# Molecular docking approaches of biomolecules extracted from red seaweed Kappaphycus alvarezii against hemolysin protein of bioluminescence disease-causing bacteria Vibrio harveyi

**Krishnamoorthy Sivakumar** ( ksivafbt@gmail.com )
ICAR–Krishi Vigyan Kendra, Tamil Nadu Veterinary and Animal Sciences University (TANUVAS)
**Sudalayandi Kannappan**
ICAR–Central Institute of Brackishwater Aquaculture (CIBA)
**Balakrishnan Vijayakumar**
University of Madras

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## Abstract

Molecular docking is suited a major approaches in structural biology and computer-assisted drug design against the protein of disease-causative agents. Marine resources are an unmatched reservoir of several natural biological products, which exhibit structural features that have not been found in a terrestrial organism. Herein, it was performed that molecular docking with various compounds/ ligand molecules identified by Gas Chromatography and Mass Spectrometry (GC-MS) analysis from the marine red seaweed *Kappaphycus alvarezii* extract against the hemolysin protein of bacterial disease-causing shrimp pathogen, *Vibrio harveyi*. Further, extracts of *K. alvarezii* were evaluated against *V. harveyi* infection during *Penaeus monodon* larviculture. This study correlates with various virulence factors including the hemolysin protein of *V. harveyi*. Among the compounds docked, an inhibitory effect was observed based on docking scores and found the highest binding affinity/inhibiting activity in Cyclotetracosane. Extract of *K. alvarezii* at 200 µg $mL^{-1}$ showed reductions in the *V. harveyi* counts that leads to a 29.70% reduction in the cumulative percentage mortality (CPM) caused by postlarvae and during larviculture. Using statistical analysis, significant differences ($p < 0.05$) were observed in the growth and virulence factors of *V. harveyi* during shrimp larviculture trials. While considering these findings, it was determined that *K. alvarezii* extract can be replaced as an alternative bio-agents by protecting against *V. harveyi* infections and possibly other aquatic pathogenic bacteria in shrimp farming systems.

## Introduction

In shrimp grow-out practices and hatchery systems, marine bacterial pathogenic *Vibrios* causes diseases [1], among them, *Vibrio harveyi* is one of the foremost disease-causing agents, causing severe mortality in shrimp hatcheries and grow-out systems [2]. *V. harveyi* is a Gram-negative, bioluminescent bacterium, that produces many virulence factors such as bioluminescence, proteases, exopolysaccharides, cell surface, hydrophobicity, bacteriocins, phospholipases, lipases, siderophores, chitinases and hemolysins [3]. Hemolysin is an important exotoxin that lyses erythrocytes through two different modes of action [4], including cell pore-forming protein (Fig. 1) [5, 6] and phospholipase enzyme activity [7]. Virulent isolates of *V. harveyi* showed enterotoxin activities and also evidenced infection with brine shrimp (*Artemia*) larvae [8]. The pathogenic *V. harveyi* is associated with bioluminescence disease in shrimp grow-out practices [2]. *V. harveyi* was also reported to cause mortality in shrimp larval stages up to 80–100% [9]. As remedial measures against bioluminescence disease, the utilization of antimicrobial agents will be effective as synthetic chemicals, showing more resistance among bacterial pathogens [10]. For these issues, it is essential to explore an alternative product that should be biodegradable and eco-friendly to the aquaculture environment. Therefore, marine organisms represent a varied reserve of bio-actives which could help in the management of a wide range of aquaculture diseases [11]. Under this situation, it may be solved by obtaining alternative biological compounds from marine seaweeds, which could be biodegradable and eco-friendly. A recent expansion of drug development research has spotted the development of new antimicrobial products against disease-causing agents.

The effects of many herbal extracts on growth, survival rate and immunoprotection against pathogenic *V. harveyi* in the tiger shrimp, *Penaeus monodon* were investigated [12]. The shrimps fed on diets for 60 days with the extraction of 2.5 mL $kg^{-1}$ showed significantly ($p < 0.001$) higher survival rate (76%) specific growth rate (4.26 ± 0.11%) and better food conversion ratio (1.5) than the other groups. Extracts of the red seaweed *Gracilaria fisheri* and furanone eradicate *V. harveyi* and *V.parahaemolyticus* biofilms and ameliorate the bacterial infection in shrimp have been evaluated [13]. The crude garlic extract, when fed through feed with the larviculture system of *P. monodon* for 30 days trial, a significant reduction in the growth, and mortality of postlarvae against *V. harveyi* infections, and also reductions in the virulence factors [9]. When the crude extract of marine macro alga *Ulva fasciata*, challenged against luminescence disease causing *V. harveyi* and its virulence factors during *P. monodon* larviculture showed the inhibition and reduced mortality of postlarvae [14]. Preliminary studies were conducted with seaweed, *Padina australis* Hauck's antibacterial activity and phytochemical test against pathogenic shrimp bacteria such as *V. harveyi*, *V. parahaemolyticus* and *Aeromonas hydrophilla* and showed enhanced results in ethyl acetate-based solvent extract [15]. Further marine red seaweed, *Tricleocarpa fragilis* also revealed the control of *V. harveyi* by using ethyl acetate-based solvent fraction and also studied biomineral characterization [16]. *In-vitro* evaluation of the antibacterial and antioxidant activities of extracts of marine macro alga *Gracilaria gracilis* (especially ethyl acetate solvent extraction) with a view into its potential use as an additive in fish feed was examined and found with antibacterial and antioxidant effects safely and effectively [17]. In the crude compounds available in the algae and ethyl acetate extracts/fractions, which of the compounds inhibits the growth of microbes requires to be assessed [18, 19]. A wide range of compounds, policies and risk measurement methodologies including animal studies are covered, to identify mixtures of concern, gaps in the regulatory structure, and data requirements are to be conceded out. Besides, the current and potential future use of new tools (*in-silico* tools, adverse outcome pathways, toxicokinetic modelling, etc.) in the risk assessment of pooled effects were studied [20]. Under this situation, molecular docking (*in-silico* analysis) is one of the best tools and is cost-effective to identify the effect of the individual molecule (ligand) performing with target diseases without scarifying the animals and before isolating or bulk production, etc [21].

Currently, molecular docking is involving a vital role in *in-silico* drug development. The advantage of the tools is that they require less investment in resources and time in comparison to wet lab studies [21]. In modern drug designing, molecular docking is the main tool in structural biology and computer-assisted drug design, which is generally made between a small molecule and a target macromolecule and is often referred to as ligand-protein docking [22, 23]. Ligand-protein docking is mostly intended to predict the predominant binding mode(s) of a ligand with a protein of known three-dimensional (3D) complex structure. Homology modelling is the best alternative to construct a reasonable (3D) model of the target, owing to a lack of an experimentally recognized crystal structure of a given protein [24]. The models established will be suitable for applications like agonists or antagonists [24]. Moreover, generating binding energies in these docking studies, the position of the ligand in the protein/enzyme binding site can be visualized and the drug-likeness of compounds can be evaluated with the help of Lipinski's rule of five [25].

Marine seaweeds are a source of biologically active natural products, which can be utilized in pharmaceuticals, food industries, and cosmetics [11]. The red seaweed *Kappaphycus alvarezii* is one of the larger edible seaweed which is highly demanded its polysaccharides and found the antibacterial activities of seaweeds like *K. alvarezii* and *Kappaphycus striatum* against many infectious pathogens [26]. Similarly, the extracts of *K. alvarezii* and *Ulva lactuca* showed antagonism against various human pathogenic bacteria [27]. Selvin et al. [28] reported the efficacy of seaweed *U. fasciata* to extract on the management of shrimp *P. monodon* juveniles against bacterial (*Vibrio fischeri, V. alginolyticus, V. harveyi* and *Aeromonas* sp.) infections. The extract of marine microalgae

*Skeletonema costatum* was evaluated against the *V. harveyi* infection during shrimp *P. monodon* larviculture and found improved survival on postlarvae (PL), reductions in growth and virulence factors of *V. harveyi* [29]. Henceforth, it is significant to study the pathogenic cum virulence mechanisms of *V. harveyi* and determine different bio-inhibitors to facilitate the favourable approach through molecular docking analysis for controlling *V. harveyi* in the shrimp grow-out and hatchery systems.

