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A PROTEIN-LEGAND INTERACTION OF CYANOBACTERIAL TOXIN MICROCYSTIN LR, A NOVEL INHIBITOR OF BACTERIAL PATHOGEN

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Abstract:

Search for alternative chemicals with less harmful effects on environment has received serious concern among scientists to look for biocides of plants and microbial origin. Antibacterial properties of a cyanobacteria, *Mycrocystis aeruginosa*, isolated from Ganga River, was evaluated to control bacterial leaf spot disease of chilli (*Capsicum annuum* L.) caused by *Xanthomonas vesicatoria*, a gram negative bacteria. The toxin, microcystin LR, was evaluated for its antibacterial activity by *in silico* methods using two molecular markers. Foliar spray with ethanol extract of *Microcystis* showed significant effects ($p<0.01$) in controlling bacterial leaf spots. Cell extract at a concentration of 100 mgL^{-1} reduced chilli leaf spots by 60% whereas at 500 mgL^{-1} the effectiveness exceeded 90%. Laboratory trials showed concentration dependent effect on growth of the pathogen. Molecular docking studies showed that DNA gyrase was inhibited by the algal toxin with binding energy of -7.29 Kcal/mol. Binding interaction of the microcystin with DNA gyrase showed three hydrogen bonds at amino acid residues Arginine 1232, Serine 1240 and Glycine 1239. Microcystin was further evaluated with penicillin binding protein (PBP) of the bacteria. Microcystin interacts also with PBP with hydrogen bonding at Glycine 469, Serine 334, Lysine 348 and Arginine 473 at binding energy of -6.99 Kcal/mol. The study showed microcystin as a novel inhibitor against pathogenic *X. vesicatoria* and has relevance in eco-friendly control of bacterial pathogen.

Keywords: Algal toxin, Bacterial pathogen, DNA gyrase, Microcystin, Protein-legand interaction.

Introduction

Cyanobacterial blooms, which contain a number of toxin producing species, may cause mal-odors and death of aquatic animals. Human illness and livestock deaths associated with cyanobacterial toxins have been reported in many countries (Ishida et al., 1997; WHO, 2003; Codd et al., 2005; Ahn et al., 2007). A record outbreak of blooming *Microcystis* occurred in Lake Erie in 2011, which in part, was related to the wettest spring on record (NOAA, 2015). The bloom outbreak reduced fish populations, fouled the beaches and influenced the local tourism industry that generates over 10 billion dollars of revenue each year. The toxin, Microcystin LR (MC-LR), is produced by *Microcystis aeruginosa*, an unicellular and colonial cyanobacterium, commonly

grow in freshwater lakes and ponds (Barrios-Llerena et al., 2007; Martin et al., 2017). They cast their distinctive hues on water surfaces when conditions are favorable for the algal growth (Moreira et al., 2016). Cyanobacterial microcystin production, although a natural phenomenon, is regulated predominantly by light intensity and the concentration of nitrate in the environment (Xie et al., 2016).

Chemically, these toxins are cyclic hexapeptides produced through non-ribosomal peptide synthases, composed of seven amino acids, five of which are non-protein and two are protein amino acids. These two protein amino acids of the toxins distinguish from one another, while the other amino acids are more are less constant among the variants. Structural variations have been identified at all seven positions of polypeptide ring. The MC-LR, the most commonly known cyanotoxin in environmental samples, presents a leucine (L) and an arginine (R), respectively, in X and Y positions of the cyclic polypeptide. The co-occurrence of the two L-aminoacids is used in the nomenclature of the variants. The position 1, which contains D-ala, is relatively conserved. Microcystin contains several uncommon non-proteinogenic amino acids such as dehydro-alanine derivatives and the uncommon β amino acid ADDA. Microcystin covalently binds to and inhibits the protein phosphatases PP1 and PP2A, and thus, causes pansteatitis. Microcystins are chemically stable over a wide range of temperature and pH due possibly to their cyclic structure. Because of their cyclic structure, they are difficult to be broken down by standard proteases like pepsin, trypsin, collaginase and chymotrypsin.

