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

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ABSTRACT

Shrimp culture systems are being affected by bacterial diseases particularly *Vibrios*. Use of therapeutic agents in shrimp culture has lead to the resistance among aquatic bacteria. Accordingly, health management becomes of major importance in aquaculture. Under this situation, developing bio-inhibitors from natural marine resources which are most appropriate, which is considered as an unmatched reservoir of several biological products. Molecular docking is suited to a major approach in structural biology and computer-assisted drug design against the proteins of disease-causative agents. In the present study, marine macroalga *Kappaphycus alvarezii* was used for developing inhibitors against bioluminescence disease-causing pathogenic bacteria *Vibrio harveyi*. *K. alvarezii* was collected from the Mandapam, Tamil Nadu, India. Ethyl acetate based extract of *K. alvarezii* was tested against the growth and virulence factors of *V. harveyi* during *Penaeus monodon* larviculture. Further *K. alvarezii* extract was subjected to GC–MS analysis to identify the biomolecules. The homology modelling of the virulent protein, hemolysin of *V. harveyi* was designed during this study. Hence, it was aimed at molecular docking against the biomolecules identified from *K. alvarezii* extract. During shrimp larviculture, the extract of *K. alvarezii* (200 μg mL^{-1}) exhibited a reduction on Cumulative Percentage of Mortality (29.70 %) in postlarvae against the challenge of *V. harveyi* infection. Among the compounds docked, an inhibitory effect was observed based on docking scores and were found the highest binding affinity/inhibiting activity in Cyclotetracosane with the least energy required with a docking score of binding energy −7.66 kcal/mol, inhibition constant 0.002 mM and intermolecular efficiency −0.32 kcal/mol. Using statistical analysis, significant differences ($p < 0.05$) were observed between 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 protecting against infections caused by *V. harveyi* and possibly other aquatic pathogenic bacteria in shrimp farming systems.

1. Introduction

Aquaculture is being one of the fastest-growing food production sectors which is representing over 50 % of world fish production. It delivers more than 17 % of animal protein along with capture fisheries and aids he livelihoods of 12 % of the world's population. Among the aquaculture industries, shrimp culture is ranking one of topmost and lucrative industry, providing employment opportunities and bringing foreign exchange to India. Though this industry faces many problem, disease incidences are causing the major losses for every year. In shrimp grow-out and hatchery systems, marine pathogenic *Vibrios* cause diseases [1], among them, *Vibrio harveyi* is one of the important disease-causing agents, causing severe mortality [2]. It is a Gram-negative, bioluminescent bacterium, that produces many virulence factors such

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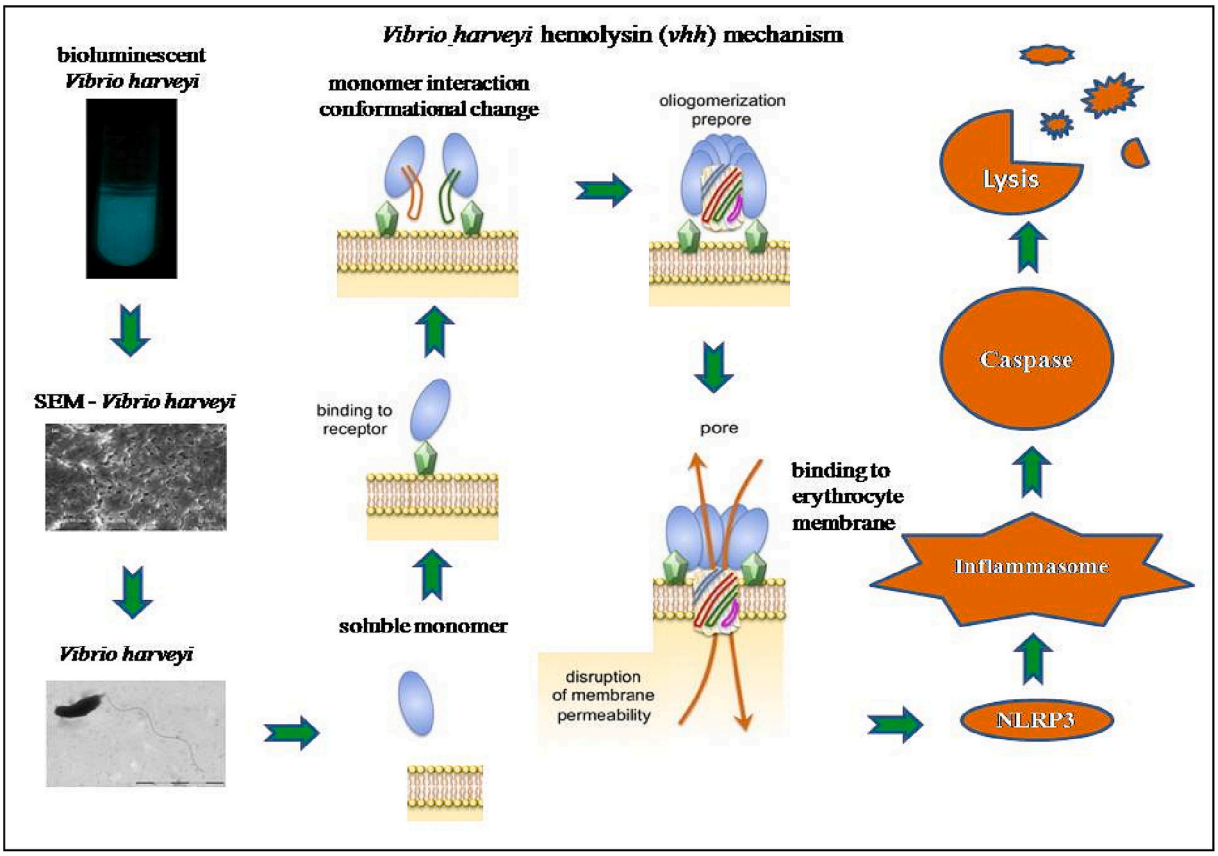

Fig. 1. Schematic diagram of mechanistic action of *V. harveyi* hemolysin (*Vhh*) on host cell [5,6].

