



Antibacterial activity of a novel compound isolated from *Bacillus licheniformis* for treating bacterial infections in fishes: An in-silico approach

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Abstract

Aeromonas hydrophila is a fish pathogen which is widely associated with diseases related to freshwater fishes. *Vibrio parahaemolyticus* is a major globally emerging marine pathogen. Seven novel compounds were extracted from the ethyl acetate extract of *Bacillus licheniformis*, a novel marine bacterium isolated from marine actinomycetes. The compounds were identified using Gas Chromatography-Mass Spectroscopy (GC–MS). Only one bioactive compound having potent antibacterial activity was virtually screened to understand its drug-like property according to Lipinski's rule. The core proteins, 3L6E and 3RYL from the pathogens, *A. hydrophila* and *V. parahaemolyticus* were targeted for drug discovery. In the present in-silico approach, Phenol,2,4-Bis(1,1-Dimethylethyl) a potent bioactive compound present in *Bacillus licheniformis* was used to prevent the infection due to the two pathogens. Further, using this bioactive compound, molecular docking was done to block their specific target proteins. This bioactive compound satisfied all the five rules of Lipinski. Molecular docking result revealed the best binding efficacy of Phenol,2,4-Bis(1,1-Dimethylethyl) against 3L6E and 3RYL with – 4.24 kcal/mol and – 4.82 kcal/mol, respectively. Molecular dynamics (MD) simulations were also executed to determine the binding modes as well as the stability of the protein–ligand docking complexes in the dynamic structure. The in vitro toxicity analysis of this potent bioactive compound against *Artemia salina* was carried out, revealing the non-toxic nature of *B. licheniformis* ethyl acetate extract. Thus, the bioactive compound of *B. licheniformis* was found to be a potent antibacterial agent against *A. hydrophila* and *V. parahaemolyticus*.

Keywords Phenol,2,4-Bis(1,1-Dimethylethyl) · Bioactive compound · Molecular docking · Molecular dynamics and simulation · Drug-like nature · In vitro toxicity

Introduction

Aeromonas hydrophila falls under the genus *Aeromonads* which is a rod-shaped gram-ve, non-spore forming, and facultatively anaerobic bacterium found widely in the aquatic environment [1]. *A. hydrophila* is a mesophilic foodborne fish pathogen in aquaculture that can eventually lead to mass

mortalities. *A. hydrophila* is an inhabitant of benthic sediments, inland water environments and especially in freshwater microflora of fishes [2].

Vibrio parahaemolyticus is a gram-negative, facultative anaerobic bacterium found in temperate, tropical coastal regions encountered in aquaculture. It is the main marine pathogen responsible for substantial economic losses in the aquaculture industry [3]. Consumption of half cooked, raw or seafood such as prawn, shrimp, fish, and shellfish which are contaminated is considered as the most common cause for *V. parahaemolyticus* infection [4].

A study was conducted by Zhou et al. [5] on the emerging *A. hydrophila* causing infection in freshwater whiteleg shrimp, *Litopenaeus vannamei*. This was the first study reported on the shrimp against the pathogenic bacteria *A. hydrophila*. They suggested a synergistic effect against *A.*

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hydrophila. Similarly, a study was conducted recently by Rathinam [6] on the *Aeromonas* infection in fishes using scientometric mapping across the world. They revealed that *Aeromonas* infection has the ability to cause major economic losses to fish farmers around the world. Thus, more research work is required to prevent this infection.

A recent study on the resistance mechanism of a pufferfish, *Tetraodon nigroviridis* against *V. parahemolyticus* infection was carried out by Jiang et al. [7]. They performed a multi-omic study and explored the molecular mechanisms and immune responses of *T. nigroviridis* to *V. parahemolyticus* infection. The results obtained from the multi-omic study showed higher consistency that indicated the reliability of generated sequencing data using the quantitative real-time polymerase chain reaction (qRT-PCR).

Bacillus licheniformis falls under the category of marine *Actinomycetes* which is a gram-positive bacterium. The present research was carried out to check the potency of the bioactive compound, Phenol,2,4-Bis(1,1-Dimethylethyl) extracted from *B. licheniformis* against the pathogens, *A. hydrophila* and *V. parahemolyticus*. In the current study, we extracted a novel bioactive compound, Phenol,2,4-Bis(1,1-Dimethylethyl) from the ethyl acetate extract of *B. licheniformis* and identified it using GC–MS. We carried out an in vitro toxicity analysis on *Artemia salina* against this novel bioactive compound to reveal the non-toxic nature of *B. licheniformis* ethyl acetate extract. In addition, we also performed an in-silico study using molecular docking to check the binding efficacy of Phenol,2,4-Bis(1,1-Dimethylethyl) against two proteins, 3L6E and 3RYL from the pathogens, *A. hydrophila* and *V. parahemolyticus*, respectively. ADMET analysis was also carried out to check the drug-likeness of the antibacterial compound and also to confirm whether it obeys all Lipinski's rule of five. Further, molecular dynamics and simulation studies were carried out to check the stability of the drug.

Materials and methodology

Extraction of compound from *Actinomycetes*

Bacillus licheniformis was inoculated into International Streptomyces Project-2 (ISP-2) medium/ Starch Casein broth for preparation of the inoculum. Later, the broth culture was kept in a rotary shaker incubator and harvested for 7 days consecutively at 30 °C. Further, it was filtered through a Whatman No. 1 filter paper and the filtrate was centrifuged at 10,000 rpm for 20 min at 4 °C. The cell free supernatant (CFS) of *Bacillus licheniformis* was extracted 2–3 times with ethyl acetate in 1:1 ratio using a separating funnel. The solvent layer was collected. The liquid extract from the solution was evaporated using the rotary

evaporator at 60 °C for 60 min to concentrate the extract solution [8]. The bioactive compounds were obtained from the concentrated ethyl acetate extract of *B. licheniformis*.