## Materials And Methods

### V. haveryi bacteria isolation

*V. harveyi* bacterial strains were isolated from the infected *P. monodon* larvae (Indian Council of Agricultural Research (ICAR) - CIBA, Muttukadu Experimental Station, Chennai, India). The water samples collected from *P. monodon* larviculture tanks were enriched with alkaline peptone broth (pH – 8.4 ± 0.2) (Hi-Media, India) and shaker incubated at 28°C for 24 h. The inoculums were serially diluted with normal saline (0.85%) and surface spread on Thiosulphate citrate bile-salt sucrose agar medium (TCBS) (Hi-Media, India) and seawater complex (SWC) agar (Hi-Media, India). Luminescent colonies were observed after 24 h of incubation in SWC and TCBS agar. After the 16–24 h, only luminous colonies were selected and streaked in Tryptone Soya Agar (TSA) (Hi-Media, India) slants supplemented with 2% NaCl (Hi-Media, India). The agar plates were also incubated as detailed above. Further, they were confirmed using phenotypic and genotypic characterization. *V. harveyi* isolates (n = 50) were characterized by [30, 31] using standard morphological, physiological, and biochemical tests. The isolates were streaked on TSA plates and incubated at 28°C for 24 h. The colonies were initially characterized by Gram staining, oxidase, catalase and oxidation/fermentation tests. *V. harveyi* isolates were further subjected to various tests such as motility, methyl red (MR)/ Voges-Proskauer (VP), salt and temperature tolerance, indole, O/129 sensitivity, citrate, urease, nitrate reduction, amino acid decarboxylase, pigment production, bioluminescence, amylase production, gelatin hydrolysis, lipase hydrolysis, hemolysis and sugar fermentation for phenotypic characterization. References strains of *V. alginolyticus* (MTCC 4182) and *V. harveyi* (MTCC 3438) were obtained from MTCC and used for this study as negative and positive control respectively. The confirmed isolates were maintained in Luria-Bertani (LB) broth (Hi-Media, India) supplemented with 2% NaCl, and 15% glycerol (SRL, India) and stored at – 80°C. *V. harveyi* isolates were reconfirmed by detecting the presence of the hemolysin (*vhh*) by PCR method as genotypic characterization [32]. The primers used in PCR were as follows: forward primer: 5'-CTTCACGCTTGATGGCTACTG-3', and reverse primer: 5'-GTCACCCAATGCTACGACCT-3' and the final product of 235 bp was obtained from the *vhh* gene DNA sequences. 25 µl of the reaction mixture was carried out for each isolates in PCR and it contains 2.5 µl of 10X PCR buffer (100 mM Tris-HCl pH 9.0, 15 mM $MgCl_2$, 500 mM KCl, 0.1% (w/v) gelatin), 200 µM of concentrations of each dNTPs, 10 picomoles of each primer, 1.5 U of Taq polymerase, the 2 µl of sample DNA and 1.25 µl of Dimethyl sulphoxide (DMSO) (Hi-Media, India). The Thermocycler (Applied Biosystems, USA) was used for the PCR with 30 reaction cycles of initial denaturation at 95°C/5 min; 95°C/1 min, 50°C/1 min, 72°C/1 min and a final extension at 72°C/5 min. The PCR products were separated on a 1% agarose gel and stained with ethidium bromide (0.5 µg $mL^{-1}$) and photographed using a gel documentation system (Bio-Rad, USA). The reference strains of *V. alginolyticus* (MTCC 4182) and *V. harveyi* (MTCC3438) were used as a negative and positive control, respectively. The pathogenicity of *V. harveyi* isolates was re-confirmed [33] by spotting cells in 3% blood agar (Hi-Media, India) and the zone formation as hemolysis around the cell spot was confirmed as the pathogen.

## Seaweed Collection And Crude Extract Preparation

The red seaweed *K. alvarezii* was collected in the intertidal zone of the Mandapam region (Latitude 9.2886°N; Longitude 79.1329°E), Ramanathapuram District, Tamil Nadu, India. The seaweeds were dried under shade, pulverized and used for extraction. Seaweed powder and ethyl acetate (SRL, India) (1:10 ratio) were made for the extraction of compounds from *K. alvarezii* and shaker incubated at 30°C at 50 rpm/96 hrs and extracted for compounds called as "cold extraction method" [34]. Subsequently, the extracts were filtered by Whatman filter paper (No.1), then rotary evaporated at 30°C/3 hrs and later stored in darkness at 4°C. The resultant extract was liquefied with 5 mg/ml of 0 % (v/v) DMSO.

## Inhibitors Preparation For Molecular Docking

The resultant crude ethyl acetate extract of *K. alvarezii*, identified through Gas Chromatography and Mass Spectrometry (GC–MS) analysis for phytochemical constituents was used as ligand molecules in docking. GC-MS analysis was performed by using Agilent GC-MS-5975C (Agilent 5975C TAD Series GC/MSD System, USA) with the triple-axis detector equipped with an autosampler. The GC column used was a fused silica capillary column (length 30 m x diameter 0.25 mm x film thickness 0.25 µm) used with helium at 1.51 ml/ for 1 min as a carrier gas. The mass spectrometer was operated in the electron impact (EI) mode at 70 eV in the scan range of 40-700m/z. The split ratio was adjusted to 1:10 and the injection volume was 1 µl. The injector temperature was 250°C; the oven temperature was 70°C/3 min, which rose to 250°C @14°C $min^{-1}$ (total run time, 34 min). The temperature of the transfer line and the ion source was set to a value of 230°C and the interface temperature at 240°C, respectively. Full mass data was recorded from 50–400 Dalton per second and the scan speed was 2000. Mass start time was at 5 min and end time at 35 min. Peak identification of crude *S. costatum* extract was performed by comparison with retention times of standards/homologous series and also the mass spectra obtained were compared with those available in the NIST libraries by an acceptance criterion of a match above a critical factor of 80% and also run with a homologous series to determine retention indices of compounds [35]. PUBCHEM (http://pubchem.ncbi.nlm.nih.gov) is a 3D structural database, organized as three linked databases within the NCBI's Entrez information retrieval system. The 3D GC-MS compounds (inhibitors) were downloaded from this database. These 3D structures were converted to PDB (Protein Data Bank) format using PyMOL (0.99rc6) software [36] and these PDB files were used for docking.

### Homology modelling of V. harveyi hemolysin protein

The 3D structure of the hemolysin protein of *V. harveyi* was not available (as of June 2021) in the Protein Data Bank (PDB; http:www.pdb.org). The primary sequence of the hemolysin protein of *V. harveyi* was obtained from the Protein sequence ID AAG25957.1 from NCBI. However, there was no suitable template

available for the hemolysin of *V. harveyi*. Therefore, the sequence was used to build the 3D model of protein structure using Phyre2 (Protein Homology/AnalogY Recognition Engine, Version 2.0) [37]. Hence, the PDB id of the 3KVN_A (1-311) template has been selected and the modelled structure was validated in the Phyre server for structural similarity analysis against the PDB database [38]. Phi/Psi dihedral angle for the predicted model was validated using the Ramachandran plot from PROCHECK [39]. The modelled structure has 5.29% of amino acid residues in outliers of the Ramachandran plot.

## Hemolysin Protein Preparation And Actives Site Prediction

The structure modification was done in hemolysin protein for docking studies like the addition of hydrogen atoms, assigning correct bond orders, fixing the charges, and orientation of groups. Following this, optimization of the amino acid orientation of hydroxyl groups, and 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 a default value of 0.3 Å [40]. For docking, the active site (binding site) of hemolysin protein was determined 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* [41].

## Docking Protocol

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). While docking and extending the PDBQT format of the PDB file was used as a coordinate file, which includes atomic partial charges. All the hydrogen atoms of the macromolecule (protein) were added using the AutoDock Tools software (Version 1.5.2 revision 2) for the preparation of the target protein hemolysin (unbound target), which is a necessary step in calculating partial atomic charges. Kollman charges were calculated for each atom of a macromolecule by using Auto Dock 4.0. During the docking, the grid dimensions were 60 × 60 × 60 Å with points separated by 0.375 Å, and the grid centre was set to 1.778, 36.282, and 81.809 for X, Y, and Z respectively, which covered all the active sites 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 the computation of drug-likeness properties. The drug-likeness scores of the compounds were evaluated with the rules of Lipinski's rule. During the docking process, a maximum of 10 conformers or poses were considered for each compound. In the present study, the best interaction(s) pose was shown by protein and ligand molecule along with the least binding energy exhibited (kcal/mol) that was considered as the highest inhibiting activity of the respective compound and compared to the control molecule Oxytetracyline-dihydrate (OTC) (Hi-Media, India). The binding energy of the individual compound was decided based on the amount of free energy required concerning 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 parameters results such as inhibition constant (mM) and intramolecular efficiency (kcal/mol) were also considered along with binding energy exhibited, since they are directly proportional to binding energy [23]. PyMOL and UCSF Chimera (Version 1.8.1) software [42] 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 with Intel® Core™ i3-2310M CPU@2.10GHz of a 32-bit operating system of Lenovo, with 2.00 GB RAM. AutoDock 4.0 was compiled and run under Microsoft Windows 7 operating system.