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]. The crucial virulence factors of *V. harveyi* are extracellular protease, outer membrane protein, *V. harveyi* hemolysin (*Vhh*), phospholipase and secretion system, etc., which function in different phases of the disease process [8]. Among them, *Vhh plays* a vital role in the pathogenicity of the host. *Vhh* is a protein with a molecular mass of ~47.3 kDa. Based on the previous study, this toxin is capable of affecting erythrocytes, fin epithelial cells and gill cells, which induces ultra-structural changes, apoptosis, rupture, and triggers inflammatory responses and pathological changes in tissues in turn [8]. Also reported that the most highly pathogenic bacteria in the study was *V. harveyi* VIB 645, which produced extracellular products with the highest titer of hemolytic activity to both Atlantic salmon and rainbow trout erythrocytes [9]. In addition, recombinant *vhh* induced various apoptotic features (such as apoptotic bodies, chromatin condensation, an increased number of TUNEL-positive apoptotic cells, and caspase-3 activity) in the Japanese founder gill cell line [9]. Virulent isolates of *V. harveyi* showed enterotoxin activities and also evidenced infection with brine shrimp (*Artemia*) larvae [10].

The pathogenic *V. harveyi* is associated with bioluminescence disease in shrimp grow-out practices [2] and also causes mortality in shrimp hatcheries up to 80–100 % [11]. The major threats to sustainable aquaculture is the existence of emerging pathogens. It also poses a severe problem to several cultured marine and brackishwater fin fishes such as Asian seabass, Groupers, Cobia, etc. [12]. As remedial measures against bioluminescence disease, the utilization of antimicrobial agents will be more effective compared with synthetic chemicals, viewing more resistance among the bacterial pathogens [13]. For these issues, it is essential to reconsider an alternative product that should be biodegradable and eco-friendly for the aquatic environment. Therefore, marine organisms signify a varied reserve of bioactives that could help in controlling a wide range of diseases occurring in aquaculture [14]. Under this situation, it may be resolved by obtaining unique 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 [15]. The shrimp fed on herbal diets for 60 days with extracting 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, which eradicate *V. harveyi* and *V. parahaemolyticus* biofilms and ameliorate bacterial infection in shrimp [16]. The crude garlic extract, when fed through the larviculture system of *P. monodon* for a 30 days, a significant reduction in the growth, and mortality of postlarvae against *V. harveyi* infections, with reductions in the virulence factors was noticed [11]. When the crude extract of marine macro alga *Ulva fasciata*, was evaluated against luminescent *V. harveyi* during *P. monodon* larviculture, it showed inhibition, and reduced mortality in postlarvae [17]. Preliminary studies were made 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 exhibited enhanced results in an ethyl acetate-based solvent extract [18].

Novel antibacterial substances were also discovered in microalgae, with *Coccomyxa onubensis* fatty acid extracts inhibiting *E.coli* and *P. mirabilis* [19]. Recently, micro algae *Nannochloropsis* have been shown to be rich in polyunsaturated fatty acid compounds (PUFAs),

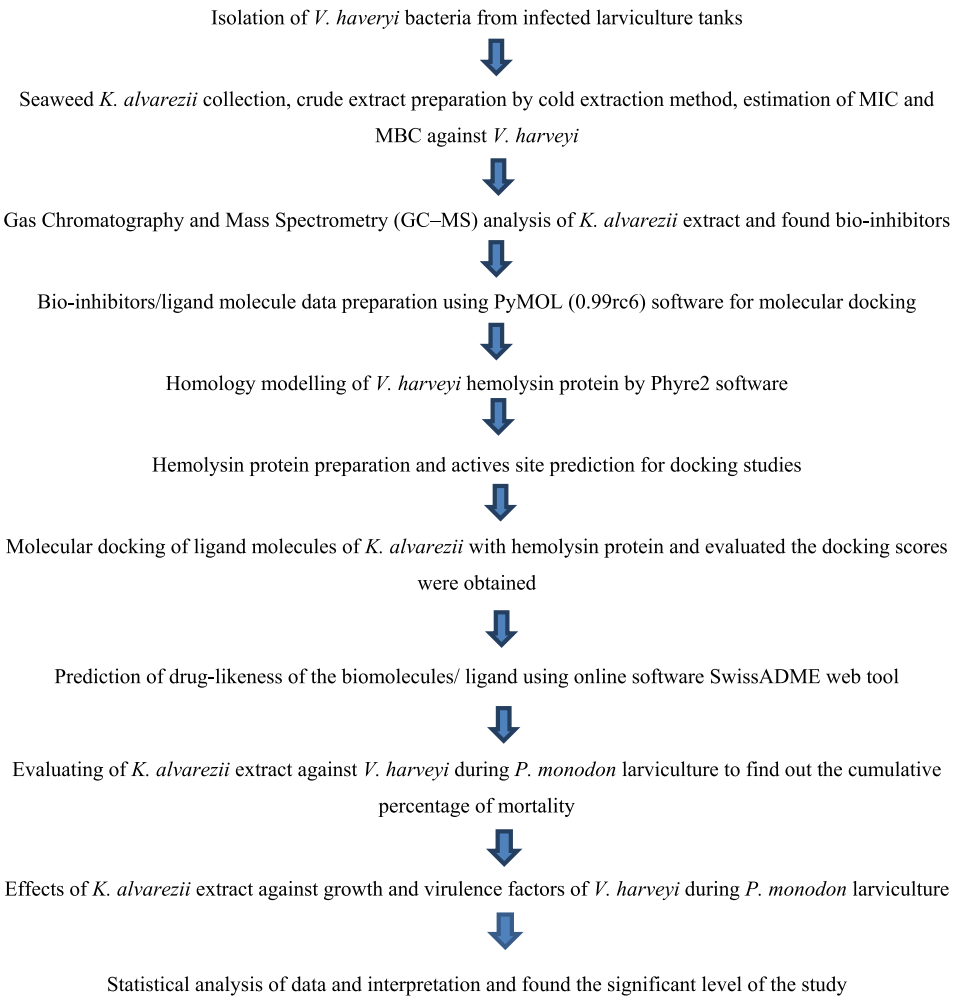

Fig. 2. The flow chart of the present study of molecular docking approaches of biomolecules extracted from *K. alvarezii* extract against hemolysin protein of bioluminescence disease causing *V. harveyi* bacteria.

carotenoids, polyphenols, vitamins, and while the microalgae strain of *Isochrysis* sp. was shown to be high in fucoxanthin and DHA, both microalgae had been used in aquaculture [20,21]. The microalgae *Thalassiosira weissflogii*, on the other hand, has antibacterial properties due to its high presence of substances such as betain, lipids, phospolipid, PUFA, polyunsaturated fatty acids, fucoxanthin (FX), and eicosapentaenoic acid (EPA) [22]. The compound chlorellin extracted from *Chlorella* which has essential antimicrobial activity against bacteria. The antimicrobial capacity was equal to that of ampicillin and oxacillin to inhibit *Staphylococcus* spp. [23]. Further, GC–MS analysis of the major compounds of *Chlorella* spp. including phenol (18.5 %), hexadecanoic acid (18.25 %), phytol (14.43 %), 9,12-octadecadienoic acid (13.69 %), and bicyclo[3.1.1]heptane (7.23 %), which have been described to possess antimicrobial activity against Gram-positive and Gram-negative bacteria. Hence, *Chlorella* spp. methanol extracts showed promising antibacterial activity [24].

