Characterization and Pharmacological Evaluation of Microcystin Variants from *Anabaena doliolum* and *Nostoc spongiaeforme* Isolated from Shekhawati Region of Rajasthan

THESIS

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By

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Under the Supervision of

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CERTIFICATE

This is to certify that the thesis entitled **"Characterization and Pharmacological Evaluation of Microcystin Variants from *Anabaena doliolum* and *Nostoc spongiaeforme* Isolated from Shekhawati Region of Rajasthan"** submitted by R. Ashwin Kumar, ID No. 2004PHXF416P for award of Ph.D. Degree of the Institute, embodies original work done by him under my supervision.

SKverma

Signature in full of the Supervisor

Name in capital block letters: **S.K.VERMA**

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Abstract

The crude extracts of two cyanobacterial strains, isolated from water bodies of Shekhawati region of India, and identified as *Anabaena doliolum* and *Nostoc spongiaeforme,* showed high level of hepatotoxicity and tumor promoting activity in *in-vivo* mouse bioassay model. A dose of 100 µg of total protein/kg of body weight showed restlessness, spasmodic leaping, slow movement and loss of co-ordination within 6 hours of intraperitonial (i.p.) administration in Swiss albino mice. Further purification of crude extracts with TLC and HPLC techniques showed the presence of toxic cyclic peptides of microcystin family. The chronic toxicity studies with sub lethal dose of Ad spot-3 (TLC spots) from *A. doliolum* have shown to cause colon tumors, whereas sub lethal dose of Ns spot-3 (TLC spots) from *N. spongiaeforme* induced the intestinal tumors. An increase in oxidative stress (MDA level), serum ALT, ALP, AST and LDH level was also found in both the cases. The compounds of these spots were characterized by MALDI-TOF and FTIR analysis. They were identified as dihydromicrocystin-LR, [D-Asp3] microcystin-WR, microcystin-LR, microcystin-FR, microcystin-RR and [D-Asp3] microcystin-HtyR. The synthesis of these microcystin variants were influenced by various nutrimental factors such as phosphate, nitrate and temperature. Increasing concentration of nitrate (0mM to0.5mM) or decrease in phosphate concentration (0.5mM to 0mM) increases the synthesis of less toxic microcystin peptides. Lower temperature (15°C) significantly increases the synthesis of more toxic variants and a temperature range of 22-26°C was found to be suitable for the synthesis of different variants of microcystin in *A. doliolum* and *N. spongiaeforme.*

The mice dosed with the sub lethal concentration of 0.8µg/kg body weight of microcystin-FR were subjected to a battery of behavioural depression models such as tail suspension test (TST), force swim test (FST) and elevated plus maze test (EPM). 40% decreased immobility was seen in

both TST and FST in mice treated with microcystin-FR as compared to control. The EPM test showed that the mice treated with microcystin-FR spent 89% more time in open arm as compared to untreated control. The microcystin-FR does not have interfered with the normal locomotory activity of animals. A dose of 8 mg/kg body weight of Cyclosporin-A (CsA) was used to suppress the hepatotoxic effect of microcystin-FR in all the above experiments as CsA provided 100% protection over the hepatotoxic nature of microcystin-FR. The results are discussed in light of the inhibition of PP_2A and transport of biogenic amine by microcystin-FR. In order to investigate the reason behind the difference in the severity of microcystin variants, and antidepression activity, docking studies were performed and analyzed with PP_2A receptor. The study infers the bending orientation or anticlockwise rotation of fatty acid side chain (ADDA group) of microcystin variants as compared with microcystin-LR in PP_2A receptor determine the severity of toxicity. The lower toxicity of microcystin-FR might due the presence of bulky phenylalanine which not only forms a nonbonding interaction with Cys-269 but also makes the benzene ring of ADDA moiety to rotate in anticlockwise direction to make nonbonding interaction with Valine 189 & Proline 190, His 191 of PP_2A. The above *in-silico* hypothesis was supported by decrease in 12.5% of liver weight to the animal dosed with 0.8µg/kg body weight of microcystin-FR, as compared to the other variants.

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List of Abbreviations

ALT	-	Alanine transaminase
AST	-	Aspartate transaminase
ALP	-	Alkaline Phosphatase
LDH	-	Lactate dehydrogenase
MDA	-	Malandialdehyde
R_t	-	Retention time
R_f	-	Retention factor
i.p	-	Intraperitonally
TLC	-	Thin layer chromatography
HPLC	-	High performance liquid chromatography
FST	-	Force swim test
TST	-	Tail suspension test
EPM	-	Elevated maze test
CsA	-	Cyclosporin-A
MALDI TOFF	-	Matrix assisted laser desorption ionization-Time of flight
FTIR	-	Fourier transform infrared spectroscopy
ROS	-	Reactive oxygen species
LPS	-	Lipopolysaccharide
TEM	-	Transmission electron microscopy
TBARS	-	Thiobarbituric acid reactive species
CsA	-	Cyclosporin-A
PP_2A	-	Protein phosphatase 2A

PG	-	Propylene glycol
McyFR	-	Microcystin-FR

Chapter I

Introduction

The prokaryotic and eukaryotic micro algae of semi-desert regions show high degree of tolerance to drought and extreme temperature. Cyanobacteria are photoautotrophic prokaryotes which comprise of a large species of widespread occurrence with diverse morphological, physiological and biochemical properties. Cyanobacterial flora of Rajasthan has been remarkably suited to develop an attributes and strategies, which enable them to colonize and survive under extreme habitats. These organisms are able to interact with their niche, develop certain survival mechanisms and either exploit or modify their attributes to make them more suitable under extreme temperature and water stress conditions (Tiwari *et al.*, 2005). While secondary metabolism has a rather restricted distribution, the primary metabolism is universal among microbes. The end products of primary metabolism are energy and intermediates for synthesis of essential macromolecules like lipids, proteins and nucleic acids. In well regulated, metabolically efficient microbes such as *E. coli*, the intermediates and end products of primary pathways do not accumulate and they do not possess the genetic apparatus to make secondary metabolites (Shimizu 2003). On the other hand, the cyanobacteria possess genes for producing secondary metabolites in addition to the genes necessary for growth. In such organisms, certain steps of primary metabolism lack regulation (Burja *et al.*, 2001). This results in over synthesis of intermediates and end products of primary pathways. Under metabolic stress, these accumulated pools of intermediates may induce subsidiary pathways to form secondary metabolites. The biochemical and molecular genetic analysis of the biosynthesis of numerous secondary metabolites has revealed that a limited number of core biosynthetic pathways are responsible for the formation of the majority of compounds. The structural diversity of natural products generated by cyanobacteria appears to be predominantly a result of modifications and combinations of reactions from primary metabolic pathways (Harada 2004, Rahman & Choudhary 2005). The bioactive secondary metabolite of cyanobacteria includes potential toxins, enzyme inhibitors, anti-tumour,

antibacterial, antifungal, antiviral, antibiotics (Burja *et al.*, 2001, Harada 2004, Katircioglu *et al.*, 2004). Many of these natural active compounds have large demands. In some cases, demand can be met by the total synthesis of the active metabolite, but in many cases, this is not a viable option due to relatively expensive and poor quality of raw materials, leading to low overall yield. The advantage of cyanobacteria is that sustainable supply of a target metabolite can be achieved by molecular and bioprocess techniques (Burja *et al.*, 2001).

The medicinal qualities of cyanobacteria were first appreciated as early as 1500 B.C, when *Nostoc* species were used to treat gout, fistula and several forms of cancer (Burja *et al.*, 2001). During 1990's, scientists had begun to screen extracts of cyanobacteria on *Nostoc, Anabaena, Lyngbya, Synechocystis, Spirulina*, and *Nodularia* for various biological activities in fresh water and marine cyanobacteria, which led to high incidence of novel biologically active compounds. The species such as *Spirulina* and *Nostoc* have been used as a source of protein and vitamins for humans and animals. The products of cyanobacteria contains 40.2% of lipopeptides, 5.6% of pure amino acid composition, 4.2% fatty acids, 4.2% macrolide and 9.4% are amides. Lipopeptides are a potent bioactive group of compounds with approximately 85% being active of which are toxins 41%, anticancer 13%, antibiotics 12%, enzyme inhibitors 8%, antiviral 4% and antifungal 18%. The remaining activities are as tumor promoter, herbicide, antimycotic, antimalarial, antimicroalgal, cell-differentiation promoter, cardio active compounds and sunscreen pigment (Burja *et al.*, 2001, Jaiswal *et al.*, 2008, Katircioglu *et al.*, 2004, Harada 2004).

The presence of high proportion of toxins (bioactive molecule) produced by *planktonic* species of cyanobacteria in cyanobacterial flora attracted scientists to study its nature, its role, factors contributing its synthesis and its mode of actions. These toxic compounds may be regarded as an evolutionary response to the pressure exerted by competing organisms, fungi and grazing animals in particular (Babica *et al.*, 2006). Eutrophication of water bodies has been a constant problem

for water authorities due to its association with the formation of cyanobacterial blooms (Carmichael 1994, Smith & Haney 2006, Smith *et al.*, 2010). Moreover use of chemicals for purification of water like chlorine, copper sulphate (algicides) can lyse the cyanobacterial cells to release the toxins into the surrounding water body (Wiegand & Pflugmacher 2005, Zurawell *et al.*, 2005). Cyanotoxins in drinking water can disrupt aquatic ecosystems and cause serious public health problems (Sivonen 1990, Carmichal 1994, Codd *et al.*, 1999). Numerous poisoning events that have resulted from the consumption of contaminated water through chronic ingestion, inhalation of droplets or contact with nasal mucous membranes, dermal contacts through bathing or recreational activities such as wading, swimming, skiing and canoeing (Ray and Bagchi 2001, Agrawal *et al.*, 2006, Wiegand & Pfugmacher 2005, Volk 2006, Backer *et al.*, 2008, Westrick *et al.*, 2010). To create a comprehensive cyanotoxin contingency plan, it is necessary to be familiar with the physical and chemical properties of the toxin, the nature of the cyanotoxin, i.e., intracellular or extracellular, growth and diurnal cyanobacteria bloom patterns and effective treatment processes (Katircioglu *et al.*, 2004).

The toxin producing cyanobacteria includes *Anabaena, Aphanizomenon, Cylindrospermopsis, Microcystis, Nodularia, Oscillatoria, Lyngbya, Scytonema and Tolypothri* (Codd & Poon 1988, Burja *et al.*, 2001, Harada 2004). Other genera including *Coelosphaerium, Fisherella, Gloeotrichia, Gomphosphaeria, Symploca* and *Trichodesmium* have also been found to be toxic but, as yet, no toxin has been isolated and characterized from these genera (Burja *et al.*, 2001). Chemically they are mainly cyclic peptides, alkaloids and lipopolysaccharides. Based on their site of action the Cyanotoxins are divided into following four groups:

Neurotoxins

Aphantoxins, anatoxins-A, saxitoxins, neosaxitoxin are known neurotoxins reported from the strains of *Anabaena, Aphanizomenon, Cylindrospermum and Oscillatoria* respectively. They are generally alkaloids which are well known for its neurotoxicity with low molecular weight (Harada 2004, Jaiswal *et al.*, 2008). Anatoxins and its variants inhibit transmission at the neuromuscular junction by molecular mimicry of the neurotransmitter acetylcholine and inhibition of acetyl cholinesterase activity (Rahman & Choudhary 2005). The enzyme acetylcholinesterase catalyzes the hydrolysis of the neurotransmitter acetylcholine and it has been an attractive target for the treatment of Alzheimer's disease and other possible therapeutic application in the treatment of Parkinson's disease and Myasthenic gravis (Harada 2004, Rahman & Choudhary 2005). These compounds are potent post-synaptic depolarizing neuromuscular blocking agent that causes hypersalivation and death within minutes to hours in animals. They are only known naturally occurring organophosphate (Codd *et al.*, 1999). Anatoxin-A (Fig. 1a) is the structural analogue of cocaine and binds to nicotinic acetylcholine receptors which lead to respiratory failure within few minutes. It can induce apoptosis in non-neuronal cells. Toxin-treated thymocytes showed all the typical morphological and biochemical features of apoptosis including DNA fragmentation, generation of ROS and caspase activation, which are more pronounced than in Vero cells. Exact mechanisms of anatoxin induced apoptosis are not known (Rao *et al.*, 2002). Homoanatoxins-A (Fig. 1b) is a potent neuromuscular blocking agents that causes severe paralysis convulsion and death by respiratory arrest. Jamaicamide from *Lyngbya majuscule* also shares the same site of action as compared to other neurotoxins stated above (Edwards *et al.*, 2004).