Gas chromatography—mass spectrometry (GC–MS) analysis

GC–MS analysis of the *B. licheniformis* ethyl acetate extract was performed using a GC–MS (Agilent 6890/Hewlett-Packard 5975) consisting electron impact (EI) mode. The active compounds extracted from the *B. licheniformis* extract were later identified using the GC–MS. TurboMass version 5.4.2. software was used to handle the chromatograms and mass spectra. The identification of the bioactive compounds was performed by comparing retention times from the chromatograph with the authentic compounds, and the spectral data present in the National Institute Standard and Technology (NIST) library database of those corresponding compounds [9]. The interpretation of the mass spectra analysis was carried out using the NIST library that was used to search for the spectrum of several unknown compounds from the *B. licheniformis* [10].

Protein database

Universal Protein Resource (UniProt) is a comprehensive, high-quality, accessible protein database that provides information about the functional annotation and stability of the protein sequence [11]. Most of the entries made in the UniProt were obtained from Genome Sequencing Projects. It contains loads of information associated with the biological functioning of the proteins. It was obtained from published literature and is maintained by the UniProt Consortium since 2008. This protein database was formed with the collaboration of the Swiss Institute of Bioinformatics (SIB), Protein Information Source (PIR), and the European Bioinformatics Institute (EBI). In the present in silico study, this database has been used to collect information related to the protein targets [11].

Microorganism used in the study

B. licheniformis was used to extract these bioactive compounds. The bioactive compounds present in this bacterium were reported to have significant antibacterial properties against fish pathogens like *Aeromonas hydrophila* and *Vibrio parahemolyticus*. Some of the bioactive compounds have anti-bacterial, anti-cancerous, and anti-oxidant properties [11].

Structural database

Protein Data Bank (PDB) is a universal platform that acts as a repository for 3D structures of nucleic acid complexes, and proteins. Research Collaboratory for Structural Bioinformatics (RCSB PDB) is a structural database used in structural biology, molecular biology, computational biology, and so on. It builds upon the data by gathering information and creating resources and tools in research and education. The RCSB PDB helps in curating and annotating the PDB data. In this in-silico study, proteins with PDB IDs, 3L6E and 3RYL were used as protein targets [11, 12].

Compound database

PubChem is a freely accessible open chemistry database. It is maintained by the National Center for Biotechnology Information (NCBI) which is a part of the National Institute of Medicine, a component of the National Institute of Health (NIH). It consists of information regarding substances and small molecules. In addition it also contains chemically modified structures and macromolecules. The bioactive compounds that have been used as ligand in this study were collected from PubChem [13].

Computational analysis using Swiss ADME

Swiss Absorption, distribution, metabolism and excretion (ADME) is a web tool that is freely accessible to a pool of fast and robust models [14]. It allows free access for the computation of physicochemical descriptors as well as prediction of ADME parameters, medicinal chemistry likeliness, drug likeliness, and pharmacokinetic properties of small molecules to help in drug delivery [15]. The PubChem server was accessed to get information regarding canonicals Simplified molecular input line entry system (SMILES). The bioactive compounds are typed or pasted directly in SMILES format as input. In the present study in-silico approach was adopted to find the physicochemical properties (molecular weight, lipophilicity, polarity, water solubility), Bioavailability score, and drug-like nature (Lipinski's rule of five) of the bioactive compounds [11, 14].

Evaluation of in vitro toxicity of ethyl acetate extract of Actinomycetes using *Artemia salina*

The toxicity of the *B. licheniformis* ethyl acetate extract was tested in brine shrimp [16, 17]. The eggs of *Artemia salina* were hatched in seawater at pH 7.8 for the bioassay.

The eggs were added to natural seawater in a glass tank with continuous aeration. The nauplii (larvae) of *A. salina* were collected after 24 h of incubation at 26–30 °C room temperature using a Pasteur pipette. The larvae were brought to one side of the chamber by attracting with a light source.

1 mL of natural seawater was added into each of the well plates (Tarsons Pvt Ltd, India). 10 hatched *A. salina* were added into each of the wells. Then *B. licheniformis* ethyl acetate extract of different concentrations (50–300 µg/mL) were added to the wells and incubated at room temperature for 24 h. After 24 h, using the magnifying lens the mortality rate of artemia were counted. In other words, the total number of both dead as well as live organisms were counted. This experiment was observed for 24, 48, 72 and 120 h and performed in triplicates. The bacterial extract was not included in the control group. The mean value of the triplicates was calculated to determine the toxicity [9].

Molecular docking analysis

Molecular docking study was carried out to determine the binding affinity of the bioactive compounds of *B. licheniformis* with the core proteins using the software AutoDock 4.2 (released version 4.2.6) [18]. It was used to analyze the protein–ligand interaction so that the ligand was able to select their specific binding sites. The proteins and ligand were prepared using the PyMOL software. The downloaded protein (.pdb) file was imported in the PyMOL software where the unwanted chains, water molecules as well as the ions were removed and saved. The downloaded ligand (.sdf) file was also imported in the PyMOL software and saved in the PDB (.pdb) file format. The proteins were at first imported into the autodock software workspace and the target protein (.pdb) file was chosen. The target proteins were prepared by adding the polar hydrogen followed by Gasteiger-Marsili and Kollman partial charges. The ligands were first prepared after the active sites were fixed with specific preferential residues. Both the proteins and ligand were saved in PDBQT file format. A grid box of dimensions 90 × 90 × 90 Å was added and set over the entire position of the protein for the search. Later, the grid space of 0.375 nm was set. The Lamarckian genetic algorithm 4.2 was used to autodock the ligands with the target proteins. It was also used to generate the ligand conformers bound to the target proteins. The hydrogen bond and binding affinity efficacy were analyzed using AutoDock version 4.2, Protein–ligand interaction profiler, and PyMol version 2.3.2. The protein–ligand complex with the least binding energy was specifically selected as the most stable conformation. The protein–ligand interaction was further subjected to molecular simulation studies [9, 19].