Further marine red seaweed, *Tricleocarpa fragilis* also revealed inhibitory on *V. harveyi* by using an ethyl acetate-based solvent fraction [25]. The ethyl acetate extract from the macro algae *Gracilaria gracilis* has been evaluated for antibacterial, antioxidant activities and also used as a additive in fish feed [26]. In the crude compounds from the ethyl extract fractions of the macro algae was found inhibitory to microbes [27,28]. 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 that 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 was examined [29]. Under this condition, molecular docking (*in-silico* analysis) is one of the top tools and is cost-effective to identify the effect of the individual molecule (ligand) on target diseases without scarifying the animals and before isolating or bulk production, etc. [30].

Currently, molecular docking plays 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 [31]. In modern drug design, molecular docking plays a main tool in structural biology and computer-assisted drug design, it is generally made between a small molecule and a target macromolecule and is often referred to as ligand-protein docking [32,33]. 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 foremost alternative for constructing a reasonable (3D) model of the target, owing to the lack of an experimentally recognized crystal structure of a given protein [34]. The models constituted will be suitable for applications like agonists or antagonists [34]. Moreover, by 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 [30].

Marine seaweeds are a source of biologically active natural products, which can be utilized in pharmaceuticals, food industries, and cosmetics [14]. The red seaweed *Kappaphycus alvarezii* is one of the edible seaweed

Fig. 3. The marine macroalga *K. alvarezii* collected from the east coast, Mandapam region, Tamil Nadu, India used in the present study.

which is highly demanded for polysaccharides. This alga is a very good source of minerals and one of the most important sources of carrageenans, a family of gel-forming, viscos producing polysaccharides. Carrageenan is an additive used for thickening and preserving foods and drinks. Seaweeds like *K. alvarezii* and *Kappaphycus striatum* was proven for controlling many infectious pathogens [35]. Similarly, the extracts of *K. alvarezii* and *Ulva lactuca* showed antagonism against human pathogenic bacteria [36]. The antioxidant activity, DPPH radical-scavenging, and lipid peroxidation activity of *K. alvarezii* were measured and found significant levels [37].

The exract of *K. alvarezii* was screened for the presence of metabolites and was characterized for its association with known bioactivities. The phytochemical compounds such as phenols, flavonoids, steroids, quinones were also present apart from the primary metabolites like carbohydrates, proteins, lipids, carotenoids, ascorbic acid and also showed antioxidant activity (DPPH) [38] with phyto-pharmacological properties. During this study different polarity solvents (ethanol, ethyl acetate and chloroform) were used in the extraction of bioactive components of *K. alvarezii*, characterized by GC–MS and studied for their antioxidant, antimicrobial, and cytotoxic activity. All the *K. alvarezii* extracts exhibited good antioxidant activity and inhibitory potential against pathogenic microbes [39]. *K. alvarezii* showed the highest water solubility ($p < 0.05$), which is associated to its carrageenan compound. The antioxidant activities; DPPH and TEAC assay with total phenolic content (TPC) of this seaweed indicated that the highest TPC (19.17 ± 0.04 mg GAE/g DW) and the lowest radical scavenging activity in both TEAC and DPPH assays [40]. In addition to that the phytochemical analysis of *K. alvarezii* revealed the presence of carbohydrate, proteins, alkaloids, glycosides, flavonoids, steroid, phenolic compounds and the absence of tannin in the extracts. Also *K. alvarezii* extracts disclosed for presence of macromolecules such as carbohydrates of 18.26 g, protein 11.49 g and fat 1.01 g/100 g of DWB. RP-HPLC analysis of the extract showed the presence of a pool of phenolic acids with higher levels of sinapic acid followed by phloroglucinol. Subsequently, different solvent extracts of *K. alvarezii* were subjected to FTIR for the analysis of functional groups. The results based on the spectral data of FTIR revealed the presence of aliphatic constituents containing carbon, ketones, alkyl halides and hydroxyl groups. Thus, the phytochemical analysis of the algae showed immense potential values in terms of primary and secondary metabolites and therefore it can be used as a functional and pharmaceutical food [41]. Selvin et al. [42] reported the efficacy of seaweed *U. fasciata*, extract in antagonizing against bacterial (*Vibrio fischeri*, *V. alginolyticus*, *V. harveyi* and *Aeromonas* sp.) infections during larviculture of *P. monodon*. 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 the growth of *V. harveyi* with its virulence factors [43]. Against this background, the present experiment was focused on studying about the pathogenicity and virulence mechanisms of *V. harveyi* and determining different bio-inhibitors to facilitate a favourable approach through molecular docking analysis for controlling *V. harveyi* infections in shrimp grow-out and hatchery systems to evolve better and sustainable shrimp and more aquaculture practices.

