment (Schmidt et al. 2014). Among the virulence factors of *Vibrios*, including *V. harveyi,* hemolysin can lyse RBC and other cells by producing pores on the cytoplasmic membrane (Wang et al. 2015).

During the docking investigations, the ligand molecules/inhibitors from *U. fasciata* abiding passed Lipinski's rule of five, which showed its drug-likeness and possibility of its considerations for further pre-clinical studies. Lead optimization of the inhibitors from *U. fasciata* in the study was recognized by computation of drug-likeness properties and it was agreed about the report of Madeswaran et al. (2012) about the lead optimization of inhibitors by drug-likeness properties through computation. All the best-docked poses generated by each docking exhibited well-established bonds with one or more amino acids of SER153, HIS393, and ASP390 in the binding pocket of hemolysin protein. The top-ranked pose with the lowest docked binding affinities and high docking scores is generally used as a standard selection in most of the docking programs (Azam et al. 2013). Likely, a lower docking score was showed a higher binding affinity known as inhibiting biological activity (Oda and Takahashi 2009).

The preliminary screening helps to compare the docking score of standard drugs against ligands. Then the pre-screened ligands were validated using AutoDock version 4.0 which is more efficient (Deepthy et al. 2014). OTC was used as a reference drug in this study and compared with inhibitors from *U. fasciata*. As a general rule, it was considered that in most of the potent inhibiting mechanism of compounds, both H-bond and hydrophobic interactions between the ligands/inhibitors and the active sites of the receptor are responsible for the biological activity (Madeswaran et al. 2011). Here, the compounds of *U. fasciata* have revealed their interactions through both H-bond and hydrophobic or either H-bond or hydrophobic on the active site residues (SER153, HIS393, ASP390) of hemolysin protein. Docking analysis was done based on the selectivity by Carbohydrate Recognition Domain (CRD) of the human asialoglycoprotein receptor (ASGP-R) with monomer sugar molecules such as D-mannuronic acid, and L-guluronic acid deriving from alginates (Brown algae *Laminaria hyperborea, Ascophyllum nodosum*, and *Macrocystis pyrifera*) and L-rhamnos 3-sulfate, D-glucuronic acid, L-iduronic acid, D-xylose, and D-xylose 2-sulfate from *Ulvans* (*Ulva* sp. and *Enteromorpha* sp.). The molecule interactions are linked by H-bonds and hydrophobic interactions with ASGP-R and determined by docking scores (Massarelli et al. 2007).

Small molecules that are linked by H-bonds and hydrophobic interactions in the target protein pocket may have the ability to interrupt the conformational changes that trigger the fusion process. A flavonoid found in *Carica papaya*, apple, and even in lemon possessed anti-dengue activity and was found that it could interrupt the fusion process of the dengue virus by inhibiting the hinge region movement and by blocking the conformational rearrangement in envelope protein (Mir et al. 2016). Hence, it is suggested in most of the research findings that analyzing the lowest binding energy (highly stable) of the ligand-binding complex demonstrated by using AutoDock tools will show the effective nature of inhibition of these receptors by the unique ligands (Sliwoski et al. 2014).

During the larviculture, the *U. fasciata* extracts showed a better level of reductions in CPM of *P. monodon* postlarvae. The survival of shrimp *P. monodon* juveniles treated with *U. fasciata* extract showed a significant level ($p<0.05$). Hence, in the present finding significant difference ($p<0.05$) has been supported by Selvin et al. (2011). Besides, with significant antibacterial properties of *U. fasciata* and *U. reticulate* against human pathogenic bacteria (Chellaram et al. 2015) and *U. fasciata* against *V. parahaemolyticus* have been described (Chakraborty and Paulraj 2010). Marine algae are reported for a wide range of compounds such as PUFA, carotenoids, phycobiliproteins, polysaccharides, and phycotoxins, etc (Chu 2012) because lipids inhibit microbes by a disturbing cellular membrane (Bergsson et al. 2011) of bacteria, fungi, and yeasts. These fatty acids could affect the expression of bacterial virulence factors, which are essential for the establishment of infection. Therefore, in this study, the counting of *V. harveyi* has been reduced in growth and virulence factors against *U. fasciata* extract. Davies and Marques (2009) have shown that saturated and unsaturated fatty acids can prevent initial bacterial adhesion, subsequent biofilm formation, and later induce lysis of bacterial protoplasts.

The antioxidant and anti-hemolytic activity of many *Ulva* species were found with momentous results (Farasat et al. 2014). Recently anti-hemolytic activity exhibited by coriander leaves (*Coriandrum sativum* L.) and filamentous alga *Enteromorpha intestinalis* were reported against H_2O_2 induced hemolytic activity (Soltani et al. 2012). Hereafter, it was evident that the anti-hemolytic activity of coriander and filamentous green alga was caused due to the compounds quenched H_2O_2 before it attacked the biomolecules of erythrocyte membrane to cause oxidative hemolysis on the effects of green tea as reported by Costa et al. (2009). Further, diterpene isolated from marine brown alga *Canistrocarpus cervicornis* caused its anti-hemolytic activity, as well as fibrinogen or plasma clotting induced by *Lachesis muta* snake venom protein, was reported (Moura et al. 2011). The inhibiting activity on snake venom protein by brown alga was compared with the antagonism of *U. fasciata* inhibitors against hemolysin protein. Moreover, in the *P. monodon* larviculture trials, an extract of *U. fasciata* exhibited a lowest (non-existent) level of inhibition or virulence of hemolysis activity of *V. harveyi* on sheep blood agar than control including reductions of growth and other virulence factors.