Molecular dynamics (MD) and analysis of simulation studies

Two independent molecular dynamics simulations (2—protein–ligand complexes) were performed using GROMACS 4.3 software [9]. MD simulations were carried out to check the accuracy of docking and stability of protein–ligand complex. First, the ligand topology was prepared by implementing the Groningen Molecular Simulation (GROMOS) force field using the Prodrgr server [20, 21]. The complexes were solvated by incorporating the Single point charge (SPC) water model within the dodecahedral box. In addition, the minimization of energy per complex was carried out by steepest descent algorithm [22]. Further, using the isothermal-isobaric ensemble (NVT and NPT) each compound was equilibrated for minimum 100 ps. Subsequently, the MD simulations were performed for 100 ns with an integrated 2 fs at 300 K temperature. The root-mean-square deviation (RMSD) and root-mean-square fluctuation (RMSF) were calculated by analyzing the deviation of protein–ligand interaction system using the GROMACS toolbox [23]. Graphs were plotted using Xmgrace software in the Ubuntu. All figures obtained for 3D structure visualization were plotted using the PyMOL software. The 2D images for the protein–ligand complexes were generated by Ligplot + tool [24].

Molecular mechanics Generalized Born Surface area (MM-GBSA) Analysis

The binding free energy (ΔG_{bind}) for the ligand molecule was calculated by applying Prime molecular mechanics Generalized Born Surface area (MM-GBSA) algorithm [25] with the retrieved docked pose from the Glide algorithm [23]. The structural binding poses of the complex were scrutinized to gain better clarification of the binding mode by employing Schrödinger diagram tool for ligand interaction.

MM-GBSA can be interpreted by:

$$\Delta G_{\text{(bind)}} = \Delta G_{\text{(sol)}} + \Delta E_{\text{(MM)}} - T\Delta S$$

where, $\Delta G_{\text{(sol)}}$ is difference in the GBSA solvation energy of protein–ligand complexes and also sum of the solvation energies for ligand as well as unligated protein. $\Delta E_{\text{(MM)}}$ is the difference in minimized energy of the complexes and also sum of the minimized energies for ligand as well as unligated protein. $-T\Delta S$ is the conformational entropy change upon ligand binding. The polar energy is estimated using GB model, while the non-polar contribution is calculated generally by Solvent accessible surface area (SASA). Furthermore, it places the liquid in solution and calculates

the energy of the auto-generated ligand strain using VSGB 2.0 solvation model.

Results

GC–MS analysis

GC–MS analysis study of *B. licheniformis* ethyl acetate extract was performed. The chromatogram is represented in Fig. 1. Based on the NIST library search performed, the molecular formula, molecular weight and retention time for the bioactive compounds are represented in Table 1. A total number of seven compounds were identified in the ethyl acetate extract of *B. licheniformis*. The compounds obtained were Propanoic Acid, 2-Hydroxy-, Ethyl Ester, (S); Phenol, 2,4-Bis(1,1-Dimethylethyl); 1,2-Benzenedicarboxylic Acid, Diheptyl Ester; 1,2-Benzenedicarboxylic Acid, Butyl 2-Ethylhexyl E; Phenol, 2,4-Bis(1,1-Dimethylethyl); 1,2-Benzenedicarboxylic Acid, Butyl Octyl Ester, and E-2-Octadecadecen-1-ol.