## Prediction Of Drug-likeness Of The Biomolecules

An online software SwissADME web tool (http://www.swissadme.ch/) was followed to evaluate the information about drug-likeness properties of bio-inhibitors with the help of the Lipinski rule of five [25]. This rule helps in distinguishing between drug-like and non-drug-like molecules. It predicts a high probability of success or failure due to drug-likeness for molecules complying with two or more of the following rules; (i) Molecular mass less than 500 Dalton, (ii) High lipophilicity (expressed as LogP less than 5), (iii) Less than 5 hydrogen bond donors, (iv) Less than 10 hydrogen bond acceptors, and (v) Molar refractivity should be between 40–130.

**Evaluating of** K. alvarezii **extract against** V. harveyi **during** P. monodon **larviculture**

The plastic tubs were washed with 10 mg $L^{-1}$ of (w/v) potassium permanganate ($KMnO_4$ - Hi-Media, India). solution for 10 min and filled with 20 L of saline water (20 Practical Salinity Units, PSU). Disease-free postlarvae (PL 10 days old) of *P. monodon* procured from a private shrimp hatchery located nearby Chennai, Tamil Nadu India. The postlarvae were acclimatized at 20 (PSU) for 5 days at 29 ± 1ºC with continuous aeration. One hundred numbers of postlarvae (Average body weight 17–18 mg) were stocked in each tub. The control tub was inoculated with *V. harveyi* (10 ml of 1.80 OD) alone. The treatment tub was inoculated with *V. harveyi* and 200 µg of crude *K. alvarezii* extract per ml (2 g/10 L). The third tub was considered as another control where *K. alvarezii* extract alone was added at 200 µg $mL^{-1}$ with PL. The 4th tub was additional control for PL with neither *V. harveyi* nor OTC (at 200 µg $mL^{-1}$) was also added. The aeration was given in each tub and PL feed at 15% of body weight was given twice. The water quality parameters such as temperature, salinity, and pH were mentioned for 5 days. The mortality of PL was counted every day and represented as cumulative percentage mortality (CPM). No water exchange was given for all the tubs until 30 days. The water samples were collected from the tanks once in 5 days. No water exchange was given for all the tubs until 30 days, but sterile seawater was added to compensate for the evaporated water. The water samples were collected from the tanks once in 5 days. The total heterotrophic bacteria and luminous *V. harveyi* counts were enumerated using TSA and *Vibrio harveyi* selective agar (VHSA) medium [35]. All the experimental tubs were covered on the top to avoid the possibility of any external contaminations. For each experiment, the triplicate was maintained and average values were presented with SD [43].

### Effects of K. alvarezii extract against growth and virulence factors of V. harveyi during P. monodon larviculture

The effects of *K. alvarezii* extract against virulence factors produced by *V. harveyi* were evaluated with shrimp postlarvae every 5 days. The bioluminescent *V. harveyi* was treated against *K. alvarezii* extract. Then the *V. harveyi* was identified by VHSA medium, later inoculated into LB broth and incubated at 28°C/100 rpm/24 hrs. After, 24 hrs, the spent culture of *V. harveyi* was analyzed for virulence factors such as hemolytic and phospholipase activities, extra crude protein (Bacteriocin), luciferase, and bioluminescence, protease enzyme production and growth. Cell surface hydrophobicity was observed by the Salt aggregations test [3]. The results of the agar plate assay were evaluated and graded based on the hydrolysis of the medium around the inoculated colonies. The activity was coded by qualitative parameters like non-existent (–), weak (+), moderate (++), high (+++), and very high (++++) [33].

## Statistical analysis

Each test was performed in triplicates and the mean value was expressed in standard deviation (SD). For statistical evaluation, the one-factor analysis was followed using SPSS ver.16.0 software and assessed the significance (*p*-value) among the treatment and control.

## Results

### V. harveyi of isolation and characterization

All the isolates of *Vibrio* were identified and confirmed as *V. harveyi*, since they were Gram-negative rods, motile, oxidase-positive, fermented glucose, and utilized D-mannitol as the sole source of carbon [31]. Among the isolates tested, which were amplified with the expected PCR fragment size of 235 bp using the *vhh* gene indicating the presence of hemolysin. The *V. harveyi* isolates identified were named (*Vh1* to *Vh20*) and isolate, *Vh1* was used in the present study.

### Effects of various biomolecules obtained from K. alvarezii extract against hemolysin protein of V. harveyi

The biomolecules were known by GC–MS analysis from an extract of *K. alvarezii* as shown in Fig. 2 and Table 1. GC–MS analysis has shown a total of 34 compounds. Under molecular docking, there are 34 compounds (inhibitors) used for docking analysis against *V. harveyi* hemolysin protein and found interaction only by 16 compounds with important active site residues i.e., exhibited 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* and the remaining 18 compounds did not interacted. The results of the interactions found between the hemolysin protein of *V. harveyi* and algal inhibitors with its docking scores obtained are shown in Table 2. The different model of hemolysin protein of *V. harveyi* alone and its best interaction pose was formed with OTC as depicted in 3D structure/ view (Fig. 3). Based on the results obtained from the molecular docking from *K. alvarezii*, the best three compounds were presented. While the compounds from *K. alvarezii* had been docked with *V. harveyi* hemolysin protein, the Cyclotetracosane was revealed highest binding activity with docking scores like binding energy (-7.66 kcal/mol), inhibition constant (0.002 mM) and intermolecular efficiency (-0.32 kcal/mol) followed by Cyclohexane, 1-(1,5-dimethylhexyl)-4-(4-methylpentyl)- of binding energy (-6.58 kcal/mol), inhibition constant (0.015 mM) and intermolecular efficiency (-0.33 kcal/mol), and 1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester of binding energy (-5.07 kcal/mol), inhibition constant (0.193 mM) and intermolecular efficiency (-0.25 kcal/mol) respectively, when compare to standard drug OTC of binding energy (-5.66 kcal/mol), inhibition constant (0.071 mM) and intermolecular efficiency (-0.17 kcal/mol) in the present study in 3D structure/ view including other molecules interacted with hemolysin protein (Fig. 4). Moreover, the screening of ligand molecules for predicting the drug-likeness of the compounds was performed based on the Lipinski rule of five. The Lipinski filter analysis revealed that all 16 compounds of *K. alvarezii* possessed drug-likeness properties (Table 3).