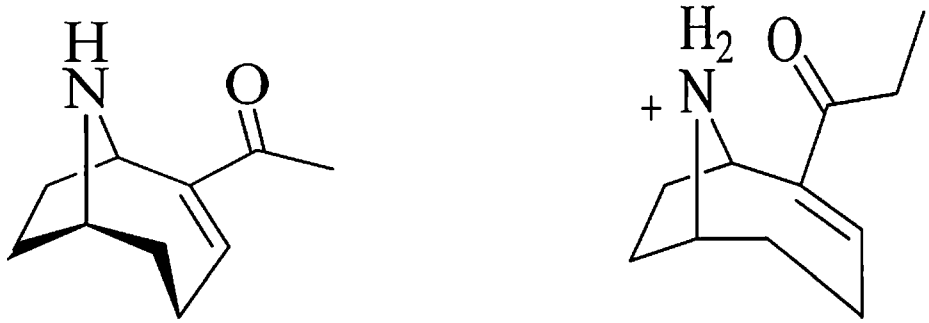


Fig. 1 (a) Structure of Anatoxins-A **(b) Structure of Homoanatoxins-A**

Saxitoxins comprises different isoforms and with varied toxicities (Fig. 2). They are well known as paralytic shellfish poisoning (PSP) toxins due to their accidental consumption in contaminated seafood. Saxitoxins (STX) and its analogue compounds (neosaxitoxin, aphantoxins) have been reported to occur naturally in marine dinoflagellates, filamentous cyanobacteria and in certain heterotrophic bacteria. In freshwater environments, STX toxins are almost exclusively associated with cyanobacteria, and the occurrence of STX producing neurotoxic cyanobacterial blooms has been increasingly reported (Carmichael *et al.*, 1997, Pomati *et al.*, 2004, Harada 2004, Jaiswal *et al.*, 2008, Pearson *et al.*, 2010). PSP toxins selectively block the voltage-gated Na^+ channels in excitable cells, thereby affecting the impulse generation in animals which can lead to death. STX binds to nerve membranes with high affinity (dissociation constant K_d=2 nM). PSP toxins block resting, active or inactive Na^+ channels equally. These toxins act from the extracellular side of the plasma membrane by occluding the entry of the Na^+ channel pore. The positively charged guanidinium group of PSP toxins interacts with negatively charged carboxyl groups at the mouth of the channel (Pomati *et al.*, 2004, Yotsu-Yamashita *et al.*, 2004, Su 2004).

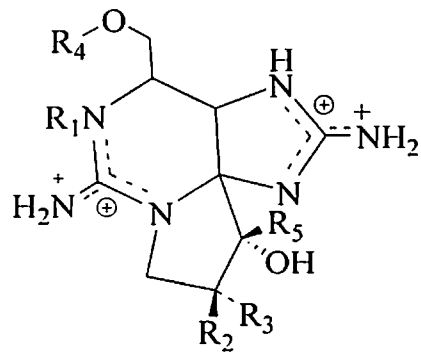


Fig. 2 General structure of Saxitoxins

Dermatotoxins

Most of the lipopolysaccharides of cyanobacteria cause severe dermatitis, oral and gastrointestinal inflammation. The lipopolysaccharide (LPS) endotoxins of cyanobacteria are generally found in the outer membrane of the cell wall where they form complexes with proteins and phospholipids (Burja *et al.*, 2001, Katircioglu *et al.*, 2004). Lipopolysaccarides, as the name implies, are condensed products of a sugar, usually a hexose and a lipid, normally a hydroxy C_{14}-C_{18} fatty acid. The many structural variants of LPS are generally phylogenetically conserved (similar fatty acid and sugar components). It has been proved that the fatty acid component of the LPS molecule elicits allergenic response (irritant) in humans and mammals (Harada 2004). In 1970, the first LPS was reported from the cyanobacterium *Anacytis nidulans,* which exhibit both pyrogenic and toxic effects. LPS of cyanobacterium are found to be 10 fold less toxic than other bacterial LPS. The considerable diversity of cyanobacteria in their chemical composition is largely related to the phylogeny (Jaiswal *et al.*, 2008). LPS toxicity leads to nausea and vomiting which are normal physiological responses to the ingestion of toxic substances. Nausea and vomiting result due to its effects over sensory motor systems, which operate to identify and rapidly expel the hazardous substances from the upper G-I tract. The two main sensory systems that direct the emetic response are local, associated with the gut mucosa (pre-absorptive response) and central, specifically the

chemoreceptive trigger zone of the area postrema, located in the dorsal surface of the medulla oblongata (post-absorptive response). Stimulation of chemoreceptors in the stomach, jejunum and ileum by irritant chemicals such as hypertonic saline, copper sulfate or mustard, or by bacterial enterotoxins, leads to the activation of vagal sensory afferent nerves to the brain. Vagal efferent processing through the enteric nervous system stimulates enteric motor neurons to affect emesis. Emetic chemoreceptors are also found in the vascular system; activation of these chemoreceptors will also initiate nausea and vomiting. Endogenous mediators of emesis such as dopamine, acetylcholine and enkephalin are reported (Katircioglu *et al.,* 2004). Prostaglandins have well-known emetic actions. Some of the other dermatotoxins which are will reported are Debromoaplysiatoxin from *Lynbya gracilis* (Katircioglu *et al.,* 2004, Jha & Xuzi-rong 2004).

Cytotoxins

Cryptophycin 114 was first isolated from *Nostoc sp ATCC* 53789 and *Nostoc sp* GSV 224, which exhibited potent cytotoxicity against human tumor cell lines and good activity against a broad spectrum of drug-sensitive and drug resistant murine and human solid tumors. Nevertheless, C-14 again appeared to be too toxic to become a clinical candidate. This led to a detailed structure function study and resulted in the isolation of Cryptophycin 815, a semi synthetic analogue, which proved to have a greater therapeutic efficiency and lower toxicity (Burja *et al.,* 2001). Cyanovirin 16, a 101 amino acid protein has recently been placed on an accelerated track for clinical development for use as a virucidal agent (Harada 2004). Cyanovirin-N (CV-N) was extracted from *Nostac ellipsosporum*, which prevent the surface receptor of HIV from undergoing the conformational changes that enable the viral and cell membranes to fuse, allowing the transfer of the viral genetic material into the host. CV-N was

found to have potent activity against all immunodeficiency viruses HIV-1, HIV-2, SIV (simian) and FIV (feline) (Diana *et al.,* 2005). The use of CV-N, Cryptophycin 114 and its analogue 815 lead to an entirely new class of antiHIV, antitumor drugs (Chaganty *et al.,* 2004 , Diana *et al.,* 2005). Some of sulfolipid of *Lyngbya lagerheimi*, Dolastatin 3, G, 11, 12 and Serinol-derived malyngamide 1-2 from *Lyngbya majuscule* also show anti-HIV activity (Davies-Coleman *et al.,* 2003). The other cytotoxins reported are diarrhetic toxin (lipopeptide) from *Microcystis* species, *Anabaena* species and *Nodularia* species, γ-butyrolactone, +-α(s)-butyramidedo-y-butyrolactone, butyrolactone 3-y and hermitamides B from *Lyngbya majuscule,* 30-methyloscillatoxin D, 31-neroscillatoxin and oscillatoxin from Oscillatoria species, hormothamnin and hormothamnione from *Hormothamnion enteromoyphoides*, kalipyrone from Tolypothrix species, borphycin and nostocyclophane from *Nostoc ellipsosporum*, micromide and guamamide from *Symploca* and tenue cyclamide from *Nostoc spongiaeforme* (Harada 2004, Burja *et al.,* 2001) .

Hepatotoxins

Hepatotoxins are the most common offender's worldwide in case of waterborne disease caused by toxins of cyanobacteria (Backer *et al.,* 2008, Smith *et al.,* 2010). The cyanobacterial hepatotoxins include cyclic peptides (Microcystins and nodularins) and cyclic guanidine alkaloid (cylindrospermopsin).

Cylindrospermopsins

Cylindrospermopsins, a cyclic guanidine hepatotoxic alkaloid first isolated from *Cylindrospermopsis raciborski.* Later it was also reported from *Aphanizomenon ovalisporum,* in Israel, *Aphanizomenon flos-aquae* in Germany and *Umezakis natan* in Japan. The toxin is a stable tricyclic alkaloid containing a guanido group linked at C7 to hydroxymethyl uracil (Fig.

3) (Saker *et al.*, 1999, Banker *et al.*, 1997, Harada *et al.*, 1994). At the hydroxyl bridge, there are two possible epimers, cylindrospermopsin and 7-epicylindrospermopsin. Because of the negatively charged sulfate group and the positively charged guanido group, the molecule is a zwitterion and very water soluble. Both naturally occurring epimers are equally toxic (Ian *et al.*, 2006). The acute toxicity studies with crude extract with LD_{50} of 200 µg/kg injected intraperitonally blocks protein synthesis of cells in kidney, liver, lungs, adrenal glands and intestine. The effect pure toxin has been identified in an outbreak of acute heptoenteritis and renal damage among the aboriginal population in Australia (Ian *et al.*, 2006, Li *et al.*, 2001).

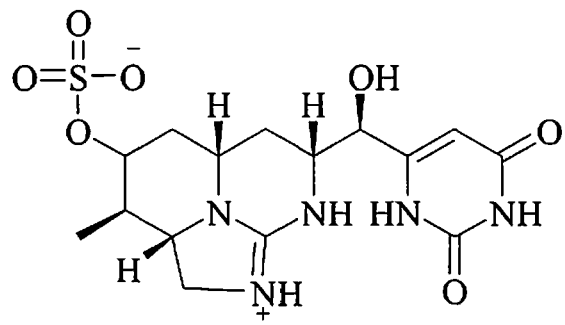


Fig. 3 Structure of Cylindrospermopsins

Cylindrospermopsin has also been found to be genotoxic in a number of *in-vitro* assay systems. Using the micronucleus assay, cylindrospermopsin caused DNA fragmentation and loss of whole chromosomes in a human white blood cell line and in mice liver (Banker *et al.*, 2001). The cell culture studies in primary mouse hepatocytes suggesting that metabolism of toxin is required to form a bioactive product for genotoxic action. The cylindrospermopsin-induced DNA fragmentation was detected by the COMET assay, an effect that could be eliminated by application of CYP450 inhibitors (Preussel *et al.*, 2006). Recent data from primary hepatocytes showed two routes of toxic action, a rapid route probably through toxicity of a CYP450 oxidation product of the toxin and a slower mechanism through the well-documented inhibition of protein synthesis, which does not require toxin metabolism. Uptake of the toxin is

relatively rapid since complete and irreversible block of protein synthesis occurs after a 1 hr exposure *in-vitro*. Inhibition of protein synthesis occurs at the ribosome during the peptide chain elongation step. Glutathione synthesis is also reduced via a CYP450-dependant mechanism, but this does not lead to an increase in oxidative stress in the cell (Banker *et al.*, 2001, Preussel *et al.*, 2006, Harada *et al.*, 1994).

Nodularin

Nodularin is produced predominantly by brackish water cyanobacteria. The first report of nodularins was in 1988, when bloom infested lake in Australia containing *Nodularia spumigena* was shown to be the cause of stock death. It is a cyclic pentapeptide, consisting of ADDA, D-glutamic acid (D-Glu), N-methyldehydrobutyrine (MeDhb), D-erythro-β-methylaspartic acid (D-MeAsp), and L-arginine (L-Arg) (Fig. 4) (Pushparaj *et al.*, 1999, Paczuska & Kosakowska 2003, Suikkanena *et al.*, 2004). Seven naturally-occurring isoforms of nodularin have been reported till date. Two of these isoforms, have variations within the ADDA residue, which reduces or abolishes the toxicity of the compound (Pearson *et al.*, 2010). The D-Glu residue is essential for toxicity of nodularin, as esterification of its free carboxyl abolishes toxicity; however substitution at position 1 has little effect on toxicity. The other two isoforms, nodularin-Har and motuporin, are variable at position 2 (Saito *et al.*, 2001). Nodularin-Har is produced by the strain *N. harveyana* PCC 7804, with the L-Arg, replaced with L-Homoarginine (L-Har) (Beattie *et al.*, 2000). The mechanisms of toxicity are relatively same as that of microcystin by inhibiting PP_1 and PP_2A with IC_{50} of 0.1nm. The hydrophobic C20 β- amino acids ADDA, present in both toxins, blocks protein phosphatases enzyme activity by interacting with the hydrophobic groove and obstructing substrate access to the active site cleft. MeDhb binds to Cys273 of PP_1A and Cys266 of PP_2A (Pearson *et al.*, 2010).