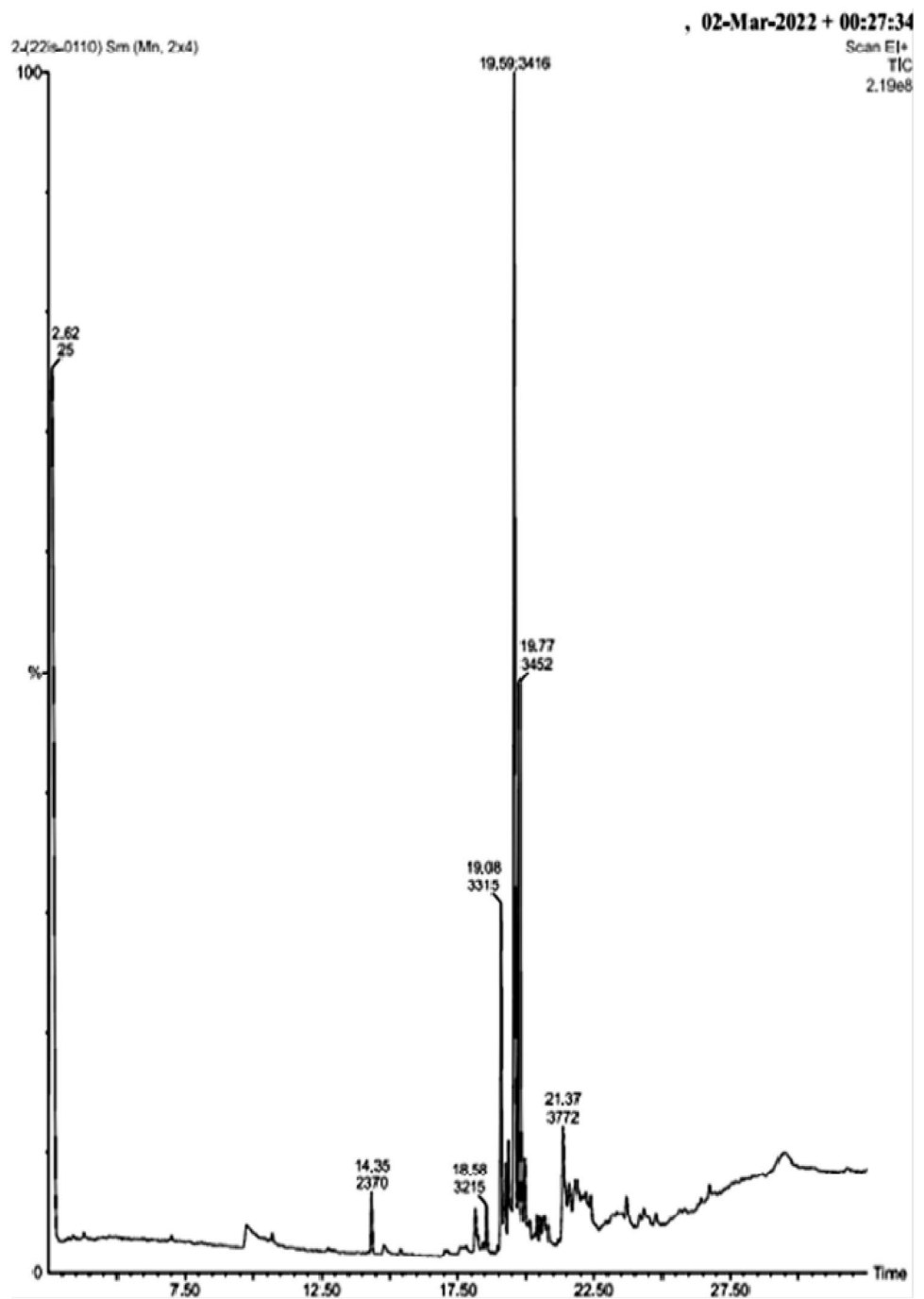
Target protein structure

Structure of two target proteins 3L6E (PDB ID) and 3RYL (PDB ID) was downloaded in PDB format from the PDB database. One novel bioactive compound was chosen to target two different proteins, 3L6E and 3RYL.

Virtual screening of the bioactive compound

Based on the parameters like drug-like nature followed by Lipinski's rule of five, the bioactive compound was virtually subjected to screening. This rule describes the ADME parameters and molecular properties which are beneficial for the pharmacokinetics of a drug in an organism's body. It is essential for the development of a pharmacologically active drug whose structure is optimized in a step-wise manner for selectivity, drug-likeness, and increased activity as per the Lipinski's rule. In general, it describes that, an orally active drug should not have more than one violation of some criteria: (a) Molecular weight (M.W) under 500 daltons (Da) (b) ≤ 5 hydrogen bond donors (c) ≤ 10 hydrogen bond acceptors (d) partition coefficient (iLOGP) of ≤ 5 (Isyaku et al. 2020). The result revealed that Phenol, 2,4-Bis(1,1-Dimethylethyl) has a molecular weight of 374.54 daltons, contains 3 hydrogen bond acceptors and 1 hydrogen donor bond, iLOGP value is 3.64, obeying Lipinski's rule of five. Therefore, according to Lipinski's rule of five, this novel bioactive compound is effective against *A. hydrophila* and *V. parahemolyticus*.

Fig. 1 GC–MS Chromatogram result of ethyl acetate extract of *B. licheniformis*



In vitro toxicity analysis of the bioactive compounds extracted from *B. licheniformis* in brine shrimps

The toxicity analysis of the *B. licheniformis* ethyl acetate extract showed that it was non-toxic to *Artemia salina*. In other words, it did not exhibit any toxic effect against the brine shrimps as the LC_{50} value of the ethyl acetate extract was observed as 200 $\mu\text{g}/\text{mL}$. After 24 h observation, 90% of the *Artemia* had survived in the highest concentration (200 $\mu\text{g}/\text{mL}$) of the ethyl acetate extract. This revealed that

the level of toxicity of the *B. licheniformis* extract was minimal. No mortality was observed after 24 h upto 300 $\mu\text{g}/\text{mL}$ concentrations. Hence, the extract was considered as non-toxic and safe for aquatic animals.

Molecular docking analysis of 3L6E and 3RYL

The procedure for docking started with 10 runs. AutoDock version 4.2 was used to analyze the binding efficacy of one bioactive compound with two different target proteins in this

Table 1 Analysis of bioactive compound activities of the ethyl acetate extract of *B. licheniformis*

| S.No | Compounds | Major peak area | Area (%) | Retention time (RT) (min) | Major activities* present |
|------|---|-----------------|----------|---------------------------|---|
| 1 | E-2-Octadecadecen-1-ol | 3772 | 19.27 | 21.37 | Antibacterial, Anticancer |
| 2 | 1,2-Benzenedicarboxylic acid, Butyl Octyl Ester | 3452 | 17.64 | 19.77 | Arachidonic acid inhibitor |
| 3 | Phenol, 2,4-Bis(1,1-Dimethylethyl) | 3416 | 17.46 | 19.59 | Antibacterial |
| 4 | 1,2- Benzenedicarboxylic Acid, Butyl 2-Ethylhexyl E | 3315 | 16.94 | 19.08 | Anticancer |
| 5 | 1,2-Benzenedicarboxylic Acid, Diheptyl Ester | 3215 | 16.43 | 18.58 | Arachidonic acid inhibitor |
| 6 | Phenol, 2,4-Bis(1,1-Dimethylethyl) | 2370 | 12.11 | 14.35 | Antibacterial |
| 7 | Propanoic Acid, 2-Hydroxy-, Ethyl Ester, (S) | 25 | 0.1278 | 2.62 | Antibacterial, Anticancer, Antidiabetic |

*Dr. Duke's phytochemical and ethnobotanical database [26]

The bioactive compounds of area % were arranged in descending order [27]

in-silico study. The binding energy distribution of 9 ligand poses was analyzed for identifying the best binding pose. Based on the binding energy, the binding affinity of this bioactive compound is predicted. If more than two compounds have similar binding energy then the hydrogen bond can also be considered. The docking results revealed that Phenol,2,4-Bis(1,1-Dimethylethyl) had the best binding affinity against 3L6E and 3RYL with -4.24 kcal/mol and -4.82 kcal/mol, respectively.

The binding pattern of 3L6E with the ligand revealed that in most of the conformations the conservation of various residues was involved in the ligand interactions. However, the interactions of the ligand were formed in different chains but with similar residue. The interaction analysis showed the presence of a conventional hydrogen bond between GLU106 and N atom of the ligand. ILE101 also forms a hydrogen bond with the ligand. A Pi-Alkyl hydrophobic interaction was observed between the ARG102 residue and the ligand (Fig. 2a and b). Table 2 represents the binding affinity of the ten ligand conformations with 3L6E.