Table 1
GC-MS profile of *K. alvarezii*

| Retention time (Min) | Name of the compound | Peak area (%) | Molecular formula | Molecular weight |
|---|---|---|---|---|
| 3.63 | Styrene | 0.34 | $C_8H_8$ | 104.14 |
| 4.03 | Anisole | 0.50 | $C_7H_8O$ | 108.13 |
| 5.30 | 1-Decene | 0.61 | $C_{10}H_{20}$ | 140.26 |
| 8.15 | 4H-1,3-Benzodioxin | 0.32 | $C_8H_8O_2$ | 136.14 |
| 8.75 | 1-Dodecene | 2.20 | $C_{12}H_{24}$ | 168.31 |
| 8.88 | 1-Dodecane | 0.25 | $C_{12}H_{26}$ | 170.33 |
| 11.68 | 1-Tetradecene | 3.52 | $C_{14}H_{28}$ | 196.37 |
| 11.78 | Tetradecane | 0.25 | $C_{14}H_{30}$ | 198.38 |
| 13.16 | Phenol, 2,4-bis(1,1-dimethyl ethyl) | 1.76 | $C_{14}H_{22}O$ | 206.32 |
| 13.89 | 1-[p-Chlorophenyl]-3-[4-[[3-[1-Pyrrolidinyl]propyl]amino]-6-[trichloromethyl]triazinyl]guanidine | 0.37 | $C_{18}H_{22}Cl_4N_8$ | 492.23 |
| 14.21 | Cetene | 4.03 | $C_{16}H_{32}$ | 224.42 |
| 14.29 | Hexadecane | 0.42 | $C_{16}H_{34}$ | 226.44 |
| 15.46 | Heptadecane | 5.58 | $C_{17}H_{36}$ | 240.46 |
| 16.07 | Tetradecanoic acid | 0.40 | $C_{14}H_{28}O_2$ | 228.37 |
| 16.47 | 1-Octadecene | 3.18 | $C_{18}H_{36}$ | 252.48 |
| 16.92 | Bicyclo[3.1.1]heptanes, 2,6,6-trimethyl | 0.84 | $C_{10}H_{18}$ | 138.24 |
| 16.98 | 2-Pentadecanone, 6,10,14-trimethyl | 0.51 | $C_{18}H_{36}O$ | 268.47 |
| 17.18 | 1,2-Benzenedicarboxylic acid, butyl 2-methyl propyl ester | 0.34 | $C_{16}H_{22}O_4$ | 278.34 |
| 17.37 | 3,7,11,15-Tetramethyl-2-hexadecen-1-ol | 0.28 | $C_{20}H_{40}O$ | 296.53 |
| 18.07 | Palmitoleic acid | 1.57 | $C_{16}H_{30}O_2$ | 254.40 |
| 18.20 | n-Hexadecanoic acid | 38.43 | $C_{16}H_{32}O_2$ | 256.42 |
| 18.52 | 5-Eicosene, (E)- | 9.39 | $C_{20}H_{40}$ | 280.53 |
| 18.78 | Oleic acid | 0.74 | $C_{18}H_{34}O_2$ | 282.46 |
| 18.87 | n-Hexadecanoic acid | 9.90 | $C_{16}H_{32}O_2$ | 256.42 |
| 19.90 | Heptafluorobutyric acid,n-tetradecyl ester | 2.19 | $C_{18}H_{29}F_7O_2$ | 410.41 |
| 19.97 | Cis-Vaccenic acid | 0.88 | $C_{18}H_{34}O_2$ | 282.46 |
| 20.07 | Cyclohexane, 1-(1,5-dimethylhexyl)-4-(4-methyl pentyl)- | 1.58 | $C_{20}H_{40}$ | 280.53 |
| 20.16 | Heptafluorobutyric acid, pentadecyl ester | 3.91 | $C_{19}H_{31}F_7O_2$ | 424.43 |
| 20.38 | E-15-Heptadecenal | 2.75 | $C_{17}H_{32}O$ | 252.43 |
| 20.76 | Oleic acid | 0.45 | $C_{18}H_{34}O_2$ | 282.46 |
| 21.56 | 2(1H)-Naphthalenone, 3,4,4a,5,6,7-hexahydro-4a-[(methylamino)methyl]-, ethylene acetal | 0.29 | $C_{14}H_{23}NO_2$ | 237.33 |
| 22.09 | Cyclotetracosane | 0.90 | $C_{24}H_{48}$ | 336.63 |
| 22.40 | Z-10-Methyl-11-tetradecen-1-ol propionate | 0.50 | $C_{18}H_{34}O_2$ | 282.46 |
| 23.20 | 5-Methylthieno[3,2-b]pyridine | 0.83 | $C_8H_7NS$ | 149.21 |

Table 2
Molecular docking parameters of bioactive compounds from *K. alvarezii* against hemolysin protein of *V. harveyi* in comparison with control molecule Oxytetracycline dihydrate

| Compounds | Binding energy (kcal/mol) | Intermolecular efficiency (kcal/mol) | Inhibition constant (mM) | H-bond interaction | Inhibitor Atom | Amino acid residue* | Distance of D….A (Å) | Hydrophobic interaction* |
|---|---|---|---|---|---|---|---|---|
| Oxytetracycline dihydrate – Control molecule | -5.66 | -0.17 | 0.071 | N-H….O<br>O-H….O | O<br>O | GLN210 NE2<br>ASP 390 OD2 | 3.013<br>3.095 | LEU176; SER164; GLN165; ALA163; ASN162; TRP166; ASP390; TRP389; VAL391; ARG167; GLN210; GLY204; SER153; HIS393 |
| 1,2-Benzenedicarboxylic acid, butyl 2-methyl propyl ester | -5.07 | -0.25 | 0.193 | N-H….O | O | GLN 210 NE2 | 3.268 | TRP166; GLN210; GLY204; ASN248; ASP156; ARG167; SER153 |
| 1-[p-Chlorophenyl]-3-[4-[[3-[1-Pyrrolidinyl]propyl] amino]-6-[trichloromethyl] triazinyl]guanidine | - | - | - | - | - | - | - | No interaction |
| 1-Decene | - | - | - | - | - | - | - | No interaction |
| 1-Dodecane | - | - | - | - | - | - | - | No interaction |
| 1-Dodecene | - | - | - | - | - | - | - | No interaction |
| 1-Octadecene | -3.95 | -0.22 | 1.27 | - | - | - | - | LEU176; TRP166; TRP389; ARG167; ASP390; HIS393; SER153; ILE160; GLN210 |
| 1-Tetradecene | - | - | - | - | - | - | - | No interaction |
| 2(1H)-Naphthalenone, 3,4,4a,5,6,7-hexahydro-4a-[(methylamino) methyl]-,ethylene acetal | - | - | - | - | - | - | - | No interaction |
| 2-Pentadecanone, 6,10,14-trimethyl | -4.17 | -0.22 | 0.873 | N-H….O | O | GLN210 NE2 | 2.798 | ILE160; ALA163; TRP166; ARG167; SER153; HIS393; TRP389; GLN210 |
| 3,7,11,15-Tetramethyl-2-hexadecen-1-ol | -4.65 | -0.22 | 0.392 | O-H….O | O | GLU208 OE1 | 2.800 | ILE160; GLN210; GLU208; SER153; HIS393; TRP389 |
| 4H-1,3-Benzodioxin | - | - | - | - | - | - | - | No interaction |
| 5-Eicosene, (E)- | -4.38 | -0.22 | 0.618 | - | - | - | - | ALA163; TRP166; TRP389; ILE160; GLN165; GLN210; SER164; SER153; HIS393; ARG167 |
| 5-Methylthieno[3,2-b]pyridine | - | - | - | - | - | - | - | No interaction |
| Anisole | - | - | - | - | - | - | - | No interaction |
| Bicyclo[3.1.1]heptanes, 2,6,6-trimethyl-, (1.alpha.,2.beta.,5.alpha.) | - | - | - | - | - | - | - | No interaction |
| Cetene | - | - | - | - | - | - | - | No interaction |
| Cis-Vaccenic acid | -3.72 | -0.19 | 1.87 | N-H….O<br>N-H….O<br>O-H….O | O<br>O<br>O | SER164 N<br>GLN165 N<br>PHE161 O | 2.918<br>3.497<br>3.074 | ALA163; GLN165; SER164; ASN162; TRP166; ARG167; GLN210; SER153; HIS393; ASP390; TRP389 |

*[O – Oxygen, N – Nitrogen, H – Hydrogen, D - Donor & 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]

| Compounds | Binding energy (kcal/mol) | Intermolecular efficiency (kcal/mol) | Inhibition constant (mM) | H-bond interaction | Inhibitor Atom | Amino acid residue* | Distance of D....A (Å) | Hydrophobic interaction* |
|---|---|---|---|---|---|---|---|---|
| Cyclohexane, 1-(1,5-dimethylhexyl)-4-(4-methyl pentyl)- | -6.58 | -0.33 | 0.015 | - | - | - | - | ALA163; SER164; ASN162; TRP166; ILE160; GLN210; TRP389; ASP390; HIS393; ARG167; SER153 |
| Cyclotetracosane | -7.66 | -0.32 | 0.002 | - | - | - | - | ALA163; SER164; GLN165; ILE160; GLN210; SER153 |
| E-15-Heptadecenal | -4.33 | -0.24 | 0.668 | O-H....O | O | SER364 OG | 2.862 | ALA163; ASN162; GLN165; SER164; TRP166; ILE160; GLN210; SER153; HIS393; ASP390; SER364; ARG167; TRP389 |
| Heptadecane | - | - | - | - | - | - | - | No interaction |
| Heptafluorobutyric acid, n-tetradecyl ester | -3.11 | -0.12 | 5.25 | - | - | - | - | ALA163; SER164; GLN165; LEU176; TRP166; ARG167; HIS393; 25SER153; VAL391; ASP390; SER364; TRP389 |
| Heptafluorobutyric acid, pentadecyl ester | -3.91 | -0.14 | 1.35 | N-H....O | O | GLN210 NE2 | 2.881 | ILE160; TRP166; SER153; GLN210; GLU208 |
| Hexadecane | - | - | - | - | - | - | - | No interaction |
| n-Hexadecanoic acid | -3.24 | -0.18 | 4.24 | N-H....O<br>N-H....O<br>N-H....O<br>O-H....O | O<br>O<br>O<br>O | ALA163 N<br>SER164 N<br>GLN165 N<br>GLN165 O | 3.017<br>2.894<br>3.031<br>2.511 | ALA163; TRP166; SER164; GLN165; LEU176; TRP166; TRP389; SER364; ASP390; HIS393; SER153; ARG167 |
| Oleic acid | -2.69 | -0.13 | 10.66 | O-H....O | O | PHE161 O | 2.717 | ALA163; TRP166; LEU176; GLN165; SER164; ILE160; GLN210; SER153; HIS393; ARG167 |
| Palmitoleic acid | -3.35 | -0.19 | 3.52 | N-H....O<br>N-H....O | O<br>O | GLY204 N<br>ASN248 ND2 | 2.856<br>3.013 | ALA163; ASN162; GLN165; SER164; TRP166; ARG167; HIS393; SER153; GLY204; ASN248; GLN210 |
| Phenol 2,4-bis(1,1-dimethylethyl) | - | - | - | - | - | - | - | No interaction |
| Styrene | - | - | - | - | - | - | - | No interaction |
| Tetradecane | - | - | - | - | - | - | - | No interaction |
| Tetradecanoic acid | -3.97 | -0.23 | 1.23 | - | - | - | - | ALA163; ASN162; TRP166; ILE160; GLN210; ASP156; GLY203; SER153; TRP389 |
| Z-10-Methyl-11-tetradecen-1-ol propionate | -3.95 | -0.20 | 1.28 | N-H....O<br>N-H....O | O<br>O | ALA163 N<br>SER164 N | 3.090<br>2.926 | SER164; GLN165; ALA163; TRP166; TRP389; SER364; ASP390; HIS393; SER153; ARG167 |