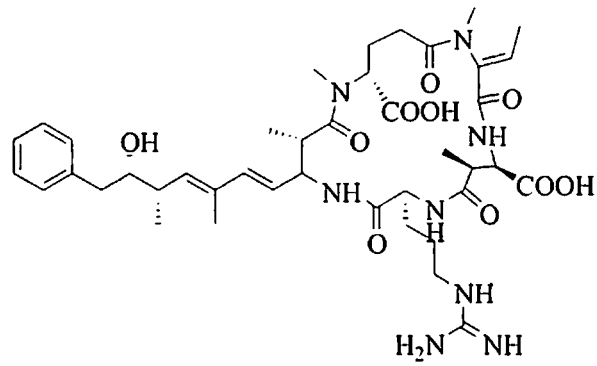


Fig. 4 Structure of Nodularin

Microcystins

Microcystins have been characterised from *Microcystis, Anabaena, Oscillatoria, Nostoc,* and *Anabaenopsis species*. About 90 structural variants of microcystins have been characterized so far from cyanobacteria. According to the world health organization, the guide line value for total microcystin (free and intracellular) is 1 µg/L (Chorus & Bartram 1999, Welker & Von-Dohren 2006, Pearson *et al.,* 2010).

Microcystin is biosynthesized via a large multifunctional protein complex consisting of non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) modules. The gene cluster encoding for microcystin (*Mcy S*) has been sequenced and partially characterized in several cyanobacterial species including *Microcystis, Anabaena, and Planktothrix* (Tillett *et al.,* 2000, Christiansen *et al.,* 2003, Rouhiainen *et al.,* 2004). In *M. aeruginosa PCC7806*, the *Mcy* S gene cluster spans 55 kb and comprises 10 genes arranged in two divergently transcribed operons, *Mcy* A-C and *Mcy* D-J (Fig. 5). The larger of the two operons, *mcy* D-J encodes for modular PKS (*Mcy* D, E, G), *Mcy* J, F, I encodes for the enzymes involved in tailoring reaction during ADDA synthesis and *Mcy* H is in fact responsible for the active transport of microcystin (ABC transporter) (Tillett *et al.,* 2000). This transporter may be responsible for the thylakoid localization of the toxin (Shi *et al.,* 1995, Young *et al.,* 2005) or for the extrusion of the toxin under certain growth conditions, including exposure to high and

red light (Kaebernick *et al.*, 2000). *Mcy* E converts the polyketide to a β-amino acid in the final step of ADDA biosynthesis (Pearson *et al.*, 2010). The smaller operon, mcy A-C encodes three NRPSs (*Mcy* A-C) (Tillett *et al.*, 2000). They are transcribed in opposite direction with a central sequences of approximaterly 900bp containing the promoter and putative regulatory *cis* element. The individual peptide synthetase catalyzes amino/hydroxyl acid activation and thioester formation reaction, the rate of reaction is determined by the growing heptapeptide chain. The production of the fatty acid side chain of the amino acid ADDA has been responsible for the function of polyketide synthase. *Mcy* E, is thought to be involved in the activation and condensation of D-Glu with ADDA (Pearson *et al.*, 2010). Thus, exchange and rearrangement of peptide synthetase modules offer the opportunity to produce novel products (microcystin).

Fig. 5 Microcystin synthetase of *Microcystis aeruginosa*, 55kb (*mcy S*)

Chemically microcystin (MCYST-XZ) is cyclo(-ADDA-D-Glu-Mdha-D-Ala-L-X-D-MeAsp-L-Z) where X and Z represent variable L-amino acids, ADDA is 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid, D-MeAsp is 3-methylaspartic acid, and Mdha is N-methyl-dehydroalanine. The most common and potent variant is microcystin- LR containing amino acid leucine (L) and arginine (R) at X and Z position (Fig. 6) (Sivonen *et al.*, 1990). Other variants differ in methyl group and amino acid in the ring, resulting in marked differences in toxicity and in hydrophilic and hydrophobic property. The ADDA moiety is responsible for toxicity by binding to the protein phosphatases (Carmichael 1992, Harada *et al.*, 1990). The stereochemistry of 4, 6 dienes in ADDA group and level of methylation in

various structures of cyclic peptides are reported to influence the level of toxicity and site of action (Gupta *et al.*, 2003, Namikoshi *et al.*, 1998)

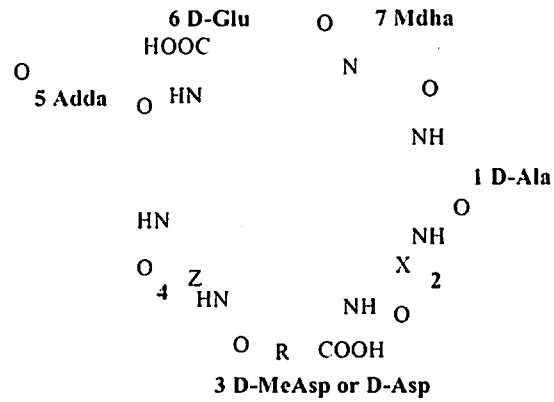


Fig. 6 General Structure of Microcystin

The molecular mechanisms of microcystins toxicity are being investigated and advances have been reached in the knowledge of the activity of this cyclic peptide at cellular and molecular level. The site of action of microcystins in hepatocytes leads to disrupting the cytoskeleton of hepatic cells leading to massive hepatic bleeding (Agrawal *et al.*, 2006, Ashwin *et al.*, 2011, Madhu *et al.*, 2006) is due to increase in reactive oxygen species (ROS). In severe cases, hepatic bleeding may lead to death or chronic intoxication by promoting hepatic tumors. The up-take studies on rat hepatocytes especially in human hepatocarcinoma cell line $HepG_2$ (Zegura *et al.*, 2003) shown that microcystin-LR more specific to hepatocytes and also indicate necessity of bile acid transporters for its site of action (Runnegar *et al.*, 1991). The tumour promoting nature of microcystin results due to the inhibition of protein phosphatase 2A activity in cellular membrane and cytosolic fractions. The inhibition of protein phosphatase activities results in disturbing the cellular phosphorylation balance and causes continual activation kinases (Ericksson *et al.*, 1990 a & b, Runnegar *et al.*, 1993, Gehringer 2004) which plays a significant role in cell growth (tumour formation) (Fig. 7).

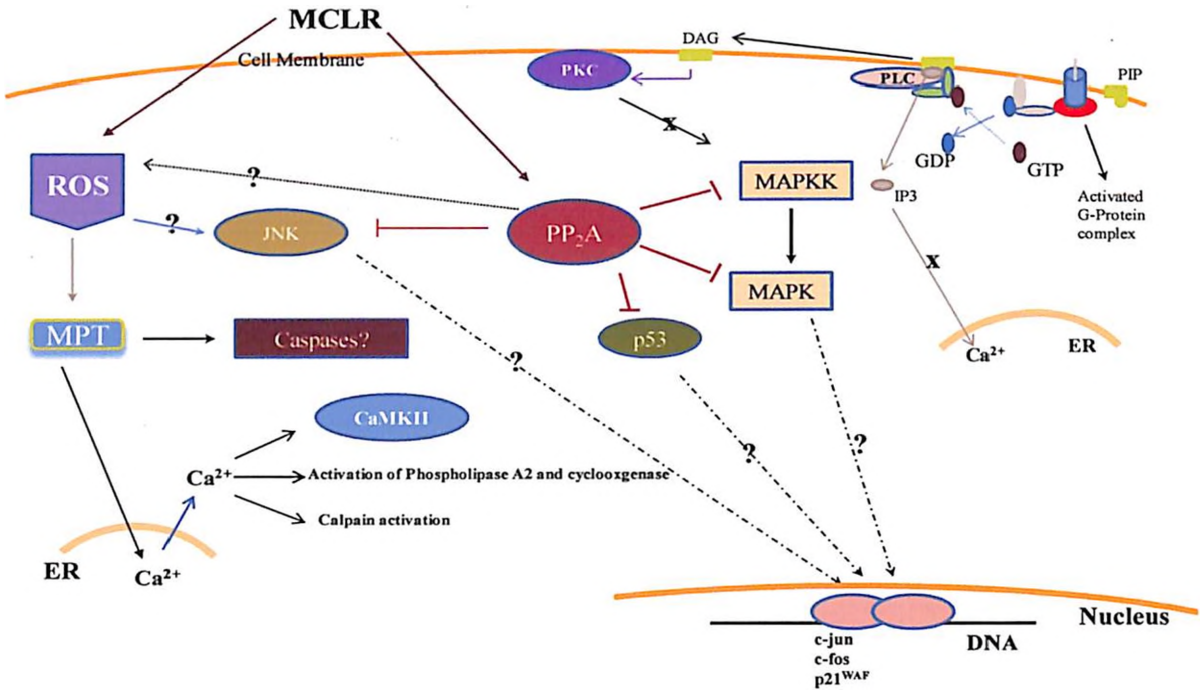
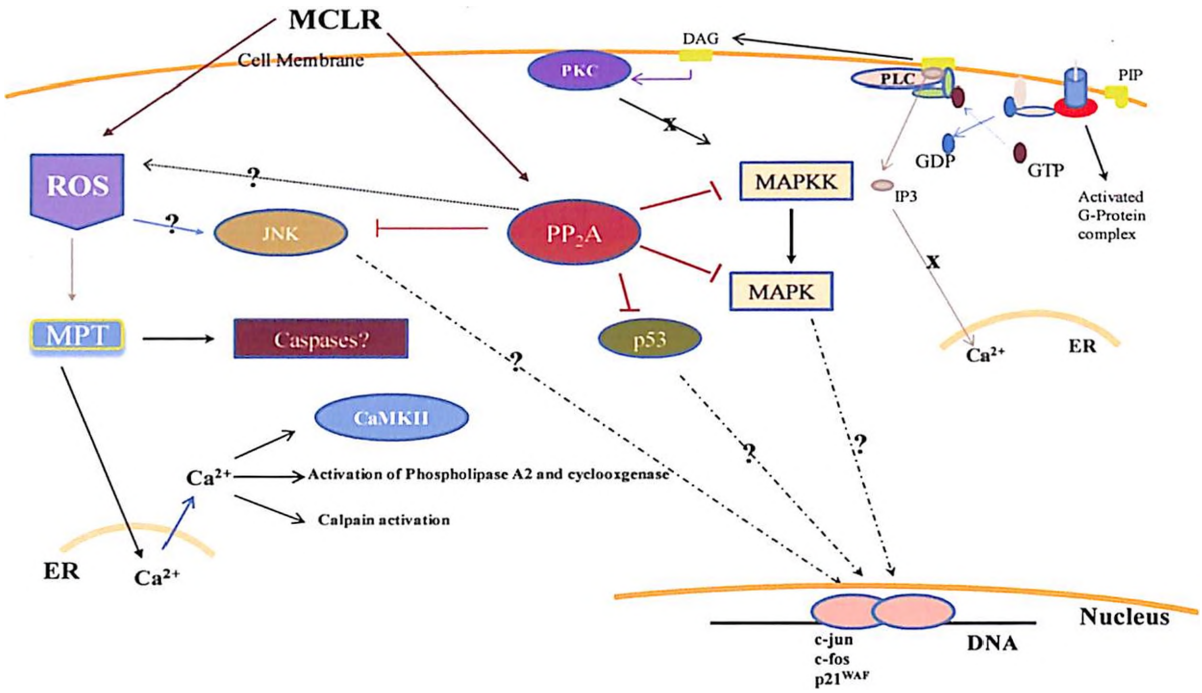


Fig. 7 The proposed scheme illustrates cellular events upon Microcystin-LR (MCLR) exposure. The MAPK pathway is usually activated by means of receptor tyrosine kinases activated by G-linked receptors which activate the cleavage of Phosphatidyllinositol 4,5-biphosphate (PIP) to diacylglycerol (DAG) and IP3 by PLC. IP3 triggers the release of Ca^{2+} from the endoplasmic reticulum (ER), but this does not occur for microcystin-LR as microcystin-LR inhibited vasopressin-induced Ca^{2+} release. DAG release and increased Ca^{2+} are known to activate Protein kinase C (PKC) activation of MAPKK which in turn activates MAPK. PKC was not found to play a role in microcystin-LR induced kinase activation *in-vivo*. PP_2A regulates the phosphorylation of both MAPKK and MAPK so inhibition of this protein phosphatase (PP) by microcystin-LR would allow for the continual activation of both these kinases. Once activated MAPK is translocated to the nucleus where it activates transcription factors such as c-jun and c-fos, which initiate transcription of genes necessary for growth and differentiation. Increased phosphorylation of p53 occurs in microcystin-LR exposed hepatocytes. Microcystin-LR induces the formation of reactive oxygen species (ROS) which triggers mitochondrial permeability transition (MPT) prior to the release of Ca^{2+} from the endoplasmic reticulum which activates CaMKII, phospholipase A2, cyclooxygenase and the calpains. ROS are known to activate by PP_2A-regulated kinase like JNK which leads to increased c-jun activity. Cytochrome c release from the mitochondria after MPT usually results in activation of the caspase cascade, however the role of caspases in microcystin-LR-induced apoptosis remains to be clarified (Gehringer 2004).