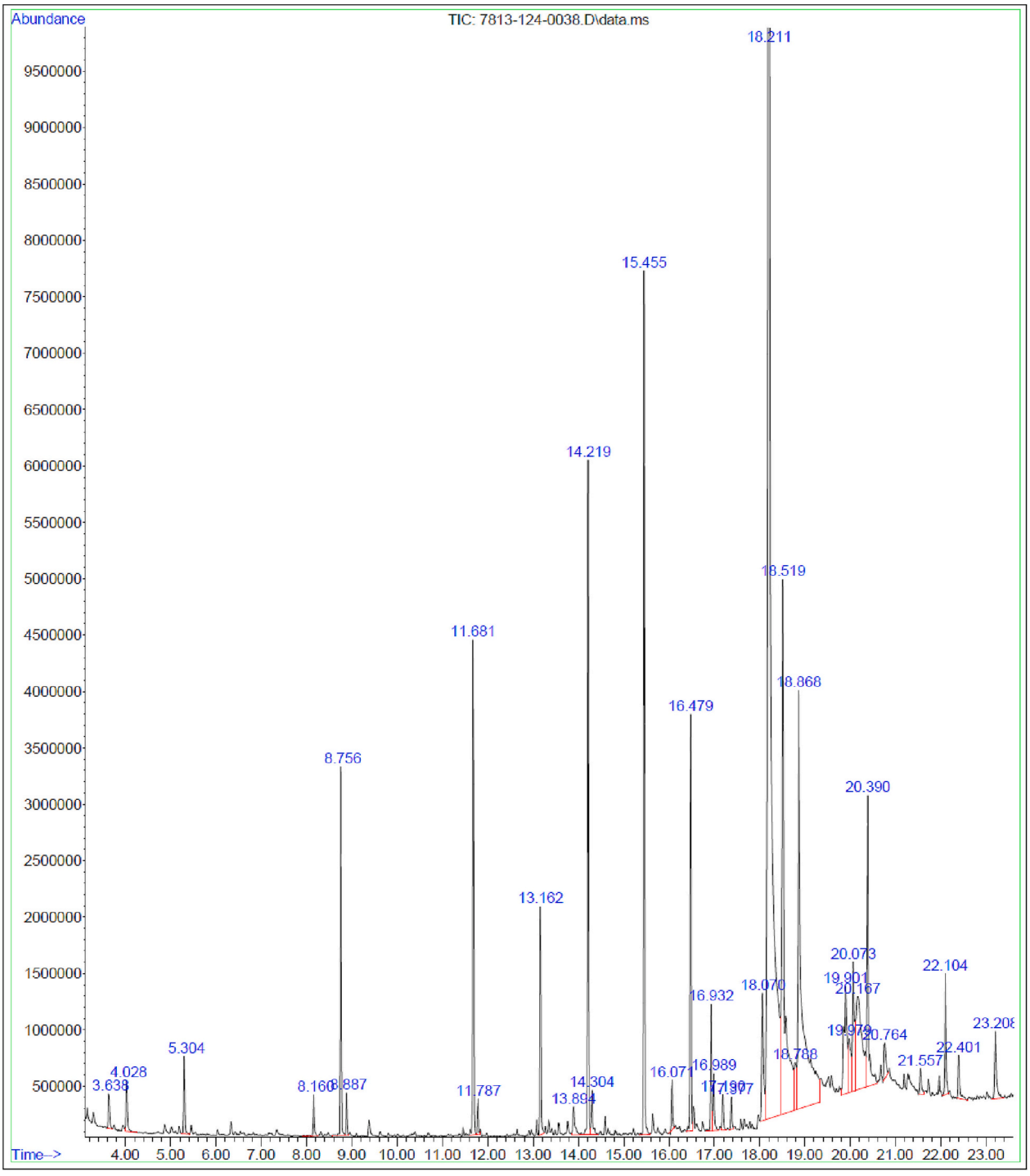

Fig. 4. GC–MS chromatogram of the crude extract of *K. alvarezii.*

2. Materials and methods

2.1. V. haveryi bacteria isolation

The *V. harveyi* bacterial strains were isolated from the infected postlarvae of *P. monodon* larviculture tanks (Indian Council of Agricultural Research (ICAR - CIBA, Muttukadu Experimental Station, Chennai, India)). The standard morphological and biochemical tests were used to identify the *V. harveyi* bacterial strains [44]. The strains were pre-enriched in alkaline peptone water (APW) and then 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). Bio-luminous colonies spread in SWC agar, then incubated at 30 °C for 20 h and observed in a dark room. The isolates were compared with *V. alginolyticus* (MTCC-4182) and *V. harveyi* (MTCC - 3438) strains as negative and positive control respectively, and then further was confirmed by PCR [45]. The confirmed *V. harveyi* isolates were named as (*Vh1* to *Vh20*) in the study. The pathogenicity of *V. harveyi* isolates, confirmed 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 [46]. The flow chart of the present study is showed in Fig. 2.

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-dimethylethyl)	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-methylpropyl 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	Heptafluorobutyricacid,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-methylpentyl)-	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

2.2. Seaweed collection, crude extract preparation, estimation of MIC and MBC

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 (Fig. 3). The alga was washed in 1 % $KMnO_4$ (w/v) to eliminate epiphytes, sand and other extraneous matters. The seaweeds were dried under shade, pulverized, and powdered for extraction. The solvent extraction method recovers almost all the compounds from the algae powder. The organic solvent, ethyl acetate (SRL, India) was used for the powdered *K. alvarezii*. These powders were extracted separately at 30 °C, a process called as Cold extraction method [47]. The extract was prepared by taking 1.0 g of shade dried powder mixing with 10.0 mL of solvent (1:10 ratio) and shaker incubated at 30 °C for 96 h at 50 rpm. Subsequently, the extracts were filtered by Whatman filter paper (No. 1), then rotary evaporated at 30 °C/3 h under vacuum and stored in darkness at 4 °C for further use. The resultant extract were liquefied with 5 mg/mL of 30 % (v/v) DMSO. The minimum inhibitory concentration (MIC) and minimum bactericidal activity (MBC) of *K. alvarezii* extract was estimated against to *V. harveyi* by dilution in broth method [48].

2.3. Preparation of inhibitors 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 × diameter 0.25 mm × 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 40–700 *m/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 for 3 min, which rose to 250 °C @14 °C min^{-1} (total run time, 34 min). The temperatures of the transfer line and the ion source were set to a value of 230 °C and interface temperature at 240 °C, respectively. Full mass data was recorded from 50 to 400 Da 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 (2020) 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 [49]. The GC–MS analysis was performed in triplicate during this study. 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) downloaded from this database. These 3D structures were converted to PDB (Protein Data Bank) format using PyMOL (0.99rc6) software [50] and these PDB files were used for docking.

2.4. 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) [51]. Hence, the PDB id of the 3KVN_A (1-311) template has been selected, and the modeled structure was validated in the Phyre server for structural similarity analysis against the PDB database [52]. Phi/Psi dihedral angle for the predicted model was validated using the Ramachandran plot from PROCHECK [53]. The modeled structure has 5.29%of amino acid residues as outliers in the Ramachandran plot.