*[O – Oxygen, N – Nitrogen, H – Hydrogen, D - Donor & 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 3
Predicting drug-likeness of biomolecules from *K. alvarezii* based on the Lipinski rule of five by the SwissADME web tool

| Compounds | Molecular mass < 500 Dalton | High lipophilicity (expressed as LogP < 5) | Less than 5 hydrogen bond donors | Less than 10 hydrogen bond acceptors | Molar refractivity between 40–130 |
|---|---|---|---|---|---|
| 1,2-Benzenedicarboxylic acid, butyl 2-methyl propyl ester | 278.34 | 3.62 | 0 | 4 | 77.84 |
| 1-Octadecene | 252.48 | 7.20 | 0 | 0 | 88.17 |
| 2-Pentadecanone, 6,10,14-trimethyl | 268.00 | 6.01 | 0 | 1 | 85.39 |
| 3,7,11,15-Tetramethyl-2-hexadecen-1-ol | 296.00 | 6.36 | 1 | 1 | 95.56 |
| 5-Eicosene, (E)- | 280.00 | 7.82 | 0 | 0 | 94.35 |
| Cis-Vaccenic acid | 282.46 | 5.70 | 1 | 2 | 89.94 |
| Cyclohexane, 1-(1,5-dimethylhexyl)-4-(4-methylpentyl)- | 280.00 | 7.08 | 0 | 0 | 91.98 |
| Cyclotetracosane | 336.00 | 9.36 | 0 | 0 | 110.80 |
| E-15-Heptadecenal | 252.44 | 5.50 | 0 | 1 | 83.56 |
| Heptafluorobutyric acid, n-tetradecyl ester | 410.41 | 7.47 | 0 | 9 | 90.40 |
| Heptafluorobutyric acid, pentadecyl ester | 424.44 | 7.84 | 0 | 9 | 95.20 |
| n-Hexadecanoic acid | 256.42 | 5.20 | 1 | 2 | 80.80 |
| Oleic acid | 282.46 | 5.65 | 1 | 2 | 89.94 |
| Palmitoleic acid | 254.41 | 4.94 | 1 | 2 | 80.32 |
| Tetradecanoic acid | 228.37 | 4.45 | 1 | 2 | 71.18 |
| Z-10-Methyl-11-tetradecen-1-ol propionate | 282.46 | 5.50 | 0 | 2 | 89.45 |

**Evaluation of** K. alvarezii **extract against** V. harveyi **during** P. monodon **larviculture**

When *K. alvarezii* extract was challenged against *V. harveyi* during *P. monodon* larviculture for 30 days. A reduction of CPM on PL was noticed as 29.70% as compared to control (76.30%). Likewise, in other controls, CPM against extract on PL showed (29.56%), with PL alone (28.39%) and PL challenged with OTC (46.80%) observed. The weight of the PL for both the control and treatments was observed without much weight difference. On the 30th day, the average weight of PL was 271.5 mg and 267.9 mg for control and treatment, respectively. The total heterotrophic and *V. harveyi* counts were observed for every sample. Luminescent *V. harveyi* counts were observed in VHSA medium under a darkroom. The maximum decrease on *V. harveyi* counts were observed on 5th, 10th, 15th, 20th and 25th days, and the mean values for treatment were $6.55\times10^4$ CFU $mL^{-1}$, $3.21\times10^4$ CFU $mL^{-1}$, $3.15\times10^3$ CFU $mL^{-1}$, $4.85\times10^3$ CFU $mL^{-1}$ and $8.30\times10^3$ CFU $mL^{-1}$ as compared to control ($1.17\times10^5$ CFU $mL^{-1}$, $2.62\times10^5$ CFU $mL^{-1}$, $7.40\times10^4$ CFU $mL^{-1}$, $2.02\times10^4$ CFU $mL^{-1}$and $2.53\times10^4$ CFU $mL^{-1}$ respectively). The water quality parameters like temperature, salinity and pH observed in every sampling were presented in Table 4. Not many changes in water quality parameters both in treatment and control were observed. But, in the treatment, and with extract alone slight brownish colour formation was noticed as compared to the control due to the unpurified nature of the extract.

Table 4
Evaluating of *K. alvarezii* extract against *V. harveyi* with a reduction in cumulative percentage mortality on *P. monodon* postlarvae

| Parameters | Days | | | | | | | Statistical analysis |
|---|---|---|---|---|---|---|---|---|
| | 0 | 5th | 10th | 15th | 20th | 25th | 30th | |
| Cumulative Percentage Mortality (CPM) | | | | | | | | |
| Control tubs with *V. harveyi* | 0 | 13.66 ± 0.30 | 26.05 ± 0.90 | 35.63 ± 1.10 | 47.33 ± 1.50 | 62.13 ± 2.30 | 76.30 ± 2.90 | $p < 0.05$ |
| Treatment tubs extract with *V. harveyi* | 0 | 7.06 ± 0.10 | 15.61 ± 0.40 | 23.36 ± 0.70 | 29.19 ± 1.30 | 38.53 ± 1.40 | 46.60 ± 1.10 | |
| Tubs with extract and PL alone | 0 | 2.39 ± 0.10 | 6.19 ± 0.20 | 12.05 ± 0.50 | 18.13 ± 0.60 | 24.69 ± 0.90 | 29.56 ± 1.00 | |
| Tubs with PL alone | 0 | 3.23 ± 0.10 | 6.03 ± 0.20 | 13.33 ± 0.50 | 17.43 ± 0.50 | 23.86 ± 1.00 | 28.39 ± 1.00 | |
| Treatment tubs (CFU mL-1) | | | | | | | | |
| Total Plate Count | $2.78\times10^{6}$ | $3.30\times10^{4}$ | $3.56\times10^{4}$ | $2.26\times10^{4}$ | $1.01\times10^{4}$ | $6.00\times10^{4}$ | $1.36\times10^{4}$ | $p < 0.05$ |
| *V. harveyi* | $2.14\times10^{6}$ | $6.55\times10^{4}$ | $3.21\times10^{4}$ | $3.15\times10^{3}$ | $4.85\times10^{3}$ | $8.30\times10^{3}$ | $9.95\times10^{3}$ | |
| Control tubs (CFU mL-1) | | | | | | | | |
| Total Plate Count | $2.59\times10^{6}$ | $1.45\times10^{5}$ | $2.74\times10^{5}$ | $1.46\times10^{4}$ | $7.80\times10^{4}$ | $1.88\times10^{4}$ | $2.06\times10^{4}$ | |
| *V. harveyi* | $1.86\times10^{6}$ | $1.17\times10^{5}$ | $2.62\times10^{5}$ | $7.40\times10^{4}$ | $2.02\times10^{4}$ | $2.53\times10^{4}$ | $1.53\times10^{4}$ | |
| The average weight of postlarvae (mg) | | | | | | | | |
| Treatment tubs | 16.90 ± 30 | 60.50 ± 40 | 121.30 ± 60 | 158.10 ± 70 | 208.50 ± 90 | 249.20 ± 80 | 267.90 ± 70 | NA |
| Control tubs | 17.50 ± 20 | 63.30 ± 40 | 126.10 ± 50 | 162.30 ± 50 | 201.10 ± 50 | 251.30 ± 90 | 271.50 ± 80 | |
| Water quality parameters for treatment and control tubs | | | | | | | | |
| Temp. (°C) | 29.00 ± 1.00 | 29.50 ± 1.00 | 29.00 ± 1.00 | 30.00 ± 1.00 | 30.00 ± 1.00 | 31.00 ± 1.00 | 30.00 ± 1.00 | NA |
| Salinity (PSU) | 20 ± 0.50 | 20 ± 0.50 | 20 ± 0.50 | 20 ± 0.50 | 21 ± 0.50 | 21 ± 0.50 | 21 ± 0.50 | |
| pH in control tubs | 8.50 ± 0.20 | 8.40± 0.20 | 8.30 ± 0.20 | 8.50 ± 0.20 | 8.30 ± 0.20 | 8.10 ± 0.20 | 8.30 ± 0.20 | |
| pH in treatment tubs | 8.30 ± 0.20 | 8.40 ± 0.20 | 8.30 ± 0.20 | 8.50 ± 0.2 | 8.30 ± 0.20 | 8.20 ± 0.20 | 8.00 ± 0.20 | |
| Values of an average of three determinations with standard deviation (SD); NA- Not applicable | | | | | | | | |