The binding pattern analysis of the ligands with 3RYL was observed in five residues ASP252, LEU249, LEU253, LEU260 and LEU268. ASP252 residue was involved in the formation of a hydrogen bond with the ligand. 2 residues of Pi-Sigma, LEU253 and LEU268 formed interactions with the ligand. Residues LEU249 and LEU260 also formed Pi-Alkyl hydrophobic interactions with the ligand (Fig. 3a and b). Table 2 summarizes the binding efficacy of the 10 ligand conformations with 3RYL.

Molecular dynamics (MD) and simulation analysis

The protein structures from the starting of the complex and their production dynamics was computed and later plotted to calculate the root-mean-square deviation (RMSD) of the atoms of 3L6E and 3RYL proteins as shown in Fig. 4a and

b. The figures reveal that both the systems deviate from their initial structures.

3L6E-ligand complex initially showed maximum fluctuation which is evident from the bigger drifts, leading to a backbone RMSD of 2 nm for 2.5 ns in the production dynamics. After that it showed fluctuation close to 2.5 nm of RMSD for another 10 ns. Later, till 50 ns, no fluctuation was seen, but after another 25 ns, a minimum fluctuation and smaller drifts were observed, resulting in a RMSD of 3–3.5 nm for a longer duration of 100 ns in the production dynamics. The flexibility of the ligand induced minor deviations in the protein. These fluctuations resulted in the disrupted structure of the protein which was observed at the end of the simulation time of 20 ns. The 3RYL-ligand complex exhibited a sharp rise and maximum deviation of RMSD close to 10 nm for the first 10–15 ns in the production dynamics. Later, numerous minor fluctuations close to 5 nm were observed for the next 10 ns. After that, it became stable and smaller fluctuations of RMSD 3 nm were observed and validated by its final convergence at 100 ns. The data revealed that the influence of the ligand is higher in 3L6E which is evident from its binding affinity result. The 3L6E-ligand complex peaks were found to be lying lower to the 3L6E protein peaks, and thus, the complex maintained its stability throughout the entire simulation. In the case of 3RYL-ligand complex, the peak of the protein 3RYL lies below the protein-ligand complex, indicating that the complex was not so stable compared to the protein.

The stability of the complex was also dependent on the root-mean-square fluctuations (RMSF) which is plotted in Fig. 5a and b. RMSF reflects the flexibility of residues around its mean position. Figure 5a and b reveals the residues from the chain in 3L6E-ligand complex and 3RYL-ligand complex showing major fluctuations. Higher fluctuations of the residues in both the proteins were noted. The 3L6E showed higher fluctuations from 0.2 to 0.6 nm

Fig. 2 **a** Binding mode of Phenol,2,4-Bis(1,1-Dimethylethyl) with 3L6E. **b** Interacting residues of 3L6E with the ligand. The pink-dashed lines indicate hydrophobic interactions (Pi-Alkyl) and green-dashed lines correspond to hydrogen bonds

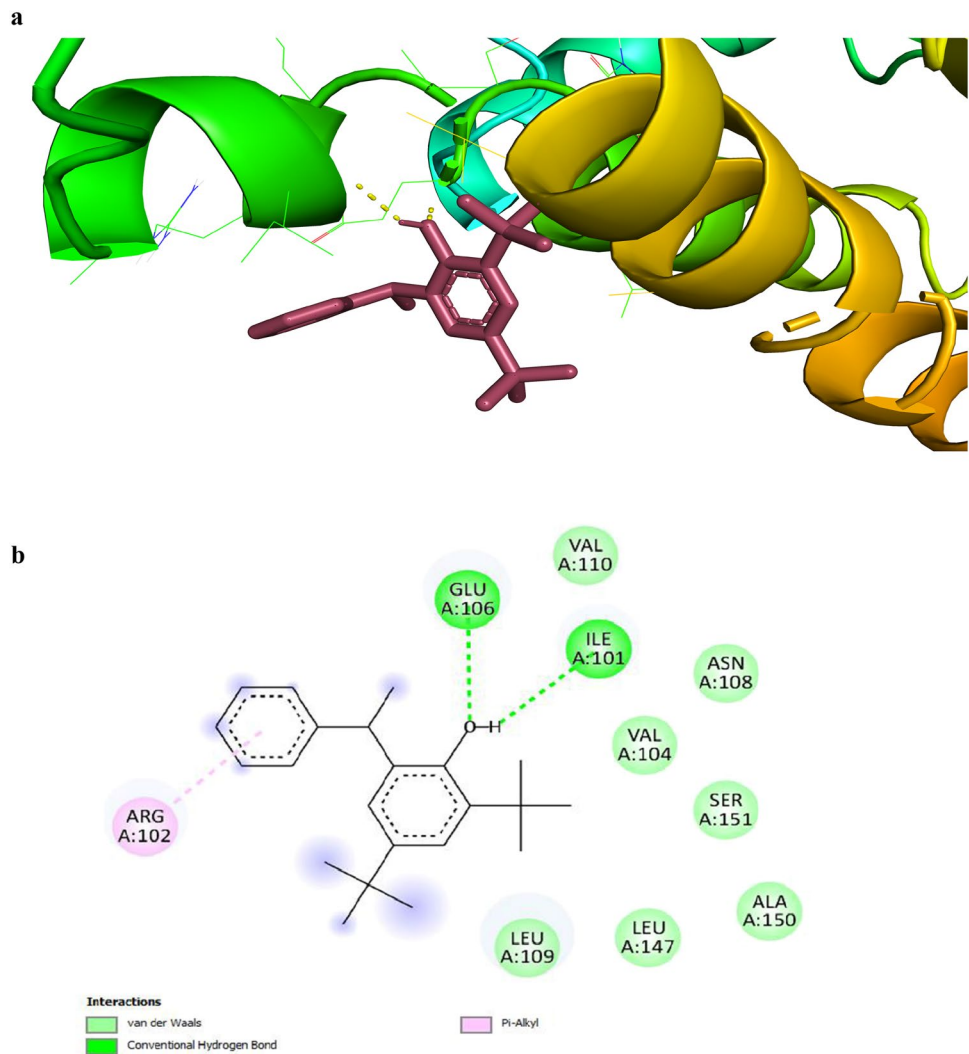


Table 2 Binding affinity of the ligand, Phenol, 2,4-Bis(1,1-Dimethylethyl) with the core proteins