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In India, considerable progress has been made in the field of natural products derived from cyanobacteria and its pharmaceutical applications. The work done on the fresh water cyanobacterial cyanotoxins are reported by Agrawal *et al.*, (2006). Rahman & Choudhary (2005), Madhu *et al.*, (2006), Suryaanshi *et al.*, (2007) and antifungal compounds by Pawar & Puranik (2007). Cyanobacterial toxins in drinking or recreational waters are hazardous to human health. The high incidence of primary liver cancer in China, Australia, Europe (Codd *et al.*, 1999, Katircioglu *et al.*, 2004, Jaiswal *et al.*, 2008) and the deaths of patients in a hemodialysis unit in Brazil (Oberholster *et al.*, 2004) are reported because of the consumption of microcystin contaminated water. Other reports are mainly focused on marine cyanobacterial secondary metabolites (Burja *et al.*, 2001). The works pertaining to microcystin toxicity are done mainly in *Microcystis* dominant phytoplankton isolated from Central India which have been found to be toxic due to high concentration of microcystin-LR (Madhu *et al.*..2006, Agrawal *et al.*, 2006). However, there is little work done so far on biodiversity of variants of cyanotoxins and their toxic contents in desert regions of Rajasthan. The existence of tough tropical climatic condition promotes seasonal toxic cyanobacterial blooms (Tiwari *et al.*, 2005). Isolation and characterization of Cyanotoxins from these organisms can lead to identification of novel compounds and help us to understand its biological significant.

Shekhawati is a semi-arid region with area of 13784 square kilometers located in the northeast part of Rajasthan, India. It is surrounded by Jangladesh, Haryana, Mewat, Dhundhar, Ajmer (Fig. 8) (Katewa & Galav 2005). The temperature ranges from sub-zero Celsius in winter to more than 50°C in summer. The summer brings hot waves of air called loo. Annual rainfall is around 450 to 600 mm. The ground water is as deep as 200 feet (60 m), and in places water is hard and salty (Batheja *et al.*, 2008). On average every third year is dry and every eighth year the region experiences famine. In such conditions growth of algae is very limited in this region.

Between the months of July and mid September is the monsoon season, during which atmospheric humidity increases. The July and August rainwater is stored in pucca tanks and used throughout the year for drinking purposes. Bawdis and Johads are traditionally constructed for storing water from rainfall. The water stored in these is very cool and used for drinking purposes which are the ideal place for the algal growth. The ideal time to collect algal samples is during fourth week of August to second week of December and during first week of March to second week of May. Although there is no toxicological evidence on human or animal lethality associated with these blooms, in a survey conducted by our lab, the animals drinking the water stored in old pucca tanks, ponds and lakes were reported to have hepatic disorders (data not shown). It triggered us to investigate the nearby water bodies for the presence of toxic cyanobacteria and to characterize the nature of toxin present. So the objectives of present work planned were:

1. To isolate toxic cyanobacterial strains from the water bodies in Shekawati region of Rajasthan
2. To isolate, purify and characterize Cyanotoxins from toxic cyanobacterial strains along with their physiochemical properties.
3. To study the pharmacological effects of toxins by *in-vivo* models.

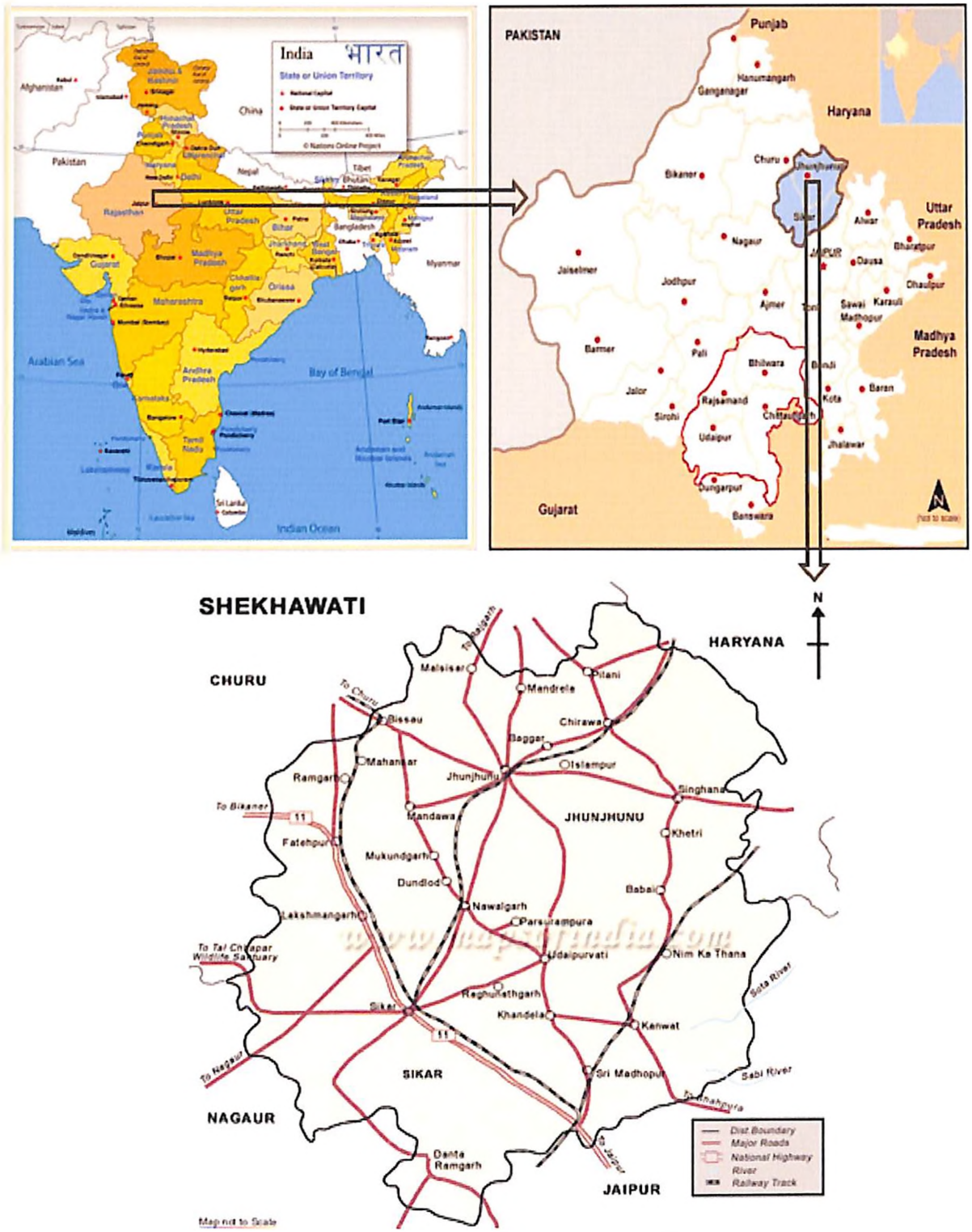


Fig. 8 Site locations of Shekhawati region

Chapter II

Materials and Methods

2.1. Isolation and Characterization of cyanobacterial strains

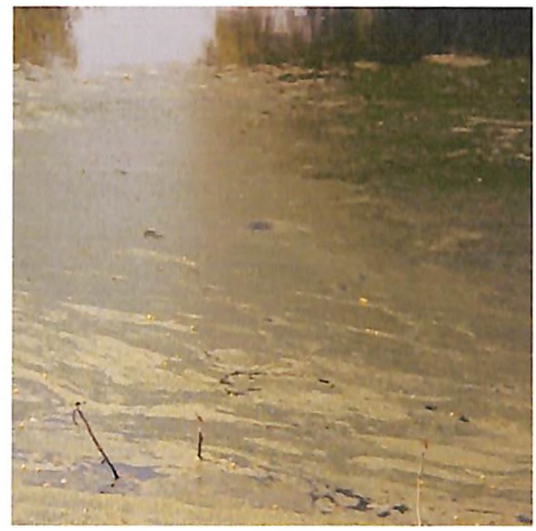
2.1.1. Sample collection

Shekawati region of Rajasthan is characterized by typical semiarid climatic conditions. The temperature in the region varies from 1°C to 48°C with an annual average rainfall of 450-600mm. Four seasonal water bodies of Pilani and nearby area were selected for collection of algal samples (Table1 & Fig. 9). Samples were collected by skimming across the water surface using 25μm plankton net. Random collections were made from different spots in a given locality in order to get maximum diversity. The samples were kept cool (25°C) and brought to the laboratory in half strength media supplemented with pond water.

Table 1 Geographic location of collection sites.

Sl. No	Location of Sample	Date of collection	Bloom formation
1	Rajgarh road pond Site-1 28°2'50.38"N 75°35'46.14"E	14.10.2006	Yes (seasonal)
2	Ram mandir talab Site-2 28°21'44.73"N 75°35'56.84"E	06.05.2006	Yes (throughout the year)
3	Shiv ganga in BITS-Pilani campus Site-3 28°21'19.68"N 75°35'17.28"E	23.03.2006	Yes (seasonal)
4	Pilani-Delhi Road Pond Site-4 28°21'49.00"N 75°36'29.76"E	18.09.2006	Yes (throughout the year)

Site-1 (Rajgarh road pond)



Site-2 (Ram mandir talab)

Site-3 (Shiv ganga in BITS-Pilani campus)

Site-4 (Pilani-Delhi road pond)

Fig. 9 Site locations for sample collection

2.1.2. Strain Isolation and Purification

Samples were initially examined under light microscope (Olympus CKX41) for preliminary morphological identification of algal species. The purification of algal strains were carried out by serial dilution followed by streaking on Chu-10 and BG_{11} plates followed by repeated sub-culturing on solid and liquid media (Chu-10 and BG_{11}). To eliminate eukaryotic contamination of cyanobacteria, cyclohexamide (100 $\mu g\ ml^{-1}$) was added to the liquid culture and kept under light (14.4Wm^{-2}) at 25±2°C for 2 days followed by serial dilution (10, 50, 100, 1000 time). 0.1 ml of diluted sample was spread over the surface of Chu-10 and BG_{11} plates and was incubated in the culture room until distinct colonies appeared. Individual colonies were picked up under binocular microscope with the help of capillary tubes and suspended in fresh liquid medium. After period of 8-10 days, 0.1ml of sample were again plated on Chu-10 and BG_{11} plates and incubated in the culture room. Distinct healthy colonies were picked up and transferred in fresh liquid medium. The above step was repeated until axenic unialgal cultures were established.

2.1.2.1. Transmission electron microscopy (TEM)

For TEM studies, the cells were washed with 0.1M Phosphate buffer (pH 7.4). Primary fixation of the sample was done by submerging in 2.5% glutaraldehyde in phosphate buffer for 6 hrs at 4°C followed by post fixation with 1.0% OsO_4 in 0.1% M phosphate buffer for 2 hrs at 4°C. The samples were washed repeatedly using same buffer, dehydrated in A-graded ethanol (50-100% v/v) and acetone to critical point drying. It was embedded on araldite and thin section of 60-70 nm were cut by an ultra microtome with diamond knives, which were then mounted on copper grids, contrasted with uranyl acetate and lead citrate and viewed under transmission electron microscope (PHILIPS Morgagni 268) at 80kV.

2.1.2.2. Strain Identification

Algal strains were identified according to morphological description of species such as trichome/filaments shape and size, cell dimensions, presence of sheath, thickness and position of akinetes/heterocysts, as described in the monograph on cyanophyta (Desikachary 1959). Further molecular taxonomic characterization was done using 16S rRNA sequencing in Dr. Radha Prasanna's lab, Microbiology Division, IARI, New Delhi. Properly identified strains were submitted to the National Center for Conservation and Utilization of Blue - green Algae, India and their accession numbers were obtained (*A. doliolum*- CCC599 and *N. spongiaeforme*- CCC598).