**Effects of** K. alvarezii **extract against growth and virulence factors of** V. harveyi **during larviculture**

During treatment, reduced growth of *V. harveyi* (OD) was observed for all the sampling days. During larviculture, *K. alvarezii* extract treatment controlled virulence factors such as luminescence, proteases, crude bacteriocin, cell surface hydrophobicity (SAT assay), hemolysis and phospholipids of *V. harveyi* in all days of samplings. The results of growth and virulence factors affected by *K. alvarezii* extract during treatment against *V. harveyi* had shown in Table 5. The reductions of growth and virulence factors of *V. harveyi* were observed against *K. alvarezii* extract in all the sampling days during larviculture than control.

Table 5
Evaluation of *K. alvarezii* extract against growth and virulence produced by *V. harveyi* during *P. monodon* larviculture

| Days | Growth and virulence factors production | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Growth (OD 600nm) | | Phospholipase activity* | | Hemolytic activity* | | Cell surface hydrophobicity SAT (M)# | | Luminescence production (CPS) | | Luciferase production (CPS) | | Crude Bacteri (OD 660nm) | |
| | Control | Treated | Control | Treated | Control | Treated | Control | Treated | Control | Treated | Control | Treated | Control | Tre |
| 5th | 1.03 ± 0.03 | 0.76 ± 0.01 | ++++ | – | ++++ | – | 0.75 ± 0.03 | 1.50 ± 0.04 | 136 ± 3.31 | 116 ± 5.11 | 137 ± 4.16 | 108 ± 2.53 | 2.11 ± 0.09 | 2.0 0.0 |
| 10th | 1.48 ± 0.05 | 1.35 ± 0.05 | ++++ | – | ++++ | – | 0.50 ± 0.02 | 2.00 ± 0.05 | 110 ± 4.01 | 105 ± 3 .36 | 123 ± 4.56 | 114 ± 1.39 | 1.98 ± 0.06 | 1.9 0.0 |
| 15th | 1.64 ± 0.05 | 1.54 ± 0.03 | ++++ | – | ++++ | – | 0.50 ± 0.01 | 1.50 ± 0.06 | 118 ± 3.56 | 110 ± 4.51 | 130 ± 5.63 | 099 ± 2.83 | 2.16 ± 0.05 | 1.8 0.0 |
| 20th | 1.35 ± 0.04 | 1.22 ± 0.03 | ++++ | – | ++++ | – | 0.75 ± 0.03 | 1.75 ± 0.04 | 150 ± 5.06 | 122 ± 3.96 | 148 ± 6.33 | 123 ± 5.57 | 1.950 ± 0.01 | 1.7 0.0 |
| 25th | 0.77 ± 0.01 | 0.73 ± 0.02 | ++++ | – | ++++ | – | 0.50 ± 0.02 | 2.00 ± 0.09 | 154 ± 1.91 | 122 ± 5.36 | 135 ± 3.69 | 100 ± 2.61 | 1.95 ± 0.07 | 1.6 0.0 |
| 30th | 1.15 ± 0.05 | 0.99 ± 0.03 | ++++ | – | ++++ | – | 0.50 ± 0.01 | 2.50 ± 0.06 | 128 ± 2.63 | 098 ± 4.03 | 129 ± 2.93 | 105 ± 1.61 | 2.37 ± 0.11 | 2.2 0.0 |
| Statistical analysis | $p < 0.05$ | | NA | | NA | | $p < 0.05$ | | $p < 0.05$ | | $p < 0.05$ | | $p < 0.05$ | |

*Activity of *V. harveyi*: – = non-existent, + = weak, ++ = moderate, +++ = high, ++++ = very high; #SAT test – (0.0 to1.0M = strongly hydrophobic, 1.0 to 2.0M = m 2.0 to 4.0M = weakly hydrophobic and > 4.0M = not hydrophobic); NA- Not applicable

# Statistical analysis

The statistical assessment was made among the treatment and control. Significant differences were found ($p < 0.05$) between the *K. alvarezii* extracts treated with *V. harveyi* and the control.

## Discussion

Hemolysin protein, which is a major pore-forming exotoxin produced by among the pathogenic *Vibrios*. It is a, which may cause lysis of RBC and other cells by producing spores on the cytoplasmic membrane. Therefore, it is recognized as an important and main virulence factor [4, 44]. The dry lab/net lab research approach predicts the ligand orientation in a complex formed by the ligand itself with proteins or enzymes [21]. The quantification of the interaction is based on the shape and electrostatic interaction of the docked complex. Many docking programs (more than 50) and tools are now in use in the field of drug research [45]. Among them, AutoDock is one of the main tools being used in the field of drug development industries, etc [46]. Hence, the AutoDock tool was aimed at the recent study against *V. harveyi* hemolysin protein. Due to the lack of an experimentally established crystal structure on the haemolysin protein produced by *V. harveyi*, the homology modelling may be the best alternative method to construct a reasonable (3D) model of the target [47] which was followed here.

While conducting the docking analysis, the ligand molecules/inhibitors from *K.alvarezii* abiding, passed Lipinski's rule of five, which showed its drug-likeness and the possibility of its considerations for further pre-clinical studies. Lead optimization of the inhibitors from *K. alvarezii* was recognized by computation of drug-likeness properties and it was agreed with the other report [48]. Besides, the hemolysin of *V. harveyi*, an important virulence determinant causing pathogenesis in marine fish, Turbot (*Scophthalmus maximus*), was further characterized, and the enzyme was identified as a phospholipase B (Hemolysis activity). The site-directed mutagenesis revealed that a specific residue, SER153, was critical for its enzymatic activity and its virulence in fish [7]. In the present findings also we have found three active site residues in our structural template/model such as HIS393, and ASP390 including SER153 in the *V. harveyi* hemolysin protein. The binding of biomolecules was established by H-bonding or hydrophobic interactions or both with important active site residues (SER153, HIS393 and ASP390) of *V. harveyi* hemolysin protein. Evaluation of seaweed sulfated polysaccharides as natural antagonists targeting *Salmonella typhi*OmpF was conducted using molecular docking and pharmacokinetic profiling. The sulfated polysaccharides showed good binding affinity compared to commercial drugs, particularly carrageenan/MIV-150, carrageenan lambda, fucoidan, and 3-phenyl lactate, ranked as top antagonists against OmpF. Further, pharmacokinetics and toxicology studies corroborated that sulfated polysaccharides possessed drug-likeness and highly progressed in all parameters [49]. In the present study, our compounds also passed Lipinski's rule of five, which showed their drug-likeness and the possibility of their being considered for further pre-clinical studies.

As a general rule, it was considered that in most of the potent inhibiting mechanisms of compounds, both H-bond and hydrophobic interactions between the ligands/inhibitors and the active sites of the receptor are responsible for mediating biological activity [50]. The anticancer activity of *K. alvarezii* against the

cancer target of *Homo sapiens* was studied by docking and found interaction with active site amino acid residues through hydrogen bond formation [51]. In this study, the inhibitors of *K. alvarezii* were exposed to 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 performed based on the selectivity by Carbohydrate Recognition Domain (CRD) of the human "Asialoglycoprotein" receptor (ASGP-R) with monomer sugar molecules from brown seaweed *Laminaria hyperborea, Ascophyllum nodosum* and *Macrocysti spyrifera* and from green algae *Ulva* sp. and *Enteromorpha* sp. The molecule's interactions are linked by H-bonds and hydrophobic interactions with ASGP-R and determined by docking scores [52]. 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. Flavonoids found in *Carica papaya*, apple, and even the lemon as the only flavone possessed anti-dengue activity that could be interrupted the fusion process of the dengue virus by inhibiting the hinge region movement and by blocking the conformational rearrangement in envelope protein [53].