| Ligand conformation | Binding energy of 3L6E (kcal/mol) | Rank | Binding energy of 3RYL (kcal/mol) | Rank |
|---------------------|-----------------------------------|------|-----------------------------------|------|
| 1 | -4.24 | 1 | -4.82 | 1 |
| 2 | -4.11 | 1 | -4.63 | 2 |
| 3 | -4.19 | 2 | -4.40 | 3 |
| 4 | -4.05 | 3 | -4.36 | 4 |
| 5 | -3.99 | 4 | -4.35 | 5 |
| 6 | -3.91 | 5 | -4.04 | 6 |
| 7 | -3.91 | 6 | -3.86 | 7 |
| 8 | -3.75 | 7 | -3.80 | 8 |
| 9 | -3.57 | 8 | -3.64 | 9 |
| 10 | -3.42 | 9 | -3.42 | 10 |

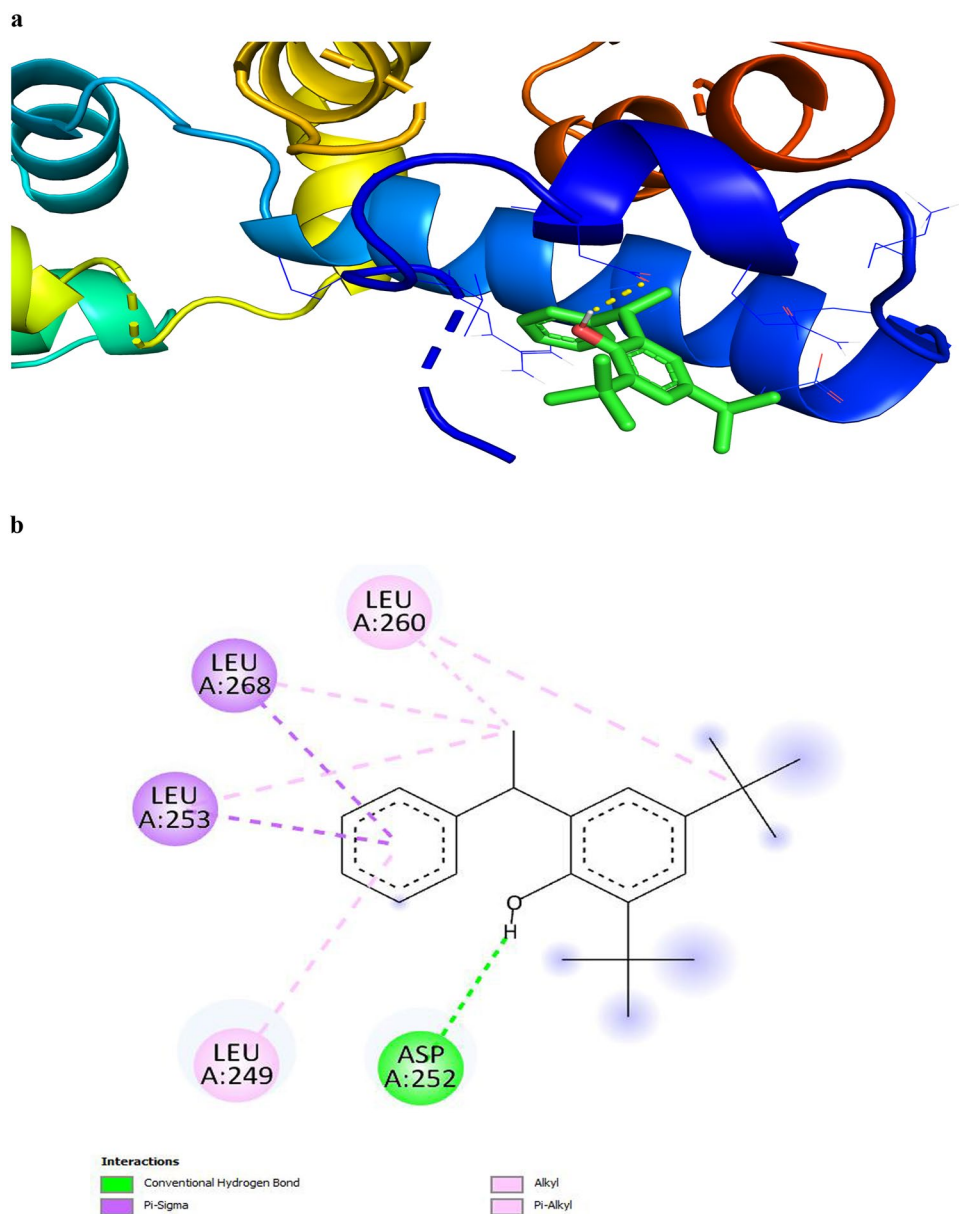
while the fluctuations were found to be 0.5 to 1 nm in the case of 3RYL protein.

The results suggest that the ligand exhibited an influence on the residues showing major fluctuations.

MM-GBSA analysis-

The calculations of free energy (ΔG) with MM-GBSA were interpreted using the VSGB solvation model. The rescoring analysis of MM-GBSA was performed to nullify the incorrect positive predictions. The binding free energy (ΔG_{bind}) values for both the complexes and their individual input for total energy are summarized in Table 3. The calculations revealed that 3L6E-complex has better binding free energy (-25.74 ± 3.04 kcal/mol) compared to 3RYL-complex (-2.90 ± 1.97 kcal/mol).