2.5. Hemolysin protein preparation and active 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 [54]. For docking, the active site (binding site) of

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

Compounds	Binding energy (kcal/mol)	Intermolecular efficiency (kcal/mol)	Inhibition constant (mM)	H-bond interaction	Inhibitor Atom	Amino acid residue[a]	Distance of D….A (Å)	Hydrophobic interaction[a]
Oxytetracyclinedihydrate–control molecule	−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; ALA163; ASN162; TRP166; ASP390; TRP389; VAL391; ARG167; GLN210; GLY204; SER153; HIS393
1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl 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 N-H….O O-H….O	O O O	SER164 N GLN165 N PHE161 O	2.918 3.497 3.074	ALA163; GLN165; SER164; ASN162; TRP166; ARG167; GLN210; SER153; HIS393; ASP390; TRP389
Cyclohexane, 1-(1,5-dimethylhexyl)-4-(4-methylpentyl)-	−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;

(*continued on next page*)

Table 2 (*continued*)

Compounds	Binding energy (kcal/mol)	Intermolecular efficiency (kcal/mol)	Inhibition constant (mM)	H-bond interaction	Inhibitor Atom	Amino acid residue[a]	Distance of D….A (Å)	Hydrophobic interaction[a]
								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	O	ALA163 N	3.017	ALA163; TRP166; SER164; GLN165;
				N-H….O	O	SER164 N	2.894	LEU176; TRP166;
				N-H….O	O	GLN165 N	3.031	TRP389; SER364; ASP390; HIS393;
				O-H….O	O	GLN165 O	2.511	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	O	GLY204 N	2.856	ALA163; ASN162; GLN165; SER164;
				N-H….O	O	ASN248 ND2	3.013	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	O	ALA163 N	3.090	SER164; GLN165; ALA163; TRP166;
				N-H….O	O	SER164 N	2.926	TRP389; SER364; ASP390; HIS393; SER153; ARG167

[a] [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].

the hemolysin protein was determined by superimposing it with esterase EstA protein from *Pseudomonas aeruginosa*. The active-site residues SER153, HIS393, and ASP390 were considered the catalytic reaction mechanisms of the hemolysin protein of *V. harveyi* [55].

2.6. Docking protocol

AutoDock 4.0 program was used to investigate the affinity of marine algal inhibitors at the binding pocket of the 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, it 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, 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 included 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 parameter 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 [32]. PyMOL and UCSF Chimera (Version 1.8.1) software [56] were used for graphical visualization, analyzing hydrogen bond interactions, and producing quality images. Hydrophobic interactions were observed between proteins and ligands using Chimera software. All the docking calculations were performed with Intel® Core™ i3-2310M CPU@2.10GHz of a 32-bit operating system from Lenovo, with 2.00 GB RAM. AutoDock 4.0 was compiled and run under Microsoft Windows 7 operating system.

2.7. Prediction of drug-likeness of the biomolecules

An online software SwissADME web tool (http://www.swissadme.ch

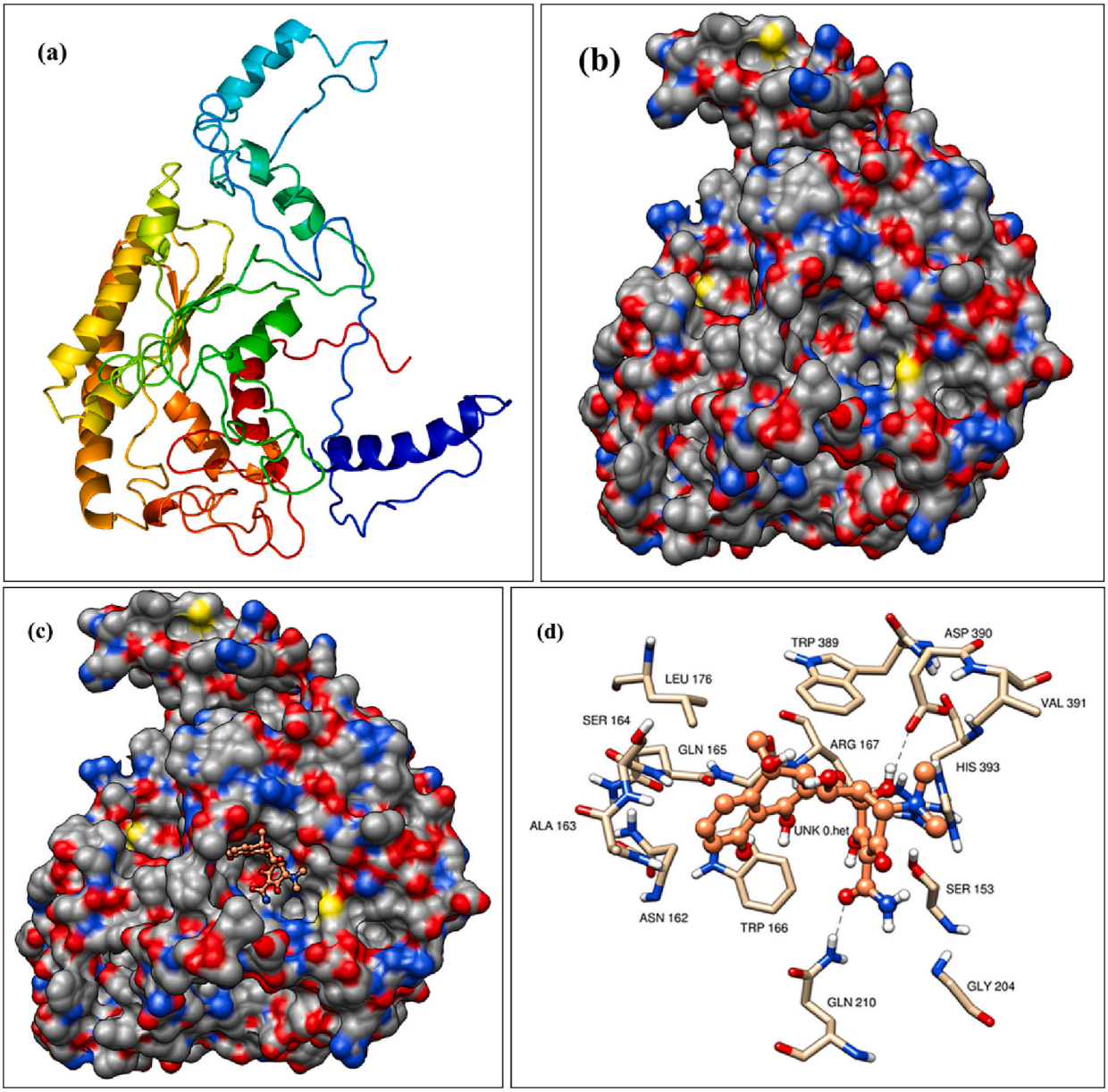

Fig. 5. The tertiary structure (a) and its surface model (b) of hemolysin protein of *V. harveyi*. The best-docked pose formed by OTC showed in (c) surface model with atoms colour, and (d) close view of docked with hemolysin protein of *V. harveyi.*

/) was followed to evaluate the information about drug-likeness properties of bio-inhibitors with the help of the Lipinski rule of five [34]. This rule helps distinguish 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 <500 Da, (ii) High lipophilicity (expressed as LogP <5), (iii) <5 hydrogen bond donors, (iv) <10 hydrogen bond acceptors, and (v) Molar refractivity should be between 40 to 130.