The top-ranked pose with the lowest docked binding affinities/ scores is generally used as a standard selection in most docking programs [22]. Likely, lower the docking score was showed a higher binding affinity and considered as better inhibiting biological activity [54]. The preliminary screening helps to compare the docking score of standard drug/control molecules against ligands. Then the pre-screened ligands were validated using Autodock version 4.0 [23](Tomi *et al.* 2016). OTC was used as a control molecule/reference drug in the current study. Thus, 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 [22].

During shrimp larviculture, *K. alvarezii* extracts showed an increased level of reductions in the CPM on the PL of *P. monodon*. The survival of *P.monodon* juveniles treated with *K. alvarezii* extract exhibited significant differences ($p < 0.01$). Therefore, the current finding significance ($p < 0.05$) was supported by another study [55]. Seaweeds and other marine organisms are possessed numerous bioactive compounds which are useful for various health benefits for humans [11]. The *in vitro* antibacterial activity of extracts of *K. alvarezii* has been reported against human bacterial pathogens [27]. It was also studied the antagonistic activities of *K. alvarezii* and *K. striatum* against several infectious agents [26]. Marine algal lipids inhibit microbes by disrupting the cellular membrane of bacteria, fungi, and yeasts [56]. These fatty acids could affect the expression of bacterial virulence factors, which are important for the establishment of infection. The saturated and unsaturated fatty acids can prevent initial bacterial adhesion and subsequent biofilm formation, and later induce the lysis of bacterial protoplasts [56].

The anti-hemolytic activity of the filamentous seaweed, *Enteromorpha intestinalis* exhibited $H_2O_2$ induced hemolytic activity was known against many pathogenic bacteria [57]. Further, diterpene isolated from brown seaweed *Canistrocarpus cervicornis* caused anti-hemolytic activity, as well as fibrinogen or plasma clotting induced by *Lachesismuta* snake venom protein, was reported [58]. The inhibiting activity of snake venom protein by brown seaweed was compared with the antagonism of *K. alvarezii* inhibitors against hemolysin protein in the present study. Moreover, in a recent shrimp *P. monodon* larviculture experiment, the extract of *K. alvarezii* showed its non-existent level inhibition or virulence of hemolysis activity of *V. harveyi* on sheep blood agar than control including reductions of growth and other virulence factors.

The highest binding affinity was revealed by the biomolecule Cyclotetracosane, which has established the docking scores like binding energy and followed by "Cyclohexane"1-(1,5-diethyl hexyl)-4-(4-methyl pentyl) and 1,2-Benzenedicarboxylic acid, butyl 2-methyl propyl ester of respectively, when compared to control molecule OTC. This report agreed with the following study; phycocyanin is the main pigment found in Micro alga, *Spirulina platensis* and has the potential effect to treat effectively type-2 diabetes mellitus by inhibiting α-amylase and α-glucosidase. Molecular docking simulations indicated that phycocyanin inhibits the enzymes by binding to the active site and causing disruption in substrate-enzyme binding [59]. Moreover, an *in-vitro* inhibition activity test showed that phycocyanin inhibits human salivary amylase by an average of 51.13% and it may contribute to making full use of phycocyanin as an anti-diabetic drug or therapeutic agent. Likely, our *in-vitro* study also shows reductions in the trials. Therefore, this recent research results also indicate that biomolecules identified from *K. alvarezii* are an efficient bio-agent against *vhh*, which can be more developed into a new drug for the treatment of *Vibrio* infection. These results support the evaluation of the present molecular docking studies from the extracts of *K. alvarezii* against the hemolysin protein of *V. harveyi* in aquaculture practices.

## Conclusions

Results were observed from the growth and virulence factors such as luminescence, luciferase, crude bacteriocin, proteases, and cell surface hydrophobicity of *V. harveyi*, the survival of PL in *P. monodon* larviculture between treatment and control. Significant differences were found ($p < 0.05$) between *K. alvarezii* algal extract treated with *V. harveyi* and control. During the present investigation, the compounds docked showed a higher binding affinity and it followed no violation of the rule by the ligands/compounds used in docking for determining the pharmacological activity of the drug. Moreover, the extract of *K. alvarezii* showed anti-hemolytic activity, a decrease in the growth and other virulence factors of *V. harveyi*. During the larviculture experiment, *K. alvarezii* exhibited an increase in the survival of *P. monodon* PL than the control. Therefore, the recent findings indicated that the red seaweed *K. alvarezii* may be provided significant scope for developing useful bioactive compounds in shrimp culture systems.

## Declarations

### Acknowledgements

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### Compliance with ethical standards

**Conflict of interest** The authors contributed equally to this work.

**Author's contribution** All the authors contributed equally to this work (idea conception, acquisition, data analysis, drafting, critical evaluation of the manuscript, and statistical analysis).

**Ethical statement** This article does not contain any study with vertebrate animals, thus it was required no animal ethic statement.

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## Figures

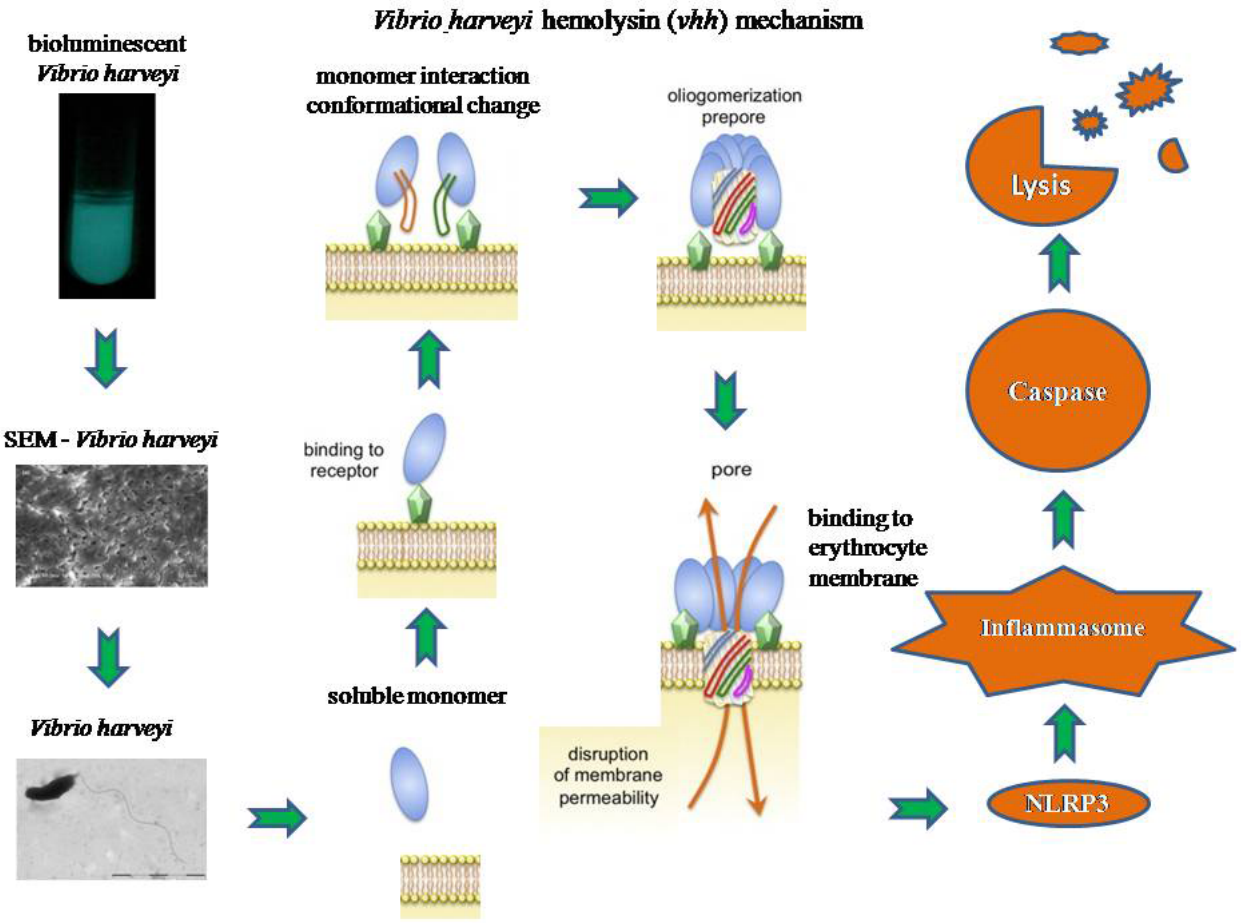


**Figure 1**

Schematic diagram of mechanistic action of *V. harveyi* hemolysin (*vhh*) on host cell [5, 6]