2.1.3. Growth media and culture conditions

The different culture media namely BG_{11} (Rippka *et al.,* 1979), Fogg's medium (Jacobson 1951), Allen and Arnon's medium (Allen & Arnon 1955) and Chu-10 (Gerloff *et al.,* 1950) were used to determine the suitable growth of isolated strains and the synthesis of toxic peptides (Table-2). These media were used in different set of experiments.

2.1.3.1. Strain Cultivation and Culture maintenance

The experimental organism's i.e *Anabaena doliolum*, *Nostoc spongiaeforme*, *Spirogyra* and *Scenedesmus* were grown axenically in BG_{11} medium (Rippka *et al.,* 1979). The cyanobacterial cultures were maintained in the media free from fixed nitrogenous compounds. All cultures were maintained in the culture room illuminated with cool day light fluorescent tubes ($14.4 Wm^{-2}$) at 25±2°C. Exponentially grown cells were harvested by centrifugation at 600 rpm at room temperature (RT) for 10mins for sub-culturing. Stock culture of axenic *A. doliolum*, *N. spongiaeforme*, *Spirogyra* and *Scenedesmus* on 1.5% agar slants were preserved under culture room condition.

Table 2 Composition of different nutrient media.

Composition	BG_{11} medium (gL^{-1})	Fogg's medium (gL^{-1})	Allen and Arnon medium (gL^{-1})	Modified Chu-10 medium (gL^{-1})
$NaNo_3$	1.5	-	1.7	-
KNO_3	-	0.25	-	-
$Ca(NO_3)_2.4H20$	-	-	-	0.232
$K_2HPO_4.3H_2O$	0.04	-	0.348475	0.01
KH_2PO_4	-	0.2	-	-
$CaCl_2.2H_2O$	0.036	0.1	0.055	0.0735
$MgSO_4.7H_2O$	0.075	0.2	0.2475	0.025
Citric acid	0.006	-	-	0.0035
Ferric ammonium citrate	0.006	-	-	-
Ferric citrate	-	-	-	0.0035
Na_2CO_3	0.02	-	-	0.02
NaCl	-	-	0.2338	-
$Na_2SiO_3.5H_2O$	-	-	-	0.044
Disodium EDTA	0.001	-	-	-
Fe-EDTA	-	-	#	-
Micronutrients	$a^{¥}$	$a^{\$}$	a*	a*
pH	7.2	5.4	8.2	6.5

Fe-EDTA was prepared by dissolving 5.2 g of EDTA and 5.4gms of $NaHCO_3$ in 100 ml of distilled water followed by boiling to remove the CO_2. To CO_2 free solution, 5 g of $FeSO_4$ was added and boiled. The solution was made up to 1liter after cooling it at room temperature. 1ml of this stock was added to 1liter of culture medium.

$a^{¥}$, $a^{\$}$ and a* Micronutrients are mentioned in Table-3

Table 3 Composition of micronutrients

Ingredients	a*Micronutrients for Modified Chu-10 and Allen and Arnon medium (mgL^{-1})	a[S]Micronutrients (A5) for Fogg's medium (mgL^{-1})	a[Y]Micronutrients for BG_{11} medium (mgL^{-1})
H_3BO_3	0.5	2.86	2.86
$ZnSO_4.H_2O$	0.05	-	-
$ZnSO_4.7H_2O$	-	0.222	0.222
$MnCl_2.4H_2O$	0.05	1.81	1.81
$CuSO_4.5H_2O$	0.05	0.079	0.079
MoO_3	0.01	0.0177	-
$CoCl_2$	0.04	-	-
$Na_2MoO_4.2H_2O$	-	-	0.390
$CO(NO_3)_2.6H_2O$	-	-	0.040

2.2. Growth

The growth of *A. doliolum* and *N. spongiaeforme* was monitored in terms of total protein or Chlorophyll *a* content.

2.2.1. Determination of proteins

The protein content was estimated by the method adopted by Lowry *et al.*, (1951) and modified by Herbert *et al.*, (1971).

The reagents used were,

Reagent A- 0.1N Sodium hydroxide (AR grade, Merck)

Reagent B- (i) 5.0% sodium carbonate (AR grade, Merck)

(ii) 5.0% copper sulphate pentahydrate (AR grade, Merck) in 1.0% sodium potassium tartrate (AR grade, Himedia).

Solution (i) and (ii) were mixed in the ratio of 25:1 (V/V)

Reagent C- 0.1N Folin-phenol reagent

0.5 ml of Reagent A was added to 0.5ml of algal sample and kept in boiling water bath for 5 mins followed by cooling under running water. 2.5 ml of reagent B was added to it and incubated for 10 mins at room temperature, followed by addition of 0.5 ml of reagent C. The intensity of blue colour developed after 15 minutes of incubation at room temperature was measured at 650 nm using UV-Visible Spectrophotometer (Jasco V640). The concentration of protein was estimated from a calibration curve prepared using lysozyme (Sigma, USA) as standard and expressed as µg protein ml^{-1} of algal culture.

2.2.2. Determination of chlorophyll *a*

Total chlorophyll *a* (Chl *a*) content of *A. doliolum* and *N. spongiaeforme* was determined by the method described by McKinney (1941). Briefly, 1 ml culture was withdrawn in 1.5 ml

micro centrifuge tube and centrifuged at 2000 rpm (Eppendorf Centrifuge 5415D) for 3 minutes; cell free supernatant was discarded. The cell pellet was added with 1000 µl of 100% methanol (AR, Merk), vortexed and incubated in dark at 4°C for 12 h. The contents were spun at 10000 rpm for 5 minutes to remove cell debris. Chl *a* in the supernatant was measured at 665 nm using UV-Visible Spectrophotometer (Jasco V640). The absorbance were multiplied with a factor of 12.7 to get the concentration of Chl *a* in µg ml^{-1} culture.

2.2.3. Determination of heterocyst frequency

Heterocyst frequency was measured by counting the number of heterocyst per 100 vegetative cells using haemocytometer under binocular microscope (Olympus CKX41). Images were captured using Olympus C-4000 camera and recorded.

2.3. Biomass Production

Commercial grade chemicals purchased from S.d. fine Pvt. Ltd were used for the biomass production of *Anabaena doliolum* and *Nostoc spongiaeforme* in BG_{11} or Chu-10 media.

2.3.1. Plastic tubs culture method

Plastic tubs of 41x29x15cm size were used for the production of selected cyanobacterial biomass (*Anabaena doliolum* and *Nostoc spongiaeforme*). The tubs were placed in the green house under the shade net (equipped with the net that could cut 50% of the light intensity) where the temperature was 30°C and the maximum light intensity was 400µmol m^{-2} s^{-1}. In each tub, 9.5 liters of BG_{11} or Chu-10 were prepared by using single distilled water and 0.5 liter of the 10 days old inoculums with total protein content of 300µg/ml was added. The culture was allowed to grow for 22 days and harvested by centrifugation (VS-2ISMTI Vision high speed refrigerated centrifuge, Vision Scientific Pvt Ltd) at 4000 rpm for 5 mins. The collected algal

samples were shade dried, weighted and stored in -20 ° C refrigerators (Cellfrost) for further analysis.

2.3.2. Baby pool culture method

Plastic baby pool of 122 x 25cm (capacity- 400 liters) and 183 x 38 cm (capacity- 750 liters) were used for the production of selected cyanobacterial biomass (*Anabaena doliolum* and *Nostoc spongiaeforme*) at mass scale. The pools were place in the green house under the shade net (equipped with the net that could cut 50% of the light intensity) where temperature was 30°C and the maximum light intensity was 400µmol m^{-2} s^{-1}. In each pool of BG_{11} or Chu-10 were prepared using single distilled water and 8 days old tub cultures were inoculated to get a OD of 0.1 µg/ml of total protein. The culture was allowed to grow for 22 days and harvested by simple filtration through plankton net of 25µm. The collected algal samples were shade dried, weighted and stored in -20 ° C refrigerators (Cellfrost) for further analysis.

2.4. Extraction of Microcystin variants from toxic cyanobacterial strains

2.4.1. Isolation of Tumorogenic compounds

The 22-days old cultures were harvested and 20g of dried cells were treated three times with 70% methanol (400 ml) for 60 mins with continuous mixing using magnetic stirrer at 4°C. The supernatant of extracts were collected by filtration using 0.4µ Whatman filter paper and concentrated by rotator apparatus (Buchi R-210). The crude extract was suspended in minimum quantity of phosphate buffer (pH 6.4) and further purified by fractionation with equal volume of solvents with increasing polarity (hexane, ether, chloroform and methylene chloride). Each fraction was checked for total protein content and *in-vivo* toxicity. The solvents used for isolation of tumorogenic compounds were of AR grade purchased from Merck.

2.4.2. Concentration and preservation of natural extracts/compounds

The extraction process described in section 2.4.1 generates large volumes containing relatively dilute amounts of toxins. These solutions were concentrated by solvent evaporation method. Evaporation was carried out using rotary evaporator at 30°C. The dried samples were suspended in minimum quantity of phosphate buffer (6.2 pH). Each fraction was checked for total protein content. All partially purified extracts/ purified compounds were further concentrated by centrifugal lyophilization and preserved at -70°C (Revco ultima II) for subsequent analysis.

2.5. Nutrimental factor determining microcystin biosynthesis

2.5.1. Nutrimental factors determining biomass production

The strains of *A. doliolum and N. spongiaeforme* were grown for 17 days in the Chu-10 medium lacking combined silicate, magnesium, phosphate, calcium or carbonate source, in the culture room illuminated with cool day light fluorescent tubes of 400 LUX at 25±2^{0}C. The pH of the media was maintained at 6.5 with HEPES buffer solution. Cells were harvested, dried (in lyophilizer) and weighed. The dried biomass of *A. doliolum and N. spongiaeforme* was taken for total protein and total microcystin estimation as described elsewhere.

2.5.2. Quantification of extracts for total Microcystins by ELISA

The microcystins were assayed with the help of Microcystin-DM ELISA kit (product no: 522015) procured from Abraxis LLC, Warminster, U.K. As per specification of vendors, 100 µl of sample was taken in Abraxis Microcystin-DM ELISA microtiter plate coated with a secondary antibody (goat anti-mouse) and 50 µl of Abraxis Microcystin-DM ELISA enzyme conjugate solution (monoclonal antibody). To this, 50µl of Abraxis Microcystin-DM ELISA antibody solution (Microcystin-HRP conjugate) was added. The contents were carefully

oscillated for 30 seconds and incubated for 90 min at RT. The wells were washed three times with 250 µl of 1x Abraxis Microcystin-DM ELISA wash buffer. The excess buffers in the wells were removed by patting the plate dry on a stack of paper towels. 150 µl of Abraxis Microcystin-DM ELISA color solution was added and the contents were shaken well and incubated for 20-25 min at RT. 100 µl of Abraxis Microcystin-DM ELISA stop solution (dilute H_2SO_4) was added and the absorbance at 450 nm was measured within 15min. The calibrations curve was prepared by using Abraxis Microcystin-DM ELISA standard solutions provided by the ELISA kit supplier (Abraxis).

2.5.3. Effect of light on microcystin synthesis

The strains of *A. doliolum* and *N. spongiaeforme* were grown in Chu-10 as well as Allen and Arnon culture medium for 17 days in the culture room illuminated with cool day light varying light intensity (50, 100, 150, 230, 300, 400, 600 and 700 LUX) at 25±2°C. Cells were harvested, dried (in lyophilizer) and their total protein content was estimated by Lowry's method as described earlier. Quantification of microcystin variants was done by HPLC with the help of UV detector at 238 nm using pure microcystin-LR as standard. The area under the curve was used to calculate the amount of microcystins variants present in the sample.

2.5.4. Effect of nitrate on microcystin synthesis

The cells of *A. doliolum* and *N. spongiaeforme* were grown in Chu-10 as well as Allen and Arnon culture medium with varying nitrate concentration (zero to 0.5 mM) for 17 days under standard culture conditions. The pH of the media was maintained at 6.5 with HEPES buffer. Cells were harvested, dried (in lyophilizer) and their total protein content was estimated by Lowry's method as described earlier. Quantification of microcystin variants was done by HPLC with the help of UV detector at 238 nm using pure microcystin-LR as standard. The area

under the curve was used to calculate the amount of microcystins variants present in the sample.

2.5.5. Effect of phosphate on microcystin synthesis

The cells of *A. doliolum* and *N. spongiaeforme* were grown in Chu-10 as well as Allen and Arnon culture medium with varying phosphate concentration (zero to 0.5 mM) for 17 days under standard culture condition. The pH of the media was maintained at 6.5 with HEPES buffer. Cells were harvested, dried (in lyophilizer) and their total protein content was estimated by Lowry's method as described earlier. Quantification of microcystin variants was done by HPLC with the help of UV detector at 238 nm using pure microcystin-LR as standard. The area under the curve was used to calculate the amount of microcystins variants present in the sample.