The changing climate conditions are likely to promote the expansion of cyanobacterial blooms. Under such condition, economic and eco-friendly use of cyanobacterial toxins bears great significance (Pandey and Pandey, 2002; Gupta and Prasanna, 2013). In a field trial, the cell extracts of three cyanobacteria were utilized for controlling leaf spot disease of chilli (Pandey and Pandey, 2002) caused by the bacterium *Xanthomonas vesicatoria*. The authors showed that the application of alcohol extracts of *M. aeruginosa* could be a highly cost-effective and eco-friendly approach controlling the leaf spot disease of chilli. This carries contextual relevance to the present study. The *X.vesicatoria* is a gram negative rod shaped bacterium causes large scale damage to chilli in dry regions of India. Because the pathogen cannot live in soil for more than few weeks and survives as inoculums in plant debris, removal of plant debris and chemical application to living plants are considered as effective control measures. Bacterial colonization of intracellular spaces leads to produce macroscopically visible symptoms including water-soaked lesions on the leaves. Infested plants can drop 50 to 100% of their foliage.

The present investigation was undertaken to study the efficacy of the cell extract of the unicellular, colonial alga, *M. aeruginosa,* towards controlling the leaf spot disease of chilli (*Capsicum annuum* L.) and to understand the mechanism of action through *in silico* gel documentation technique. For a better understanding of the inhibitory mechanism and the mode of interactions of the cyanotoxins, docking

analysis was accomplished and two primary drug-target-pathways at membrane and nucleic acid synthesis, i.e., penicillin binding protein and DNA gyrase, respectively, were chosen. Enzymes are major drug targets in drug discovery and development processes in the pharmaceutical and biotechnology industry (Tan, 2007). DNA gyrase, also known as DNA topoisomerase II, plays a critical role in bacterial cell survival and has been widely used as a drug target in antibacterial chemotherapy (Collin et al., 2011). This enzyme is composed of two subunits, namely A and B. Subunit A interacts with DNA and responsible for DNA cleavage, while the subunit B encompasses the active site of ATPase. The first mechanism involves inhibition of the gyrase enzymatic activity via competition with ATP or substrate while the latter one comprises the stabilization of the covalent gyrase–DNA complex or gyrase poisoning (Collin et al., 2011). Therefore, seeking new antibacterial agents with novel mechanisms for DNA gyrase inhibition has a great practical significance (Collin et al., 2011; Rahimi et al., 2016). Penicillin-binding proteins are responsible for peptidoglycan polymerization and insertion into pre-existing cell wall. The C-terminal penicillin-binding domain of both classes has a transpeptidase (TP) activity catalyzing the peptide cross-linking between two adjacent glycan chains. In class A, the N-terminal domain is responsible for glycosyltransferase activity, catalyzing the elongation of uncross-linked glycan chains (Emran et al., 2015).

Materials and Methods

Preparation of cyanobacterial cell extracts

The cyanobacterial samples were collected from Ganga River and clonal cultures were maintained at 24±1^{o}C temperature and 14.4 WM^{-2} light intensity with 18/6 light/dark cycle. Water extract was made by re-suspending 50 mg of freeze-dried cyanobacterial sample in 10 ml of distilled water. After a careful mixing, the sample was kept at 37^{o}C temperature for overnight. The ethanol extract of *Microcystis* sample was made by re-suspending 50 mg of sample in 10 ml of ethanol. The sample was mixed and left for 10 minutes before centrifugation at 2500 g for 6 minutes. The supernatant was transferred and the organic solvent was evaporated to dryness under a stream of N_2 gas using a reactitherm heating module at 40^{o}C. The dry residue was re-suspended in 1 ml ethanol and different concentrations were prepared. The filter paper disc (5 mm in diameter), saturated with different concentrations (25, 50, 75 and 100%) of the crude extract of *M. aeruginosa,* were kept overnight at room temperature to evaporate the ethanol. The filter discs treated with 95% ethanol and evaporated as described for the test disc was used as control. The antibacterial susceptibility was screened employing disc diffusion method.

Isolation of the pathogen and laboratory trials

The pathogen *X. vesicatoria* was isolated by the standard pour-plate technique and the sample was maintained at 30^{o}C in a bacteriological incubator. Cultures (at 30^{o}C for 24h) of *X. vesicatoria* strain were swabbed with sterile cotton on the surface of the

culture plates. The filter paper disc saturated with different cyanobacterial extracts were placed on the surface of the swabbed plates. The plates were incubated for 24h at 30°C. The solvent control was used in each case. After 24h, areas of inhibition of bacterial growth were observed as clear zone around the well and the diameter of the zone of inhibition was measured. The maximum inhibitory concentration against the test bacterial strain was compared to the standard and commercially available antibiotic, amoxycillin and sparfoxicine. Further, the bacterium was allowed to grow in the culture medium containing different concentrations of cyanobacterial cell extract and the growth was recorded as NTU at 0, 24, 48 and 72h.