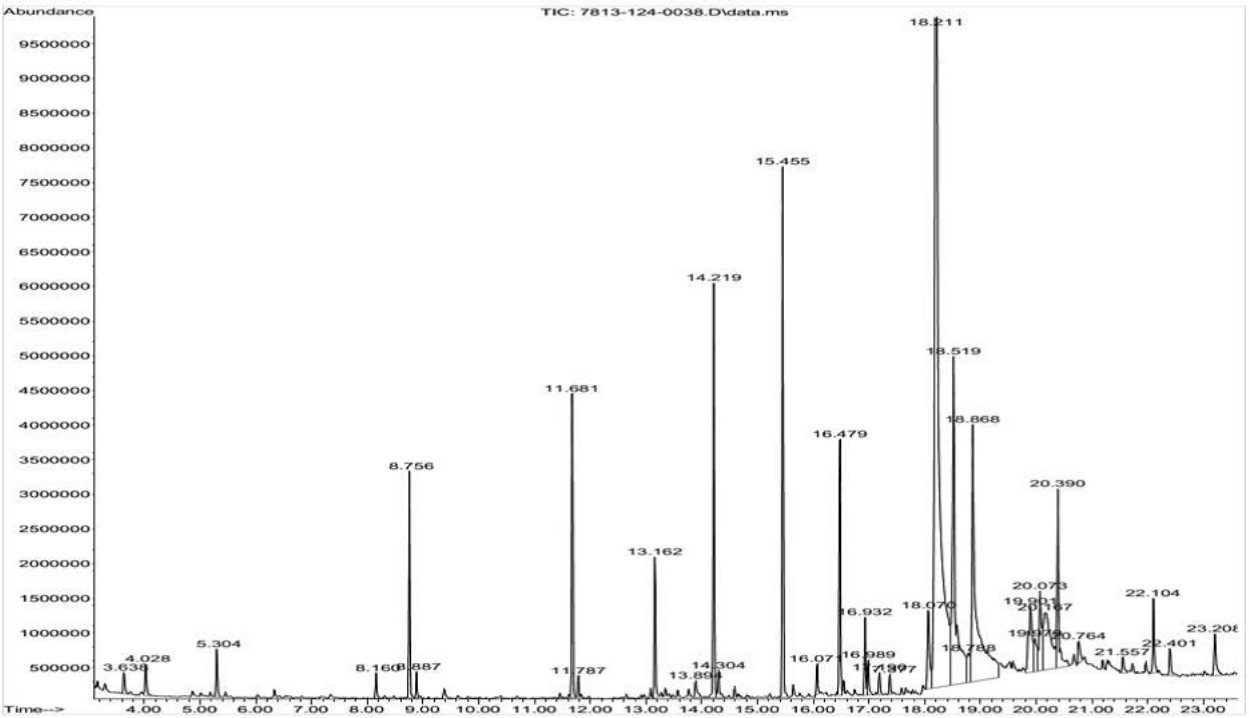


Figure 2

GC-MS chromatogram of the crude extract of *K. alvarezii*

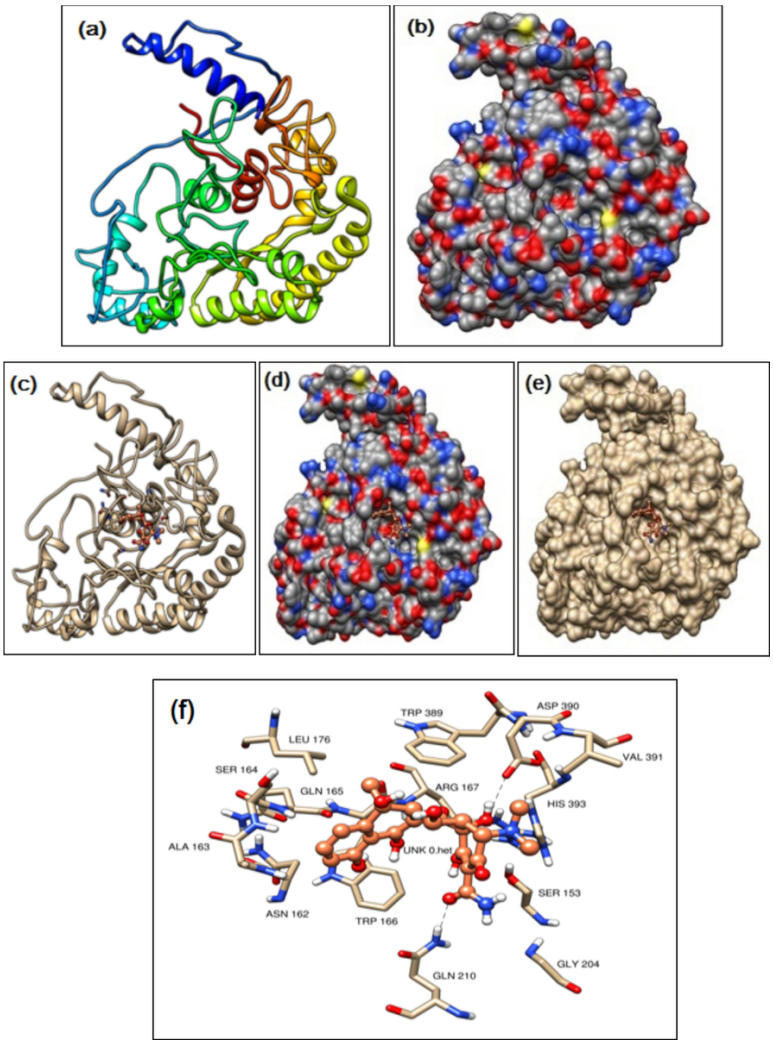

**Figure 3**

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 colour, (e) without atoms colour and (f) close view of docked with hemolysin protein

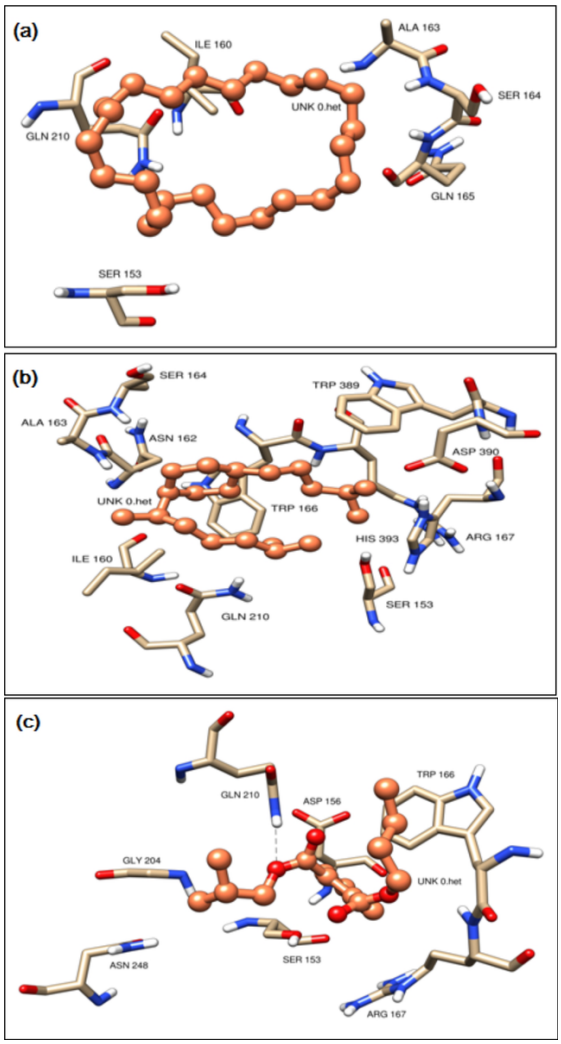


**Figure 4**

The best-docked pose of bioactive compounds from *K.alvarezii* (a) Cyclotetracosane, (b) Cyclohexane,1-(1,5-diethyl hexyl)-4-(4-methyl pentyl)- and (c) 1,2-Benzenedicarboxylic acid, butyl 2-methyl propyl ester with hemolysin protein.