Fig. 3 **a** Binding mode of Phenol,2,4-Bis(1,1-Dimethylethyl) with 3RYL. **b** Interacting residues of 3RYL with the ligand. The pink-dashed lines indicate hydrophobic interactions (Pi-Alkyl); purple-dashed lines indicate Pi-Sigma interaction, and green-dashed lines correspond to hydrogen bonds



Discussion

A pharmacologically active drug is considered to be medically effective. Nowadays, drugs having poor pharmacological properties are the prime reason of failure in the late stage of drug discovery. Hence, validation of the antibacterial properties inherent in the target bioactive compounds is necessary [28]. Marine microorganisms are unexplored and also several novel secondary metabolites are found in the oceans. They have antibacterial, and anticancer properties and hence, play a significant role in human livelihood as well as in the aquaculture industry. *A. hydrophila* is a renowned bacterial fish pathogen causing huge mass mortalities in aquaculture. Many studies have been reported on fishes and shrimps infected with *A. hydrophila* [29].

Similarly, *V. parahemolyticus* is a bacterial pathogen that has been affecting the aquaculture industry severely. In the present study, a potent bioactive compound, Phenol, 2,4-Bis(1,1-Dimethylethyl) was selected among several others that were extracted from a marine *Actinomycete*, *Bacillus licheniformis*. The result of the present investigation corroborates the result of the work of Ren et al. [30] who reported that Phenol, 2,4-Bis(1,1-Dimethylethyl) has antioxidant, antibacterial, antifungal and anticancer properties in the field of medicine. Similarly, Devi et al. [31] reported the antifungal activity and the result of molecular docking of the compound, Phenol, 2,4-Bis(1,1-Dimethylethyl) extracted from mangrove sediment derived Actinobacterium, *Kutzneria* sp. strain TSII.

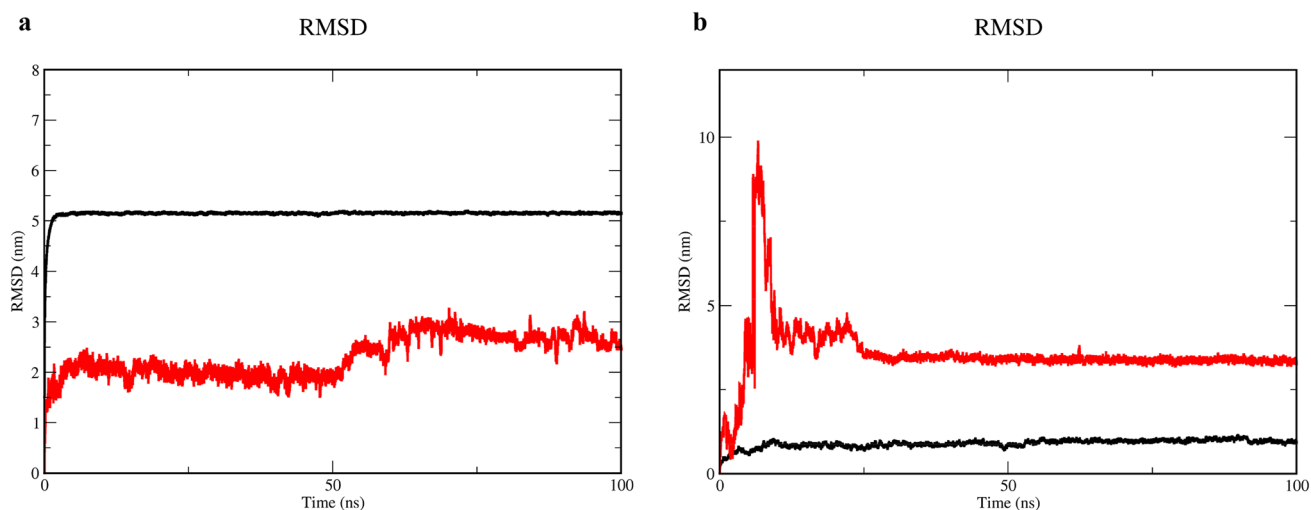


Fig. 4 RMSD plot analysis of **a** the 3L6E protein (black) and protein–ligand complex (red) showing root mean square deviation **b** the 3RYL protein (black) and protein–ligand complex (red) showing root mean square deviation

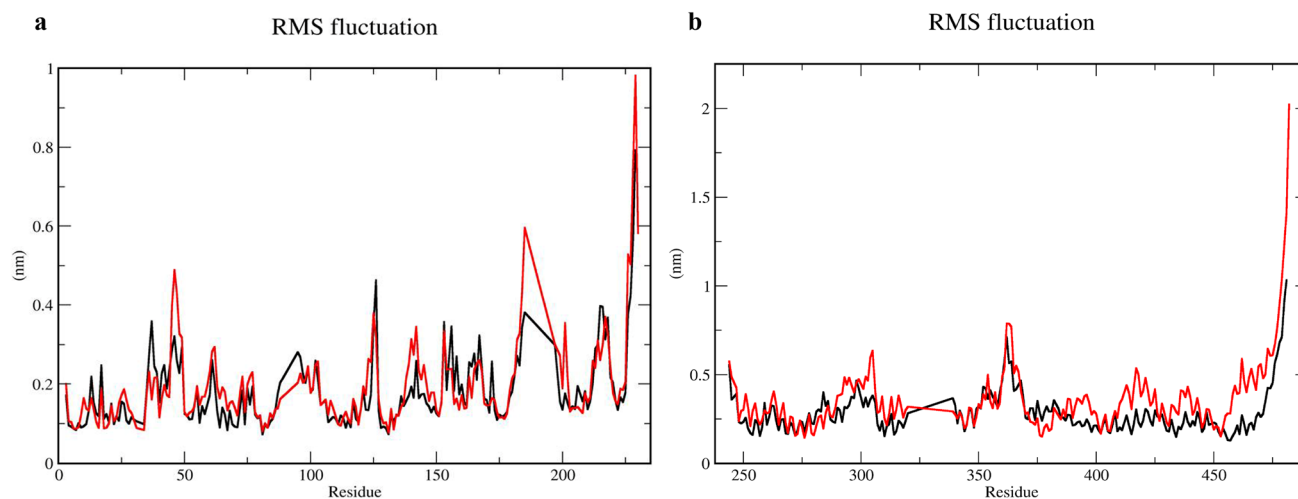


Fig. 5 RMSF plot upon binding of the ligand showing fluctuation of the residues in the core protein chains. RMSF of residues of **a** the 3L6E protein (black) chain for protein–ligand complex (red) **b** the 3RYL protein (black) chain for protein–ligand complex (red)