Field trials with cyanobacterial extracts

For the disease spread on the test plant, the pathogen *X. vesicatoria* was inoculated through a soil mix. For the field trials with respect to the test of effect of cyanobacterial extracts on leaf spot formation, a 500 g fresh cyanobacterial mass was blended in 1000 ml of 95% ethanol and left at room temperature with occasional shaking for 48h. The suspension was filtered through cheese cloth and the filtrate was evaporated at 40-50 °C to a residue on the day of experimentation. The extracts were diluted with distilled water to make 100 $mgL,^{-1}$ 200 $mgL^{-1}$and 500 mgL^{-1} concentrations for foliar spray. Cell extracts were applied using a hand operated agro-sprayer (Turbhe Polycans (P) Ltd., India).

Protein-ligand studies

The structure of Microcystin LR and that of the proteins, DNA gyrase (2X CS) and penicillin binding protein (4 WEJ) were retrieved from PubChem and PDB respectively. Molecular docking was done by AutoDock4. Autodock, which consists of two main programs, Autodock and Auto Grid, performs the docking of the ligand to a set of grids describing the target protein. Lamarckian Genetic Algorithm (LGA) is comprised of a stochastic population generator, a docking routine based on a Lamarckian genetic algorithm, and a local search function based on molecular mechanics (MM) energy minimization. The input files for Auto Grid and Autodock were created and the grid map calculation was run which was followed by docking calculation in the Autodock. These grid maps were used for Autodock docking calculations to determine the total interaction energy for a ligand with a macromolecule. The grid box size was set at 126, 126 and 126 A° (x, y, and z) to include all the amino acid residues that present in the rigid macromolecules. The spacing between grid points was 0.375 A°. The LGA was a chosen search for the best conformers. During the docking process, a maximum of 10 conformers were considered. The population size was set to be 150 and the individuals were initialized randomly. Maximum number of energy evaluation was set to be 500000; maximum number of generations 1000, maximum number of top individuals that automatically survived was set to be 1, mutation rate of 0.02 and crossover rate of 0.8. Step sizes were 0.2 Å for translations, 5.0 A° for quarter anions and 5.0 A° for torsions. Cluster

tolerance was 0.5Aº, external grid energy 1000, max initial energy 0.0, max number of retries 10000 and 10 LGA runs were performed. Autodock results were analyzed to study the interactions and the binding energy of the docked structure (Emran et al., 2015). Visualization of docked structure was done using PYMOL tool (Verma et al., 2015).

Results and Discussion

Field and laboratory trials with cyanobacterial extracts and *X. vesicatoria*

The foliar spray with cyanobacterial cell extract showed significant effect ($p<0.01$) in controlling the leaf spots (Fig. 1). High concentrations appeared more effective in reducing the number of leaf spots. For instance, ethanol cell extract at a concentration of 100 mgL^{-1} reduced leaf spot by over 60% whereas at 500 mgL^{-1} concentration, the effectiveness exceeded 90%. Efficacy of ethanol extract was ~30 to 58 % higher than those of the water extract (Fig. 1). Laboratory trials also showed significant effect of cyanobacterial cell extract in controlling disease development and growth of the pathogen (Fig. 2). Similar to the effects on leaf spots, when the efficacy of cell extract was tested in terms of zone of inhibition of the pathogen, ethanol extract showed superiority over the water extracts. The effect on the pathogen showed concentration dependence and, similar to the concentration dependence of foliar spray, the pathogen did not grow at 500 mg L^{-1} cell extract (Table 1).

Cyanobacteria (blue green algae) constitute a primitive, diverse and ubiquitous group of prokaryotes with oxygenic photosynthesis. Tremendous adaptability to varying environmental conditions together with potential protective and tolerance mechanisms help cyanobacteria to be ubiquitous in distribution. Production of a wide array of photosynthetic pigments, storage products and secondary metabolites make them a metabolically versatile group of organisms (Volk and Fulkert, 2006). In addition to their potential application in agriculture, pollution control, bioenergy sector and nutraceutical industry, cyanobacteria produce an array of biologically active compounds which have diverse effects and usages. Among the major bioactive compounds, many are pharmacologically important and used in drug development (Tan, 2007). Cyanobacteria release antimicrobial compounds, antineoplastic agents, immunomodulators and enzyme inhibitors (Prasanna, 2010; Abostate et al., 2015). Being environmentally safer, antimicrobial properties of cyanotoxins have gained importance in view of the emergence and spread of a wide array of pathogenic microbes. Our observations indicate that the cell extract of *M. aeruginosa* contains toxin with antibacterial properties for *X. vesicatoria,* where the efficacy of ethanol extract showed superiority over the water extract.