2.5.6. Effect of temperature on microcystin synthesis

The strains of *A. doliolum* and *N. spongiaeforme* were grown in Chu-10 as well as Allen and Arnon culture medium by placing it in different temperatures (15, 20 25, 30 °C) for 17days under standard culture condition. Cells were harvested, dried (in lyophilizer) and their total protein content was calculated by Lowry's method as described earlier. Quantification of microcystin variants was done by HPLC with the help of UV detector at 238 nm using pure microcystin-LR as standard. The area under the curve was used to calculate the amount of microcystins variants present in the sample.

2.6. Isolation of peptides by chromatographic techniques

Microcystins from *Microcystis* were purified using a combination of TLC, HPTLC and HPLC method as described by Namikoshi *et al.*, (1993) and Sano & Kaya (1998) with minor

modifications essential for some steps to purify the variants of Cyanotoxins from *A. doliolum* and *N. spongiaeforme*.

2.6.1. Partial purification of toxins by TLC

Fractionated extracts were partially purified by thin layer chromatography (TLC) using 10 cm long and 5 cm wide silica gel GF_{60} plates (Merck) as stationary phase and chloroform: methanol: water (60:24:16) as mobile phase. The extracts were dissolved in 100% methanol and were applied as separate spots to the plates at 1.5 cm from the bottom edge (spotting edge). The spots were dried using hair drier for 1-3 mins. The dried spots were allowed to develop by placing the plates in pre-saturated developing chamber with mobile phase reaching up to 80% height. The plates were dried at room temperature for 5 to 10 mins and were visualized under hand held UV illuminator (Banglore genei) at 234 nm and 364 nm. A part of samples were also visualized by iodine chamber method. Qualitative evaluations of total microcystins were carryout using densiometry (TLC Scanner 3 with CAS software ver.1.2.3, Camag) at 200 nm and 700 nm. The identification and approximate estimation of total microcystin were carried out by comparing R_f Values and area under the curve of both standard Microcystin-LR (Alexis, USA) and samples. All separations were performed at the room temperature 24-27°C. The spots coinciding with standard were scrap and dissolved in 100% methanol and were concentrated by centrifugal lyophilization.

2.7. Peptide identification by MALDI-TOF/MS.

The samples (10µg) were mixed with matrix α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich) in equal amount and dissolved in buffer containing acetonitrile and 0.1% trifluroacetic acid with the ratio of 1: 2. The samples were spotted on the MALDI target Anchor Chip and were allowed to crystallize. The target plate was introduced into the Ultraflex III MALDI

TOF/TOF (Bruker Daltonik GmbH) to obtain the MS spectrum. MALDI-TOF/MS was conducted by Dr. Madhurarekha, Sandor Proteomics Pvt Ltd, Hyderabad.

2.8. Quantification and purification of Cyanotoxins by HPLC

2.8.1.1. Instruments

The HPLC system employed was Jasco HPLC (Jasco Inc., Japan) with solvent delivery system of two pumps (Model PU-1580, Jasco Inc., Japan), auto Injector (Model As-1559, Jasco Inc., Japan) and UV-Visible detector (Model UV-1575, Jasco Inc., Japan). C-18 reverse phase column (Alltima 250 mm x 4.6 mm, 5 µm) equipped with a guard-column of same packing material used to separate Cyanotoxins. Data collection and integration was accomplished using BORWIN® work station (Jasco Inc., Japan). Other instruments used in the separation and purification of Cyanotoxins were Cyclo mixer (Remi, India), Bath sonicator, Millipore® filtration assembly (Waters, USA), Refrigerated centrifuge (Model CPR24, Remi, India) and Deep freeze (Vest frost, -20°C).

2.8.1.2. Preparation of Mobile phase and Samples for HPLC

TLC separated spots were quantitated by Lowry's method as described in the section 2.2.1 and dissolved in Phosphate buffer (pH 6.2). The mobile phases of 0.01 M ammonium acetate: Acetonitrile (64:36 & 72:38 v/v) mixtures, 0.01 M ammonium acetate: methanol (50:50 v/v) and trifluoroacetic acid: Acetonitrile (60:40 v/v) were prepared by using in-house millipore water. The aqueous phases were passed through the 0.22 µ millipore membrane filter (Millipore, USA). All mobile phases were degassed by sonication (Ultrasonic cleaner, Toshniwal instrument Pvt. Ltd. India.) before use. Standard Microcystin-LR (MC-LR) procured from Alexis, USA was used as reference compound. Ammonium acetate, potassium dihydrogen orthophosphate, dipotassium hydrogen orthophosphate used was of analytical

grade and purchased from Merck, India. HPLC grade solvents such as acetonitrile (ACN), trifluroacetic acid, methanol, and glacial acetic acid were purchased from Spectrochem, India.

2.8.1.3. Chromatographic conditions

The methods proposed by Namikokoshi *et al.*, (1993), Lee & Chou (2000) was modified to separate the variants of microcystin isolated from *A. doliolum* and *N. spongiaeforme*. The TLC spots, isolated from the algal cultures grown in Chu-10 were separated with 0.01 M ammonium acetate: Acetonitrile with the ratio of 64:36 (v/v) for the compounds of *A. doliolum*, whereas 72:38 (v/v) ratio was used for compounds purified from *N. spongiaeforme*. A ratio of 0.01 M ammonium acetate: methanol (50:50v/v) was used to separate compounds from *N. spongiaeforme* grown in Allen and Arnon culture medium. The flow rate was maintained at 1ml/min in all experiments. The selection of specific mobile phases for strain grown in different medias was depend on the complexity of the compounds to be separated and chromatographic peak properties of the samples. All purified compounds were collected with the help of UV detector at the wavelength of 238 nm. The microcystin-LR (Alexis, USA) was used as standard for estimating microcystin variants present in the sample. The area under the sample curve was used to calculate the amount of microcystins variants present in the sample. All purified compounds were concentrated by centrifugal lyophilization and stored at -70°C for further analysis.

2.9. Peptide Characterization by FTIR Spectroscopy

The qualitative and preliminary analysis of the chemical groups present in the HPLC purified samples was conducted with the help of FTIR spectroscopy (Shimadzu IR-Prestige 21 FTIR-Spectrometer) in solid phase on lyophilized peptides in a KBr disk. The IR spectra obtained at

4000-400cm^{-1} range was used to compare the purified microcystin variants with that of standard MCLR.

2.10. Peptide structure prediction

The identification of purified peptides was carried out by comparing the results of MALDI TOF MS as described in section 3.7 with available previous literature. The structure of these peptides is based on their elution profile in HPLC, FTIR analysis, MALDI TOF MS analysis and previous literature sources.

2.11. Screening of extracts/compounds for its *in vivo* bioactivity

2.11.1. Animals

Swiss albino mice of either sex between age group of 3 to 4 weeks weighing 18-25g were procured from Chaudhary Charan Singh Agricultural University, Hisar. They were housed in standard animal house condition (12 hours light/dark cycles; temperature: 21±2^{0}C; 50-60% relative humidity) with commercial standard mash feed (pellet chow feed) and water *ad libitum*. Following a quarantine period of 29 days the animals were randomly assigned to different groups. They were housed in laboratory cages [24 x 17 x 14 cm (l x w x h)] in groups of 6 per cage. Autoclaved husk was used as the bedding material. All studies on toxicity and neuronal activity were conducted after obtaining the approval from Institutional Animal Ethics Committee (IAEC/RES/11/1 and IAEC/RES/13/09). The animals were used only once for each experiment.

2.12. Screening of extracts/compounds for its *in vivo* toxicity

2.12.1. Determination of Acute toxicity

LD 50 or median lethal dose was determined in 4 groups containing six animals each with the dose range 500 to 2000 ng/kg body weight for purified compounds and 6 groups containing six

animals each with the dose range 0.1 to 1.0 mg/kg body weight for crude/partially purified extracts. A single dose of the samples were administered via intraperitonally (i.p) on one occasion to determine the characteristic toxicity of microcystins such as motor activity, respiration, skin colour, body weight, lethargy, swollen blood-engorged liver, pallor of ear, tail and feet as described for MCLR from *Microcystis* species (Falconer *et al.*, 1983, Runnegar *et al.*, 1991, Theiss *et al.*, 1988).

2.12.2. Determination of Sub acute toxicity

Two groups containing six animals each were dosed daily, with partially purified extracts (25µg /kg body weight)/ purified compounds (200 ng/kg body weight) to promote tumour (Nishiwaki *et al.*, 1992). These animals were injected with increased dose for every second day until toxic signs for microcystins were observed. The dose of the sample was increased in arithmetic progression in one group and geometric progression in other. The animals were maintained at the maximum tolerable dose for two weeks to develop pathological changes. They were sacrificed on 18^{th} days and autopsy was performed. During the course of study the parameters like body weight, respiration, skin & tail colour, food & water intake, locomotory activity were monitored for 4 hour every day after sample administration.

2.12.3. Determination of Chronic toxicity

Chronic dose responses of samples were studied by dosing daily for 28 days. Three dose levels were chosen for test animals present in 3 groups containing six animals each. A high dose of 300 µg/kg for partially purified extracts, 20 µg/kg for purified compounds were chosen to produce pathological changes. A low dose of 50 µg/kg for partially purified extracts, 0.75 µg/kg for purified compounds were administered for clinical response, while the third set of dose was chosen in midway such as 150 µg/kg and 10 µg/kg for partially purified extracts and

purified peptides respectively. The observations were made for 6 hrs daily after 30 mins of drug administration. The parameters like body weight, respiration, skin & tail colour, food & water intake, locomotory activity were monitored regularly. 2ml blood was collected by retro-orbital bleeding method on 28th days for serum analysis. All experimental animals were sacrificed on 28th days and autopsy was performed. The hepatocytes of test animals were weighed and preserved for further analysis.

2.12.3.1. Histopathology of animals

The liver of all animals subjected to toxicity studies were collected and perfused with normal saline. They were fixed with 10% buffered formalin. The fixed tissue were dehydrated with the ascending concentration of ethyl alcohol and cleaned with xylene. Section of 2-3 mm thickness was taken from the liver lobes with maximum diameters and processed for embedding in paraffin. Section of 4-5 µm were cut and stained with Ehrlich haematoxylin/eosin.

2.12.3.2. Determination of Hepatotoxicity by biochemical markers

Although the increase or decrease in MDL in hepatocytes and AST, ALP, AST, LDH in serum is not specific to hepatotoxicity but these parameters were used in the present study as diagnostic tool and to verify the course of toxicity of microcystin variants with respect to hepatocytes.

The serum was collected from the blood by mixing with 25 µl of 10 mM EDTA and centrifuged at 4000 rpm (Eppendroff centrifuge 5415 R) for 5 mins at 4°C and stored at -70°C (Revco ultima II) for further studies.

2.12.3.3. Estimation of Malandialdehyde (MDA) level in hepatocytes by TBARS

To liver homogenate containing 10 mg of total protein was added with 100 µl of $FeCl_2$ (1mM), 100 µl of ascorbic acid (1mM), 1 ml of CCl_3COOH (2.8% w/v), 20 µl of EDTA

(5mM), 1 ml of Thiobarbituric acid (1 % w/v in 0.05M NaOH) and heated in water bath for 1 hour. The mixture was added with 2 ml of n-Butanol (AR grade Merck) and kept aside for 5 mins. The upper butanol layer was separated and read in spectrofluoriphotometer (Shimadzu 5301PC) at 515 nm emission and 547 nm excitation wavelength. The standard calibration curve was prepared by using standard malandialdehyde (Sigma).

2.12.3.4. Estimation of serum alkaline phosphatase (ALP)

The assay was carried out as described in Spinreact LDH-LQ kit 41233. 20 µl of the sample was mixed with 1200 µl of working reagent (WR) (prepared as per manufacture's instruction). The working reagent contains dietanolamine (pH 10.4) 1mmol/L, magnesium chloride 0.5mmol/L, p-Nitrophenylphosphate 10mmol/L. The reaction mixture was incubated for 1min at RT. The initial absorbance was read at 405 nm in semiautomated biochemistry analyser (STAT fax-3300) at the interval of 1 min. The number of units of ALP was calculated by using the differences between absorbencies in the formula.