2.8. 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 ± 1 °C with continuous aeration. One hundred numbers of postlarvae (ABW 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}) being 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 every 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. Tryptone soya agar (TSA) and *Vibrio harveyi* selective agar (VHSA) was used to count the total number of heterotrophic bacteria and luminous

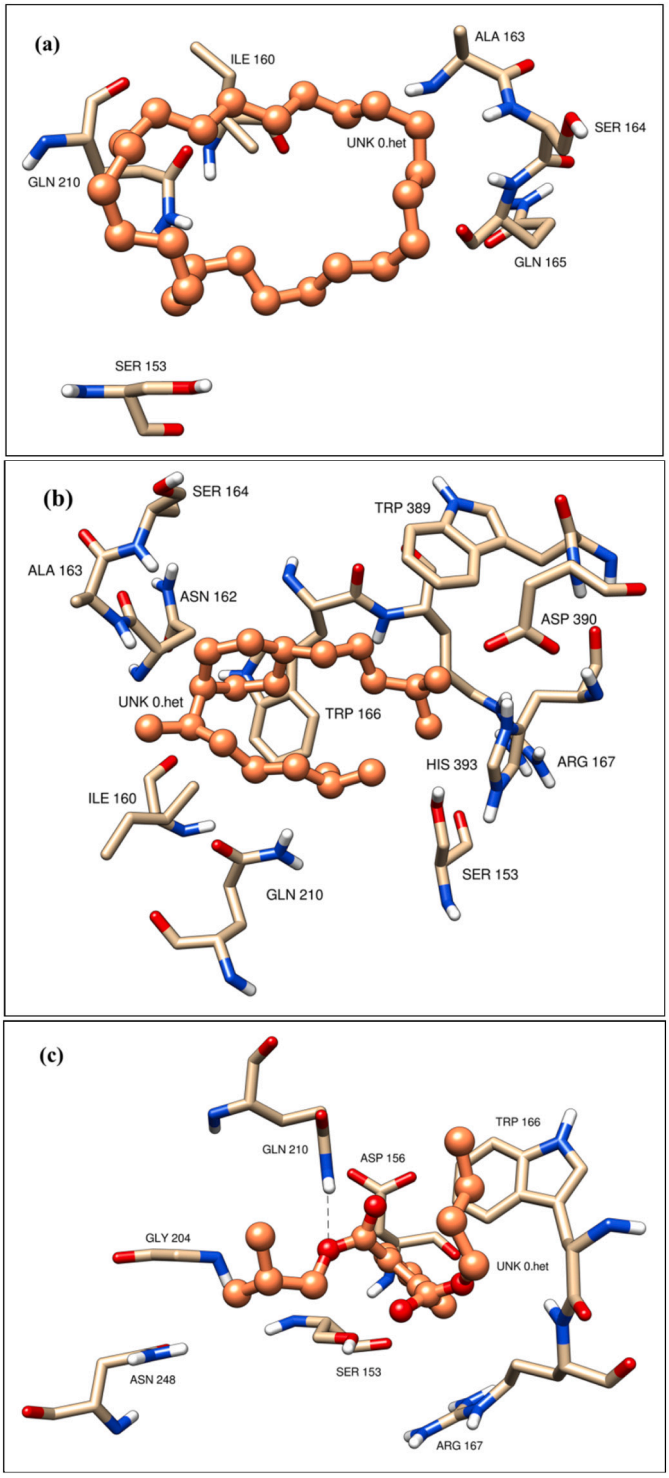

Fig. 6. The best-docked pose of bioactive compounds from *K. alvarezii* (a) cyclotetracosane, (b) cyclohexane,1-(1,5-diethyl hexyl)-4-(4-methyl pentyl)- and (c) 1,2-benzenedicarboxylic acid, butyl 2-methyl propyl ester with hemolysin protein.

V. harveyi [49]. All the experimental tubs were covered on the top to avoid the possibility of any external contaminations. For each experiment, a triplicate was maintained and average values were presented with SD [57].

2.9. 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 the *K. alvarezii* extract. Then the *V. harveyi* was identified by VHSA medium, later inoculated into LB broth and incubated at 28 °C/100 rpm for 24 h. After, 24 h, the spent culture of *V. harveyi* was analyzed for virulence factors such as hemolytic and phospholipase activities, extra crude protein (Bacteriocin), luciferase, bioluminescence, and 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 (++++) [46].

2.10. Statistical analysis

Each test was performed in triplicate 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.

3. Results

3.1. Isolation of Vibrio harveyi, characterization with MIC and MBC

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. 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. The MIC and MBC of *K. alvarezii* extract against *V. harveyi* were observed at 40 μg and 90 μg concentrations respectively.

3.2. 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 Figs. 4 and S1 and S2 and Table 1. GC–MS analysis has revealed a total of 34 compounds. Under molecular docking, there are 34 compounds (inhibitors) used for docking analysis against *V. harveyi* hemolysin protein and interaction was found 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 the hemolysin protein of *V. harveyi* and the remaining 18 compounds did not interact. The results of the interactions found between the hemolysin protein of *V. harveyi* and algal inhibitors and the 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 the 3D structure/view (Fig. 5). Based on the results obtained from the molecular docking of *K. alvarezii*, the three best 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

Table 3
Predicting drug-likeness of biomolecules from *K. alvarezii* based on the Lipinski rule of five by the Swiss ADME web tool.

Compounds	Molecular mass <500 Da	High lipophilicity (expressed as LogP<5)	<5 hydrogen bond donors	<10 hydrogen bond acceptors	Molar refractivity between 40 to 130
1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl 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

Fig. 7. *K. alvarezii* extracts challenging against *V. harveyi* during *P. monodon* larviculture in 30 days period.

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. 6). 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).

3.3. 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 (Fig. 7). A reduction of CPM on PL was noticed at 29.70 % as compared to the 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 in a darkroom. The maximum decrease in *V. harveyi* counts was observed on the 5th, 10th, 15th, 20th and 25th days, and the mean values for treatment were 6.55×10^4 CFU mL^{-1}, 3.21×10^4 CFU mL^{-1}, 3.15×10^3 CFU mL^{-1}, 4.85×10^3 CFU mL^{-1} and 8.30×10^3 CFU mL^{-1} as compared to control (1.17×10^5 CFU mL^{-1}, 2.62×10^5 CFU mL^{-1}, 7.40×10^4 CFU mL^{-1}, 2.02×10^4 CFU mL^{-1} and 2.53×10^4 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 the extract alone slight brownish colour formation was noticed as compared to the control due to the unpurified nature of the extract.