Some antimicrobial compound exhibit single activity while the others play multiple roles. Various cyclic peptides and depsipeptides of cyanobacterial origin have been reported as antibacterial agents (Ishida et al., 1997; Barrios-Llerena et al., 2007). Microcystin LR, produced by *M.aeruginosa*, is a potential inhibitor of bacterial

pathogen. Because *M. aeruginosa,* grows prolifically in nutrient rich surface waters, and it can be easily cultivated on desired scale, using cell extract of this cyanobacterium is a cost effective and eco-friendly approach in controlling leaf spot diseases in dry regions. Our observations are based on a single test crop, we suggest, for more general usages, the specificity of the cell extract/ toxin may be tested further with other crops and with other bacterial pathogens.

AutoDock results of algal toxins with bacterial DNA gyrase and PBPs

The toxin was evaluated by *in silico* method with two molecular markers. The target protein structure of bacterial DNA gyrase and PBPswere docked with, microcystin LR, which showed excellent AutoDock results as evident by their binding energies. The free energy were calculated as the sum of four energy terms of intermolecular energy (Van der Waal, hydrogen bond, desolvation energy and electrostatic energy), total internal energy, torsional free energy and unbound system energy. Molecular docking studies showed that DNA gyrase of chain B was inhibited by algal toxin with binding energy of -7.29 Kcal/mol. Microcystin LR was further evaluated with penicillin binding protein (PBP) of the pathogen. The PBP showed interaction with microcystin with binding energy of -6.99 Kcal/mol.

The possible binding modes of microcystin LR at targeted protein PBP sites are displayed in Fig. 3 and its corresponding energy values and amino acid residues involved in the interaction with various conformations obtained on molecular docking studies by Autodock 4.0 and details are listed in Table 2. Studies revealed that the inhibitory activity was influenced by polar interaction and hydrogen bonding with active site residues of bacterial penicillin binding protein. The large negative free energy of the conformation of the complexes with maximum number of H-bond formation were found to be -6.99 Kcal/mol. Serine 334, Lysine 348, Arginine 473 and Glycine 469 were found to have an interaction with inhibitor (Fig. 3). Figure 4 shows the interaction of microcystin LR with DNA gyrase with binding energy of -7.29 Kcal/mol, where, three hydrogen bonds are formed between amino acid residues Arginine 1232, Serine 1240 and Glycine 1239. Various conformations showing hydrogen bonding of DNA gyrase of bacterial origin with microcystin LR as ligand are listed in Table 3. It is evident from the Figures 3 and 4 that the bond length between amino acid residues involved in H-bond with toxin was less than 3.0 A^0 which makes this toxin as potential drug candidate. Molecular docking and inhibition studies of *Bacopa monnieri*'s potent phytochemical, leuteoline, have demonstrated that the leuteoline, which offers a strong specificity towards DNA gyrase binding site, could be a potent antimicrobial compound against pathogenic *Staphylococcus aureus* (Emran et al., 2015) suggesting that binding interaction studied of bioactive compounds should be employed for establishing more rationale structure-activity relationships useful in antimicrobial drug development.

Conclusions

The study showed microcystin as a novel inhibitor against pathogenic *X. vesicatoria* and has relevance in eco-friendly control of bacterial pathogen. Further, the interaction studies of microcystin LR with penicillin binding protein (PBP) and DNA gyrase showed that amino acid residues primarily serine, lysine, arginine and glycine were significantly involved in the inhibition phenomena. It's a unique finding in a way since this toxin was potentially active both at cell wall synthesis level as well as at nuclear level. These studies revealed for the unexploited potential of algal toxin in the treatment of bacterial pathogen. Furthermore, since the enzymes are the major drug targets in drug discovery and development in the pharmaceutical and biotechnology industry, our observations also suggest that binding interaction studied of bioactive compounds should be employed for establishing more rationale structure-activity relationships useful in antimicrobial drug development.