Average absorbance difference per minute x3300= U/L ALP

2.12.3.5. Estimation of serum alanine aminotransferase (ALT)

The assay was carried out as per the procedure described in Spinreact LDH-LQ kit 41274. 50µl of the sample was mixed with 500 µl of working reagent (WR) (prepared as per manufacture's instruction). The working reagent contains Tris (pH 7.8) 100mmol/L, lactate dehydrogenase 1200U/L, L-alanine 500mmol/L, NADH 0.18mmol/L, α-ketoglutarate 15mmol/L. The mixture was incubated for 1minute at RT. The initial absorbance was read at 405nm in semiautomated biochemistry analyser (STAT fax-3300) at the interval of 1 minute. The number of units of ALT was calculated by using the differences between absorbencies in the formula.

Average absorbance difference per minute x1750= U/L ALT

2.12.3.6. Estimation of serum aspartate aminotransferase (AST)

The assay was carried as per the procedure described in Spinreact LDH-LQ kit 41264. 50µl of the sample was mixed with 500 µl of working reagent (WR) (prepared as per manufacture's instruction). The working reagent contains Tris (pH 7.8) 80mmol/L, lactate dehydrogenase 800U/L, malate dehydrogenase 600U/L, L-aspartate 200mmol/L, NADH 0.18mmol/L, α-ketoglutarate 12mmol/L. The mixture was incubated for 1minute at RT. The initial absorbance was read at 405nm in semiautomated biochemistry analyser (STAT fax-3300) at the interval of 1 minute. The number of units of AST was calculated by using the differences between absorbencies in the formula.

Average absorbance difference per minute x1750= U/L AST

2.12.3.7. Estimation of serum lactate dehydrogenase (LDH)

The assay was carried as per the procedure described in Spinreact LDH-LQ kit 41214. 50µl of the sample was mixed with 1500 µl of working reagent (WR) (prepared as per manufacture's instruction). The working reagent contains Imidazol 65mmol/L, pyruvate 0.6mmol/L, NADH 0.16mmol/L. The mixture was incubated for 1minute at RT. The initial absorbance was read at 340nm in semiautomated biochemistry analyser (STAT fax-3300) at the interval of 1 minute. The number of units of LDH was calculated by using the differences between absorbencies in the formula.

Average absorbance difference per minute x4925= U/L LDH.

2.13. Screening of microcystin -FR for Antidepressive activity

2.13.1. Drugs

Propylene glycol (PG) was purchased from Merck (India). Amphetamine was purchased from Sigma Aldrich (India). Cyclosporin-A (CsA) and Naproxan were obtained as a gift from Sun

Pharma Limited (India); Desvenlafaxine, Bupropion were purchased from IPCA laboratories (India) and Ranbaxy research laboratories (India) respectively.

2.13.2. Animal groups

The animals were randomized into control and experimental groups with each containing six animals (n=6). All microcystin samples were dissolved in Phosphate buffer saline (PBS) (pH 7.4) and their sub-lethal concentration as established earlier in the section 2.12 were used in subsequent experiments. Cyclosporin-A was dissolved in propylene glycol and administered 30 minutes before injecting microcystin-FR in test animals. The dosage of Cyclosporin-A (8 mg/kg body weight) and the positive controls such as Bupropion (10 mg/kg body weight) and Desvenlafaxine (4 mg/kg body weight) were chosen from the previous reports (Rao *et al.*, 2004, Anthony *et al.*, 2008). All samples and controls were administered intraperitonally (i.p) only once in a day for 28 days. In case of acetic-acid induced writhing test, five groups were used including the control and standard drug. Naproxen (10 mg/kg) was used as reference drug to screen the analgesic activity of the compounds.

2.13.3. Hepatotoxicity of microcystin-FR along with Cyclosporin-A

The serum of the animals was collected on 28th day after 8 hours of the sample administration and hepatotoxicity of the test drugs were analyzed by serum aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) assays by commercially available kits (Spinreact kits) as mentioned in the section 2.12.3.6 and 2.12.3.7.

2.13.4. Histopathology

The liver of all animals was collected on 28th day and perfused with normal saline to carryout histopathology as described in the section 2.12.3.1

2.13.5. Force swim test

The animals were forced to swim individually in a glass jar (25x12x25cm) containing water column of 15 cm at a temperature of 24±3°C for a period of six minutes as described by Porsolt *et al.,* (1997). The floor of the cylinder was divided into 4 equal quadrants. A training session of 15 minutes was conducted for the mice, one day before the actual experiment. A mouse was considered to be immobile when it remains floating in the water without struggling, making only minimum forelimb movement, necessary to keep its head above the water (Fig. 10). After an initial vigorous activity for 2 minutes, each animal assumed a typical immobile posture. The total duration of immobility and number of quadrants crossed was recorded for the next 4 minutes. The changes in immobility duration were studied after administrating the drugs in separate group of animals.

2.13.6. Tail suspension test

Behavioural despair was induced by tail suspension according to the procedure of Pandey *et al.,* (2008) with slight modification. The study was conducted after 30 minutes of drug administration. The animals were suspended individually using adhesive tapes from a horizontal rod 50 cm above the flat surface of the tabletop. The point of attachment on the tail was 2 cm from the tip (Fig. 11). The duration of immobility during the six minutes observation period was recorded. Animals were considered to be immobile when they were completely motionless. The parameter recorded was in terms of number of second spent immobile.

Fig. 10 Mouse forced to swim in a jar containing water during force swim test.

Fig. 11 Mice suspended on the 30 cm long rigid iron rod horizontally at the height of 50 cm from the working surface during tail suspension test

2.13.7. Screening of microcystin-FR for Anxiolytic activity

2.13.7.1. Elevated maze test

The test was performed on the experimental protocol standardized by Ramamoorthy *et al.*, (2008) with slight modification. The anxiety of Swiss albino mice was measured by allowing the animals to move freely over an elevated maze height 50 cm above the ground. The apparatus consists of two open and two closed arms having walls (50x10x50cm), arranged alternatively with a common central platform (10x10cm). The open arm was provided with 2 mm high ledge to prevent falling of mice. The apparatus was indirectly illuminated with a ceiling-fronting lamp (60W), which was placed 100 cm above the apparatus (Fig. 12). The animals were placed at the centre of apparatus; head turned towards an open arm, after which it was allowed to walk freely for 5mins.The time spent in the open arm was recorded to evaluate anxiolytic activity of the samples/controls mice.

2.13.8. Screening of microcystin-FR for Spontaneous locomotive activity

The spontaneous locomotive activity was assessed as per method proposed by Boissier & Simon (1965) with slight modification. The mice were placed individually in a square arena (30x30cm), with wall painted black (Fig. 13). The first 2 minutes were left for acclimatization the digital locomotory scores of actophotometer were recorded for the next 10 mins in a dimly lit room. Dilute alcohol was used to clean the arena and dried completely between trials.

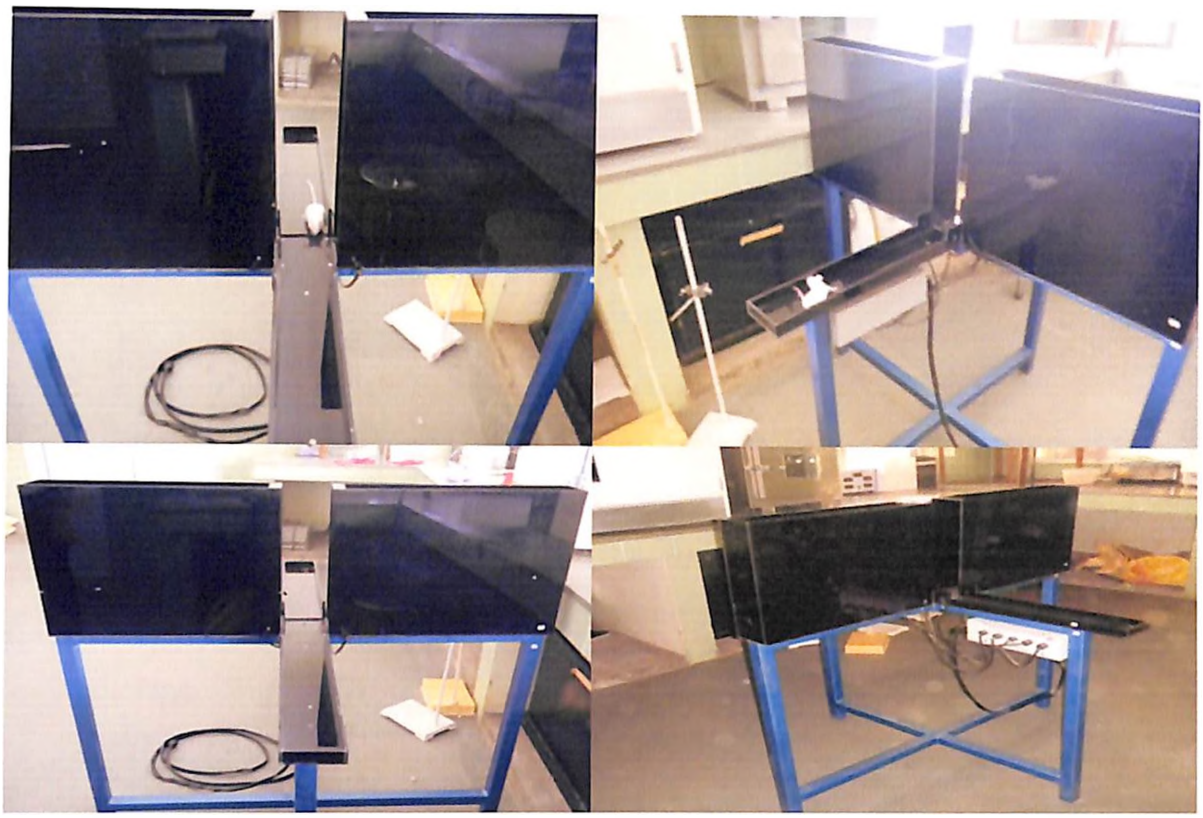


Fig. 12 Mice exploring the open arm of elevated maze during elevated pulse maze test

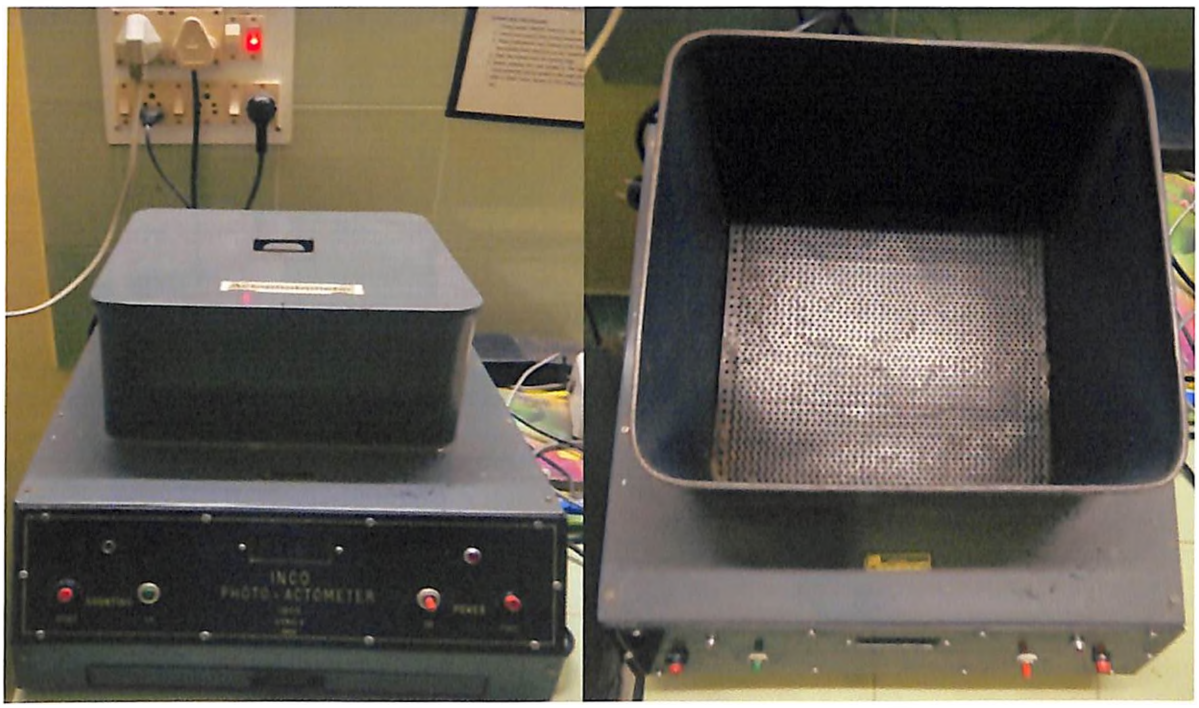


Fig. 13 Actophotometer with black square arena (30x30cm)

2.13.9. Screening of microcystin-FR for Analgesic activity

2.13.9.1. Acetic-acid induced Writhing test

The analgesic activity of the compounds was determined by acetic acid induced writhing test in mice as described by Collier *et al.*, (1968) with some modification. Mice were administered with the standard drug and test compounds, one hour prior to administration of 0.1 ml/10g of 1% v/v acetic acid. The writhing (full extension of hind limbs) were recorded for 15 minutes after five minutes of injecting acetic acid. Animals giving 20 or more wriths were selected for studying analgesic activity. The inhibition activity of individual compound as well as by the reference drugs were calculated using the formula,

% inhibition-100[1- W_t- W_c]

Where W_c represents the average writhing produced by the control group and W_t represents average writhing produced by the test group.