Table 3 Binding free energy (ΔG) of 3L6E and 3RYL complexes

| Protein ligand complex | ΔG_{sol} (kCal/mol) | ΔG_{EMM} (kCal/mol) | ΔG_{sasa} (kCal/mol) | ΔG_{bind} (kCal/mol) |
|------------------------|-----------------------------|-----------------------------|------------------------------|------------------------------|
| 3l6e-complex | 5.23 ± 2.00 | -25.74 ± 3.80 | -2.3 ± 0.35 | -25.74 ± 3.04 |
| 3ryl-complex | 0.05 ± 0.54 | 0.025 ± 0.29 | 0.013 ± 0.19 | -2.90 ± 1.97 |

Application of computational methods like molecular docking study in drug discovery has been gaining a lot of attention due to their ability in identifying and developing novel potent compounds [32]. They may later prove to be a promising antibacterial agent in the field of aquaculture and pharmaceutical research. Researchers from various areas have applied these strategies to identify potential novel

bioactive compounds against several pathogenic diseases. The present study involved molecular docking studies to identify interactions between a potent bioactive compound, Phenol, 2,4-Bis(1,1-Dimethylethyl) of *B. licheniformis* with core proteins of *A. hydrophila* (3L6E) and *V. parahaemolyticus* (3RYL). Our investigation showed that Phenol, 2,4-Bis(1,1-Dimethylethyl), isolated from ethyl acetate

extract of *B. licheniformis* has antibacterial and other pharmacokinetic properties or binding affinity with a good docking score of -4.24 kcal/mol and -4.82 kcal/mol against *A. hydrophila* (3L6E) and *V. parahemolyticus* (3RYL), respectively. Similar to our study, Devi et al. [31] used in silico method for identification of the antifungal activity of Phenol, 2,4-Bis(1,1-Dimethylethyl) isolated from the ethyl acetate extract of *Kutzneria* sp. strain TSII against F_0 ATP synthase subunit A protein of *Pithomyces chartarum* with a docking score of -5.355 kcal/mol. In our present study, both 2D and 3D structures showed the ligand with target protein, 3L6E interactions by Vander Waals, hydrogen bonding with ILE¹⁰¹ and GLU¹⁰⁶ residues, and Pi-alkyl with ARG¹⁰² residue. Similarly, it reveals the target protein, 3RYL with ligand interaction by hydrogen bonding with ASP²⁵², Pi-Sigma with LEU²⁵³ and LEU²⁶⁸ and Alkyl as well as Pi-Alkyl with LEU²⁴⁹ and LEU²⁴⁹ residues, respectively. The 2D as well as 3D structures clearly display the protein–ligand interactions by hydrogen bonding (1.80 bond length) and Pi-Pi stacking (5 bond length) with respective SER⁶⁶ and TYR⁹⁷ residues [31].

Phenol, 2,4-Bis(1,1-Dimethylethyl) fulfils all the criteria of ADMET and Lipinski's rule of 5. Abdullah et al. [33] reported the antibacterial activity of Phenol, 2,4-Bis(1,1-Dimethylethyl) identified by GC–MS from Malaysian mango kernel. There have been several reports of the compound Phenol, 2,4-Bis(1,1-Dimethylethyl) displaying anticancer [34], antioxidant [35, 36], and antifungal [37] properties.

In our current investigation, the MD simulations clearly revealed the RMSD plot for both protein–ligand complexes and the stability of ligand, Phenol, 2,4-Bis(1,1-Dimethylethyl) was shown within the binding pocket by RMSF plot. Further, the binding free energy by MM-GBSA analysis confirms the stability of both the complexes. The present study validates the antibacterial activity of ethyl acetate extract of *Bacillus licheniformis* proteins (3L6E and 3RYL) ligand (Phenol, 2,4-Bis(1,1-Dimethylethyl)) stability. Furthermore, to validate the antibacterial activity of the bioactive compound of *Bacillus licheniformis*, it is necessary to carry out in vivo and clinical studies.

Conclusion

Aeromonas hydrophila and *Vibrio parahemolyticus* pathogens infect several fishes, shellfishes, shrimps, crustaceans and is a serious problem since it affects the production of aquatic animals in the aquaculture industry. Therefore, the development of novel antibacterial therapeutics is a dire necessity for the inhibition of both these pathogens. The in vitro toxicity study revealed the non-toxic nature of the antibacterial compound against *Artemia salina*. The ADMET analysis revealed the drug-likeness of the

compound, obeying all Lipinski's rule of five. The present study analyzed the binding efficacy of Phenol,2,4-Bis(1,1-Dimethylethyl), a potent antibacterial agent against the core proteins, 3L6E and 3RYL of *A. hydrophila* and *V. parahemolyticus*, respectively. The molecular docking and simulation analysis of the core protein 3L6E compared to 3RYL with the bioactive compound displayed better potency and relative stability of the drug. The energetics related to the static interactions and dynamic studies including SASA, RMSF, RMSD revealed the binding energy of the complexes and supported the stability induced through the protein–ligand interactions. The orientation of the ligand, Phenol,2,4-Bis(1,1-Dimethylethyl) in the binding pocket was accompanied by the lowest binding energy against 3L6E and 3RYL which proves that it is a potential antibacterial therapeutic agent against *A. hydrophila* and *V. parahemolyticus*.

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