Conclusions

The results indicated that the inhibitors exhibited better docking scores and showed higher binding affinity, followed no violation of the rule by the ligands used in docking for determining drug pharmacological activity. Also found, anti-hemolytic activity, reductions in the growth and virulence factors of *V. harveyi* by *U. fasciata* extract during the experiment. In the larviculture trial, *U. fasciata* showed improved survival level on *P. monodon* postlarvae than control. Hence, the present findings indicated that the marine green alga *U. fasciata* may be a remarkable source for developing potent bio-inhibitors in shrimp disease management.

Declarations

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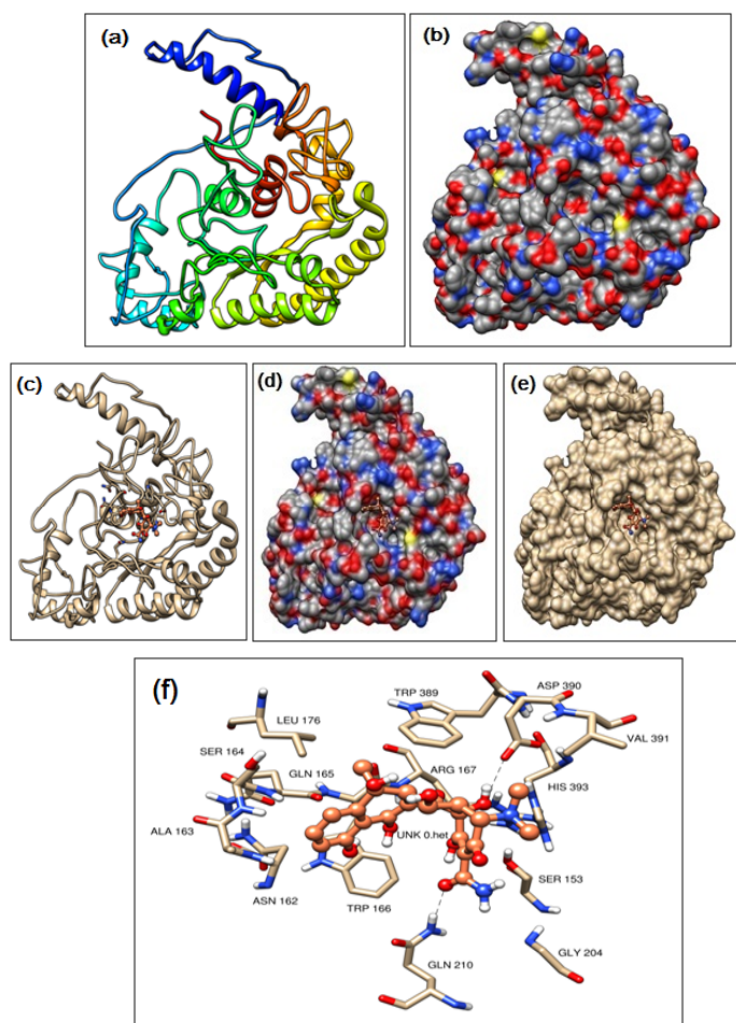
Figures

Figure 1

The tertiary structure (a) and its surface model (b) of hemolysin protein. The best docked pose formed by OTC showed in (c) tertiary structure, (d) surface model with atoms color, (e) without atoms color, and (f) close view of docked with hemolysin protein
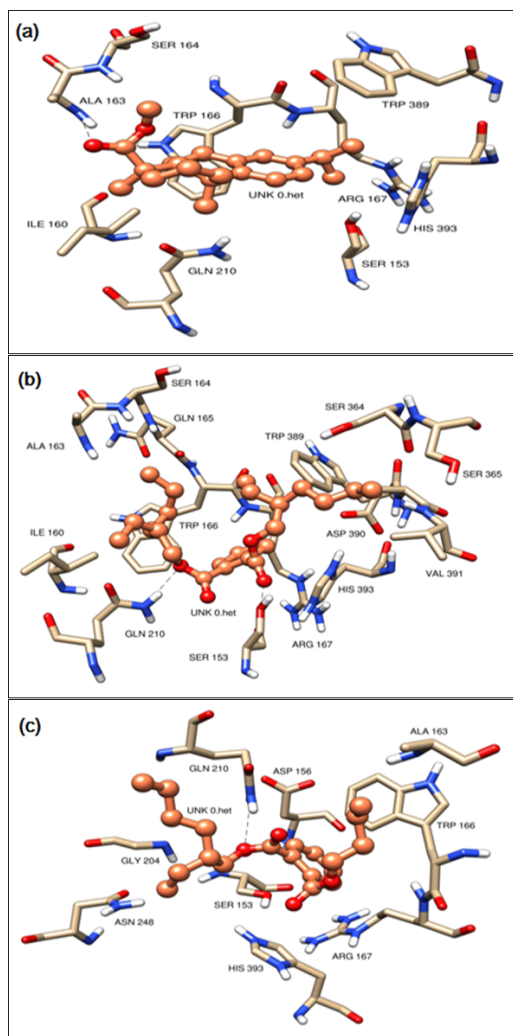
Figure 2

The best-docked pose of compounds from U. fasciata (a) Methyl dehydroabietate, (b) Bis(2-ethylhexyl) phthalate, (c) 1,2-Benzenedicarboxylic acid, butyl 2-ethylhexylester with hemolysin protein