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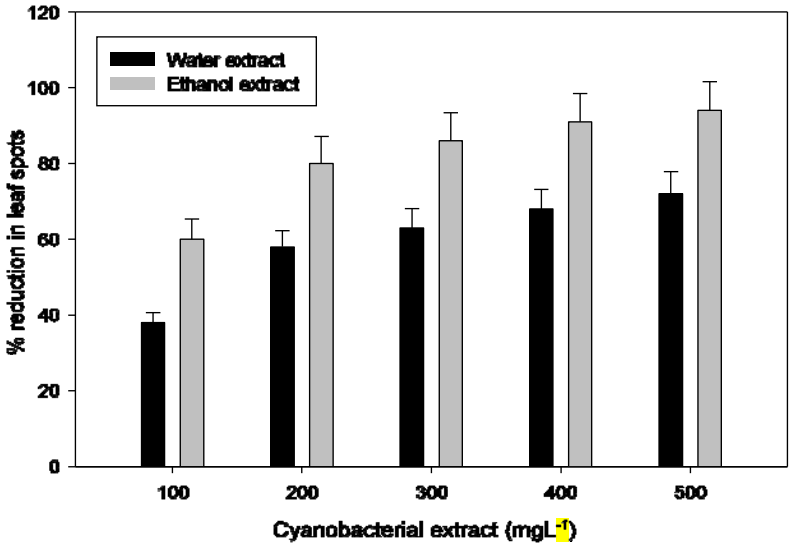

Fig. 1: Effects of cyanobacterial extracts on leaf spot disease of chilli. Values are mean (n=6)±1SE

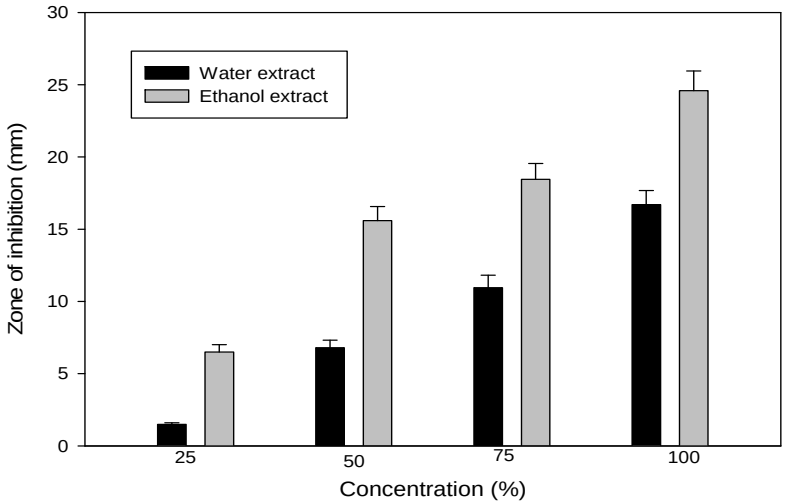

Fig. 2: Effects of cell extracts of *Microcystis aeruginosa* on growth of *Xanthomonas vesicatoria*. Effect on the pathogen is presented in terms of zone of inhibition. Values are mean (n=6)±1 SE

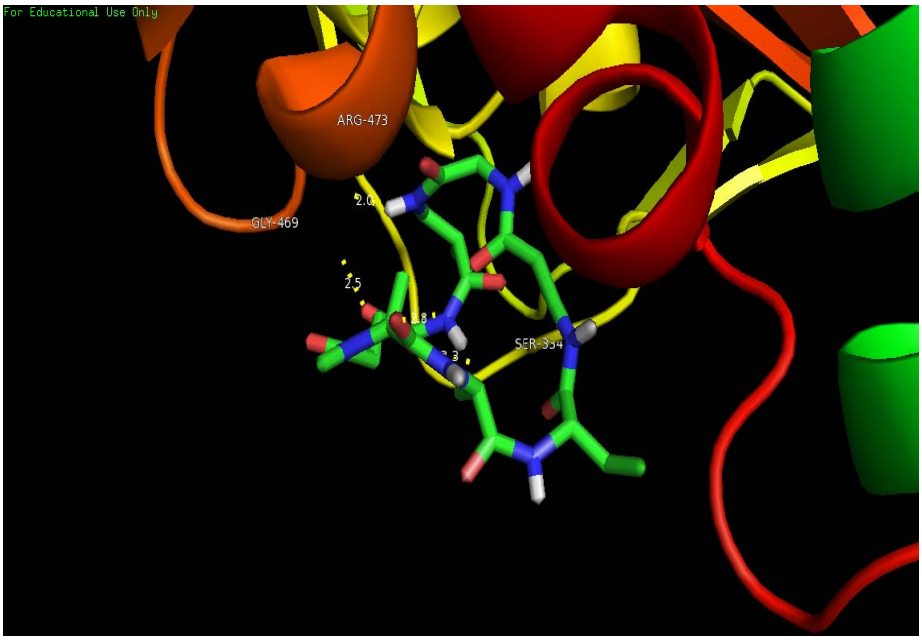

Fig. 3: Molecular docking of microcystin with penicillin binding protein showed hydrogen bonding at Glycine 469, Serine 334, Lysine 348 and Arginine 473