3.4. 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* extracts treatment controlled virulence factors such as luminescence, proteases, crude bacteriocin, cell surface hydrophobicity (SAT assay), hemolysis, and phospholipids of *V. harveyi* on all days of sampling. The results of growth and virulence factors affected by *K. alvarezii* extract during treatment against *V. harveyi* are shown in Table 5. The reductions in growth and virulence factors of *V. harveyi* were observed against *K. alvarezii* extract on all the sampling days during larviculture, compare to control. Also, in both control and treatment tubs the *V. harveyi* counts are dominating on all the days, whereas lower herterotrophic levels are lower in both the tubs as compared to *V. harveyi* counts (Figs. 8 and 9). Therefore, the heterotrophic counts did not contaminate the treatment

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	2.78×10^6	3.30×10^4	3.56×10^4	2.26×10^4	1.01×10^4	6.00×10^4	1.36×10^4	NA
V. harveyi	2.14×10^6	6.55×10^4	3.21×10^4	3.15×10^3	4.85×10^3	8.30×10^3	9.95×10^3	
Control tubs (CFU mL^{-1})								
Total plate count	2.59×10^6	1.45×10^5	2.74×10^5	1.46×10^4	7.80×10^4	1.88×10^4	2.06×10^4	
V. harveyi	1.86×10^6	1.17×10^5	2.62×10^5	7.40×10^4	2.02×10^4	2.53×10^4	1.53×10^4	
The average weight of postlarvae (mg)								
Treatment tubs	16.90 ± 30	60.50 ± 40	121.30 ± 6 0	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.

and control tubs.

3.5. 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.

4. Discussion

Gas chromatography and Mass Spectrometry (GC–MS) is an analytical technique for the quantitative determination of volatile compounds [58], and GC-MS is widely used in practical fields, such as food, environmental, forensic, and pharmaceutical analysis [59]. Aromatic characterization of heterotrophic microalgae *Crypthecodinium cohnii* has been performed using GC–MS analysis during different growth phases, and significant results were found during the study [60]. Similarly, in our findings, the volatile compounds present in the *K. alvarezii* extract was used as a ligand molecule in the docking studies. Hemolysin protein, which is a major pore-forming exotoxin produced by among the pathogenic *Vibrios,* which may cause lysis of RBC and other cells by producing spores on the cytoplasmic membrane. Therefore, it is recognized as an important virulence factor [4,61]. The dry lab/net lab research approach predicts the orientation of the ligand in a complex that the ligand forms by itself with proteins or enzymes [30]. 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 [62]. Among them, AutoDock is one of the main tools being used in the field of drug development industries, etc. [63]. 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 for 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 [64] 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 their 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 [65]. 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 we have also 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-phenyllactate, ranked as top antagonists against OmpF. Further, pharmacokinetics and toxicology studies corroborated that sulfated polysaccharides possessed drug-likeness and highly progressed in all parameters [66]. 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 [67]. 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 [68]. 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

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 600 nm)		Phospholipase activity[a]		Hemolytic activity[a]		Cell surface hydrophobicity SAT (M)[b]		Luminescence production (CPS)		Luciferase production (CPS)		Crude bacteriocin (OD 660 nm)		Protease production (OD 440 nm)	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated
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	2.01 ± 0.07	0.18 ± 0.00	0.14 ± 0.00
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	1.945 ± 0.05	0.16 ± 0.00	0.12 ± 0.00
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	1.87 ± 0.06	0.17 ± 0.00	0.13 ± 0.00
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	1.74 ± 0.03	0.13 ± 0.00	0.13 ± 0.00
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	1.66 ± 0.05	0.27 ± 0.01	0.12 ± 0.00
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	2.25 ± 0.07	0.35 ± 0.01	0.12 ± 0.00
Statistical analysis	$p < 0.05$		NA		NA		$p < 0.05$		$p < 0.05$		$p < 0.05$		$p < 0.05$		$p < 0.05$	

[a] Activity of *V. harveyi*: – = non-existent, + = weak, ++ = moderate, +++ = high, ++++ = very high.
[b] SAT test - (0.0 to1.0 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); NA- Not applicable.

Carbohydrate Recognition Domain (CRD) of the human "Asialoglycoprotein" receptor (ASGP-R) with monomer sugar molecules from brown seaweed *Laminaria hyperborea*, *Ascophyllum nodosum*, and *Macrocystis pyrifera* and 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 [69]. 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 that possessed anti-dengue activity that could interrupt the fusion process of the dengue virus by inhibiting the hinge region movement and by blocking the conformational rearrangement in the envelope protein [70].

The top-ranked pose with the lowest docked binding affinities/scores is generally used as a standard selection in most docking programs [31]. Likely, lower the docking score was showed a higher binding affinity and was considered as better inhibiting biological activity [71]. The preliminary screening helps to compare the docking scores of standard drug/control molecules against ligands. Then the pre-screened ligands were validated using Autodock version 4.0 [32]. 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 [31].

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 [42]. Seaweeds and other marine organisms possess numerous bioactive compounds that are useful for various health benefits for humans [15]. The in vitro antibacterial activity of extracts of *K. alvarezii* has been reported against human bacterial pathogens [36]. Various antimicrobial assays were performed with clinical strains of *E. coli* to analyze the antibacterial activity of *K. alvarezii* mediated silver nanoparticles [72]. It was also studied the antagonistic activities of *K. alvarezii* and *K. striatum* against several infectious agents [35]. Disc diffusion assay results indicated that the extract of red alga *K. alvarezi* was more efficient against *Bacillus cereus*. Among the fatty acids determined levoglucosenone and 4-Pyridinemethanol were present in high percentages [73]. Physicochemical impact of bioactive terpenes on the microalgae biomass structural characteristics was studied in the following algae such as Spirulina (*Arthrospira platensis*) and *Chlorella* (*Chlorella vulgaris*) and found significant results at ($p < 0.05$) level [74]. Detection of microalgae single-cell antioxidant and electrochemical potentials by gold microelectrode and Raman microspectroscopy combined with chemometrics was studied. In this study it explained the influence mechanism by which the intracellular phenolic and flavonoid contents of microalgae influence their surface electric charge. This study could be applied to the real-time functionality evaluation during microalgae cultivation [60]. Marine algal lipids inhibit microbes by disrupting the cellular membranes of bacteria, fungi, and yeasts [75]. 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 [75].

The anti-hemolytic activity of the filamentous seaweed, *Enteromorpha intestinalis* exhibited H_2O_2-induced hemolytic activity and was known against many pathogenic bacteria [76]. Further, diterpene isolated from brown seaweed *Canistrocarpus cervicornis* caused antihemolytic activity, as well as fibrinogen or plasma clotting induced by *Lachesis muta* snake venom protein, was reported [77]. Resveratrol is a naturally occurring phytoalexins, which inhibits the virulence of *V. harveyi* by reducing the activity by directly binding to the active center of *V. harveyi* hemolysin [78]. A natural flavonoid gastrodin

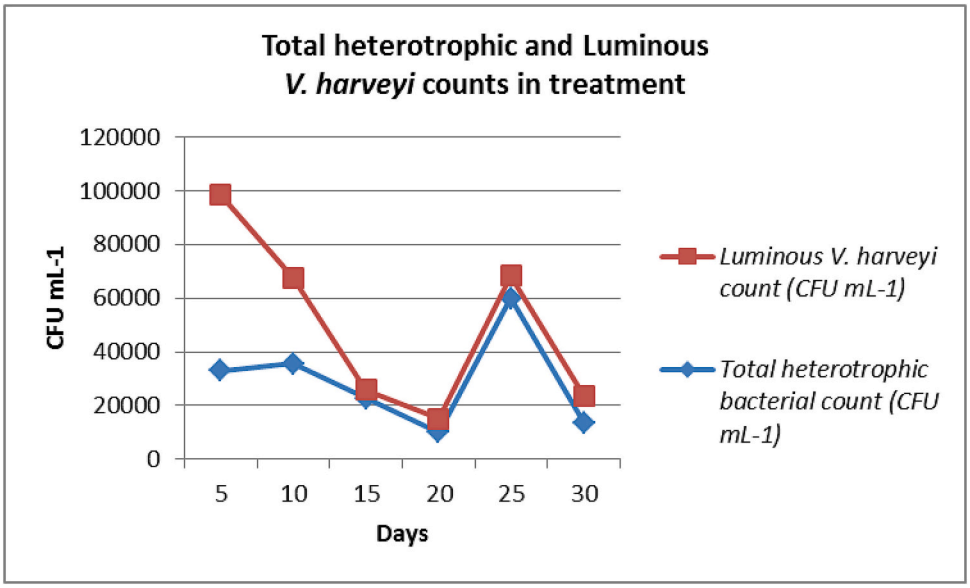

Fig. 8. Differential counts of total heterotrophic and luminous *V. haveyi* counts in treatment tubs during *K. alvarezii* extracts challenging against *V. harveyi* during *P. monodon* larviculture in 30 days period.

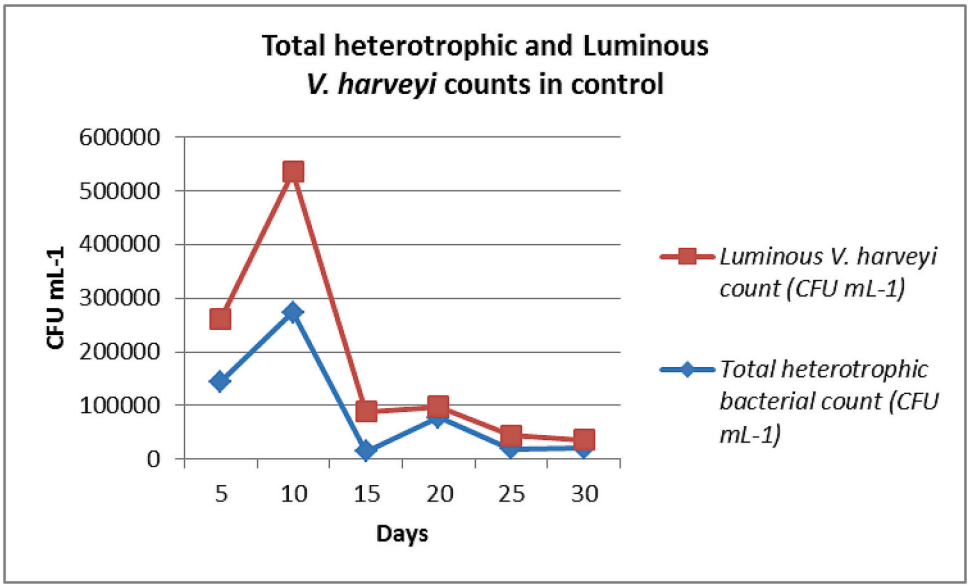

Fig. 9. Differential counts of total heterotrophic and luminous *V. haveyi* counts in control tubs during *K. alvarezii* extracts challenging against *V. harveyi* during *P. monodon* larviculture in 30 days period.

relieves *V. harveyi* infection by blocking hemolysin active centers and it acts as an effective antitoxin agent for *V. harveyi* hemolysin (*Vhh*) [8]. Futher honokiol is a natural compounds that inhibits *Vhh* virulence by reducing its haemolytic activity at non-bacteriostatic concentrations. It can effectively inhibit the damage caused by *Vhh* and *V. harveyi* both in vitro and in vivo. RT-PCR and molecular docking analysis showed that although honokiol up-regulated the transcription level of *Vhh*, it could block the active centre of *Vhh* directly and forcefully, thereby reducing the catalytic activity of *Vhh* [79]. 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-diethylhexyl)-4-(4-methylpentyl) and 1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester respectively when compared to the 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 to effectively treat 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 [80]. Moreover, an in-vitro inhibition activity test showed that phycocyanin inhibits human salivary amylase by an average of 51.13 % which 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 research results

also indicate that biomolecules identified from *K. alvarezii* are an efficient bio-agent against *Vhh*, which can be further developed into a new drug for the treatment of *Vibrio* infection. These results support the evaluation of the present molecular docking studies of the extracts of *K. alvarezii* against the hemolysin protein of *V. harveyi* in aquaculture practices.

5. Conclusions

The results indicated that the bioinhibitors showed better docking scores and showed higher binding affinity, with 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 *K. alvarezii* extract during the experiment. In the larviculture trial, *K. alvarezii* exhibited an improved survival levels on *P. monodon* postlarvae compared to the control. Hence, the present findings indicate that the marine seaweed *K. alvarezii* may be a significant scope of source for developing potent bio-inhibitors in shrimp disease management.

CRediT authorship contribution statement

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.

Declaration of competing interest

The authors contributed equally to this work.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2023.103207.

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