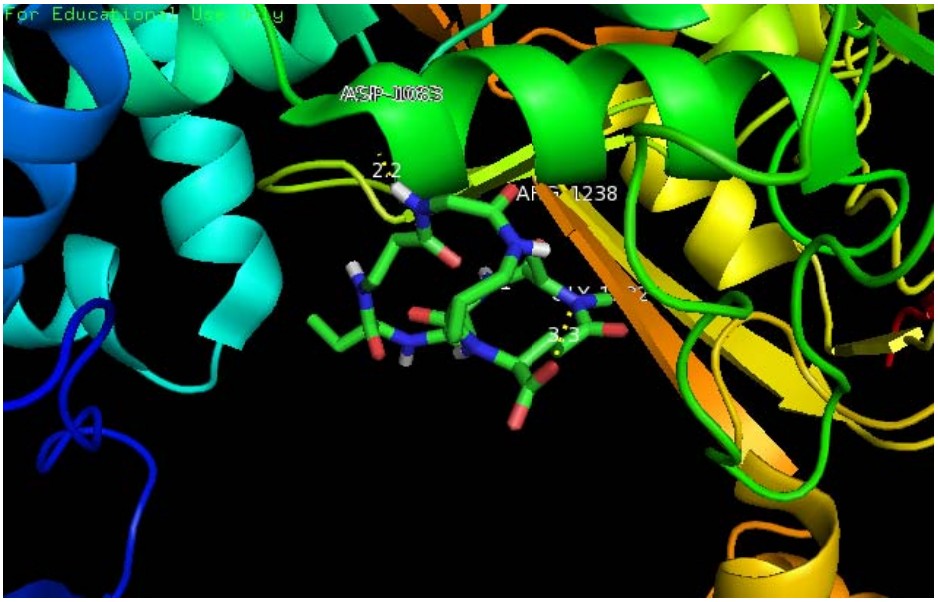

Fig. 4: Molecular docking of microcystin with DNA gyrase of bacterial origin with hydrogen bonds at Arginine 1232, Serine 1240 and Glycine 1239

Table 1: Effects of ethanol extract of *M. aeruginosa* (measured in terms of nephalo turbidity unit; NTU) on growth of *X. vesicatoria*. Values are mean (n=6) ±1SE

	Growth of *X. vesicatoria* (NTU)	
	Cell extract (mgL^{-1})	
Time (h)	100	200
500		
0	0.04±0.003	0.04±0.003
0.04±0.003		
24	1.40±0.07	0.62±0.05
0.32±0.02		
48	2.90±0.14	1.30±0.08
0.58±0.04		
72	3.55±0.24	1.30±0.08
0.58±0.04		

Table 2: Summary of molecular docking results of penicillin binding protein (PBP) with microcystin LR obtained through Autodock

Conformations	Binding energies Kcal/mol	Number of hydrogen bonds	Amino acids
1	-7.14	3	Lys 348, Arg 473, Gly 469
2	-6.99	4	Ser 344, Lys 348, Arg 473, Gly 469
3	-6.99	3	Lys 348, Arg 473, Gly 469
4	-6.87	3	Arg175, Arg 175, Leu 276
5	-6.86	3	Arg 175, Arg 175, Leu276
6	-6.83	3	Arg 175, Arg 175, Leu 276
7	-6.72	2	Arg 473, Ile 347
8	-6.71	2	Arg 473, Gly 469
9	-6.63	2	Arg 175, Leu 276
10	-6.11	2	Ala 57, Tyr 124

Table 3: Summary of molecular docking results of DNA gyrase with microcystin LR obtained through Autodock

Conformations	Binding energies Kcal/mol	Number of hydrogen bonds	Amino acids
1	-7.84	1	Tyr 1025
3	-7.83	1	Lys 1323
5	-7.8	1	Lys 1323
6	-7.78	1	Lys 1323
7	-7.4	2	Tyr 1322, Thr 1325
8	-7.29	4	Arg 1232, Ser 1240, Arg 1232, Gly 1239
9	-7.0	2	Asp 1083, Glu 1088