A Schematic representation of isolation, purification and screening of cyanotoxins (microcystin) has been depicted in Fig. 14

2.14. Statistical analysis

All data were expressed as mean ± standard error of mean (SEM). One-way ANOVA was also performed for all animal studies. The significance level was evaluated by Bonferroni's Multiple Comparison for FST, TST, EPM and Acetic-acid induced Writhing test. Dunnett's Multiple Comparison tests were carried out for all toxicological analysis to get individual comparisons of test with that of controls. Each value is a representation of the mean of six animals at significance level of $p<0.01$. The statistical software package PRISM Version 2.01 (Graphpad Sofware Inc., SanDiego, CA) was used for analysis.

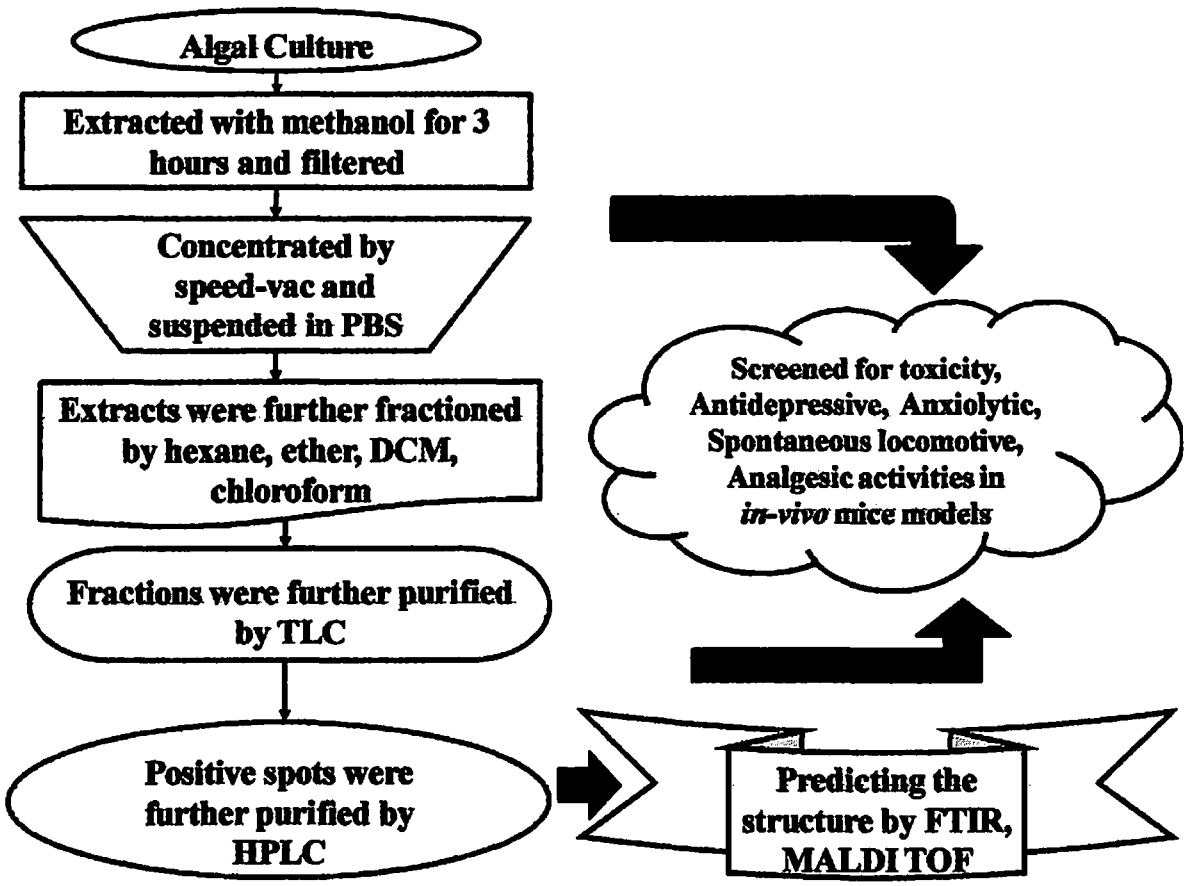


Fig. 14 Schematic representation of isolation, purification and screening of cyanotoxins from *Anabaena doliolum* and *Nostoc spongiaeforme*.

2.15. Docking of microcystin variants with PP_2A receptor:

To dock the microcystin variants with PP_2A receptor, Molegro Virtual Docker running in RED HAT LINUX operating system was used. The computational design was performed as per the following steps.

1. Microcystin-LR a molecular model of the toxin (template) was downloaded from the Protein Data Bank (PDB accession no:1EVA, Fig. 15)
2. Three dimensional structures of different variants of microcystins were generated by changing the appropriated residue as reported by Namikoshi *et al.*, 1992, Namikoshi *et al.*, 1993, Namikoshi *et al.*, 1998, Lee & Chou 2000 using Pymol software.
3. The generated structures were docked with PP_2A residues. Each variant was docked for 100 times in different poses. The pose with highest docking energy along with closer orientation to the reported docked structure of microcystin-LR (Bagu *et al.*, 1995) were considered for the selection of particular pose to proposed the mode of action.

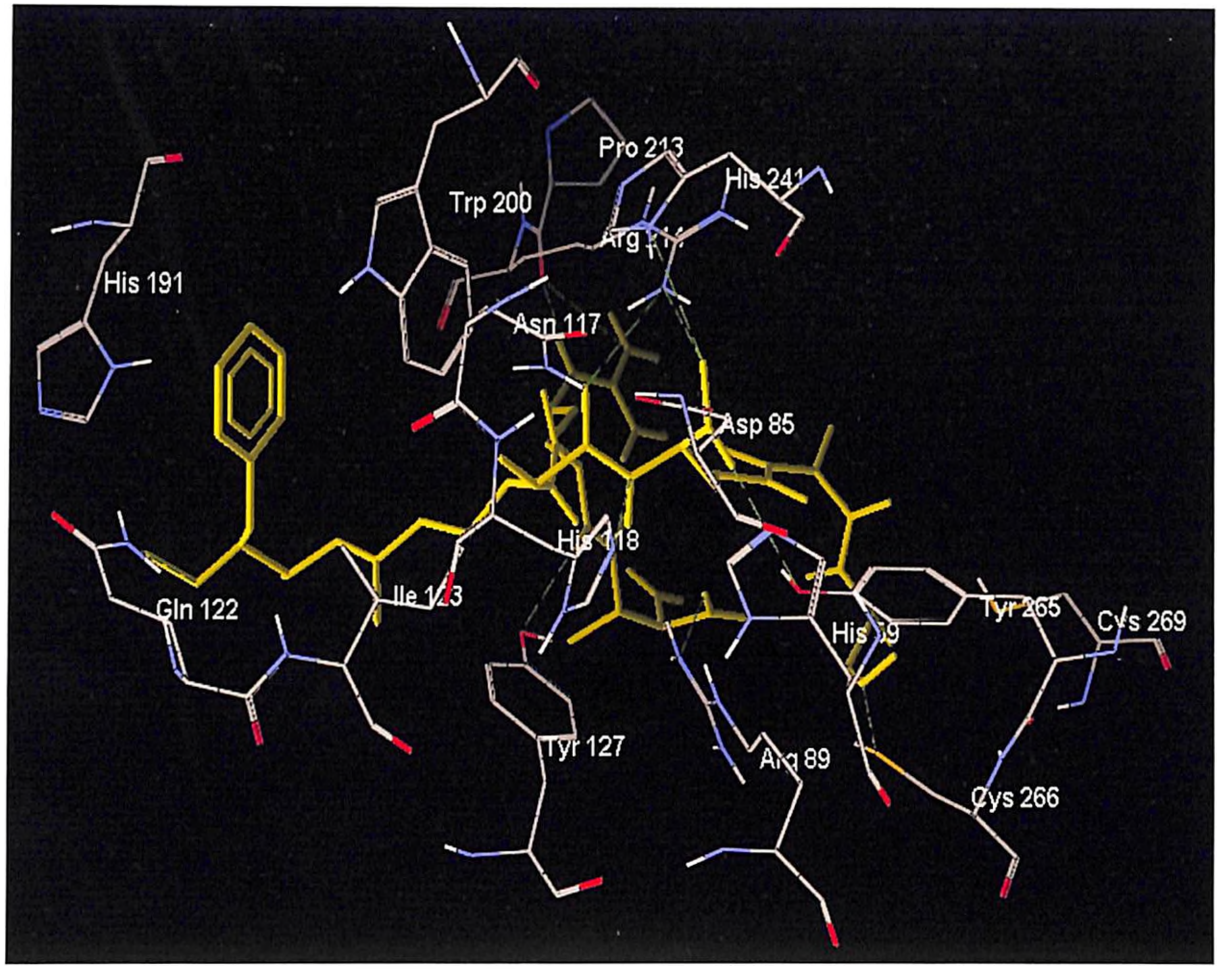


Fig. 15 Docked structure of microcystin-LR with PP_2A receptor download from PDB and reproduced in molegro virtual docker running in RED HAT LINUX operating system. The residues of PP_2A receptor which were involved in interaction with microcystin are represented as wire frame where as ligand (microcystin-LR) are represented as stick.

Chapter III

Results

3.1. Isolation and Characterization of cyanobacterial strains

3.1.1. Sample collection

The survey of four eutrophicated ponds of Pilani showed extensive bloom formation, especially during the month of February-April and September-November, 2006 (Ref. Table 1). Microscopic examination of these algal blooms reveals that the site-2 and site-4 contained *Anabaena doliolum* and *Nostoc spongiaeforme* respectively, whereas site 1 and site 3 was dominated by green algae such as *Spirogyra* sp. and *Scenedesmus* sp. Cyanobacterial strains were isolated and purified by using standard microbiological methods as mentioned in material and methods.

3.1.2. Strain identification and characterization

3.1.2.1. Morphological characteristics of *Anabaena doliolum*

The culture isolated from site-2 exhibited a characteristic of dark blue green pigmentation (Fig. 16 a, b & c). The strain was found to be filamentous with heterocysts forming at both ends of the cellular chain (Fig. 16 d & e). The old culture showed the formation of akinetes (resting cells) and their hormagonia can give rise to young trichomes that differentiated into terminal heterocysts (Fig. 16 f, g & h). Trichome lacked basal-apical polarity and is composed of barrel shaped, spherical vegetative cells which are immotile and enclosed by sheath (Fig. 16 h). Hormagonia is composed of cells that are smaller in size and cylindrical or isodiametric in shape with irregularly distributed gas vacuoles.

Taxonomic position of *Anabaena doliolum*

Phylum: Cyanophyta

Class: Cyanophyceae

Order: Nostocales

Family: Nostocaceae

Genus: *Anabaena*

Species: *doliolum*

3.1.2.2. Morphological characteristics of *Nostoc spongiaeforme*

The culture showed characteristic of light blue green colouration (Fig. 17 a, b & c) with globose, gelatinous thallus that expands to filamentous flexuous with loosely entangled distinct sheath over the periphery (Fig. 17 d, e). The filaments have barrel shaped cells with the end-cell having conical with round apex (Fig. 17 f). The older culture formed akinetes (Fig. 17 g). Spores were present in long chain of cylindrical cells with rounded ends covered with a smooth hyaline outer wall, spores were colorless initially and later become yellowish. Trichomes were loosely entangled. Heterocysts were sub-spherical with 7-8 µm broad (Fig. 17 h)

Taxonomic position of *Nostoc spongiaeforme*

Phylum: Cyanophyta

Class: Cyanophyceae

Order: Nostocales

Family: Nostocaceae

Genus: *Nostoc*

Species: *spongiaeforme*

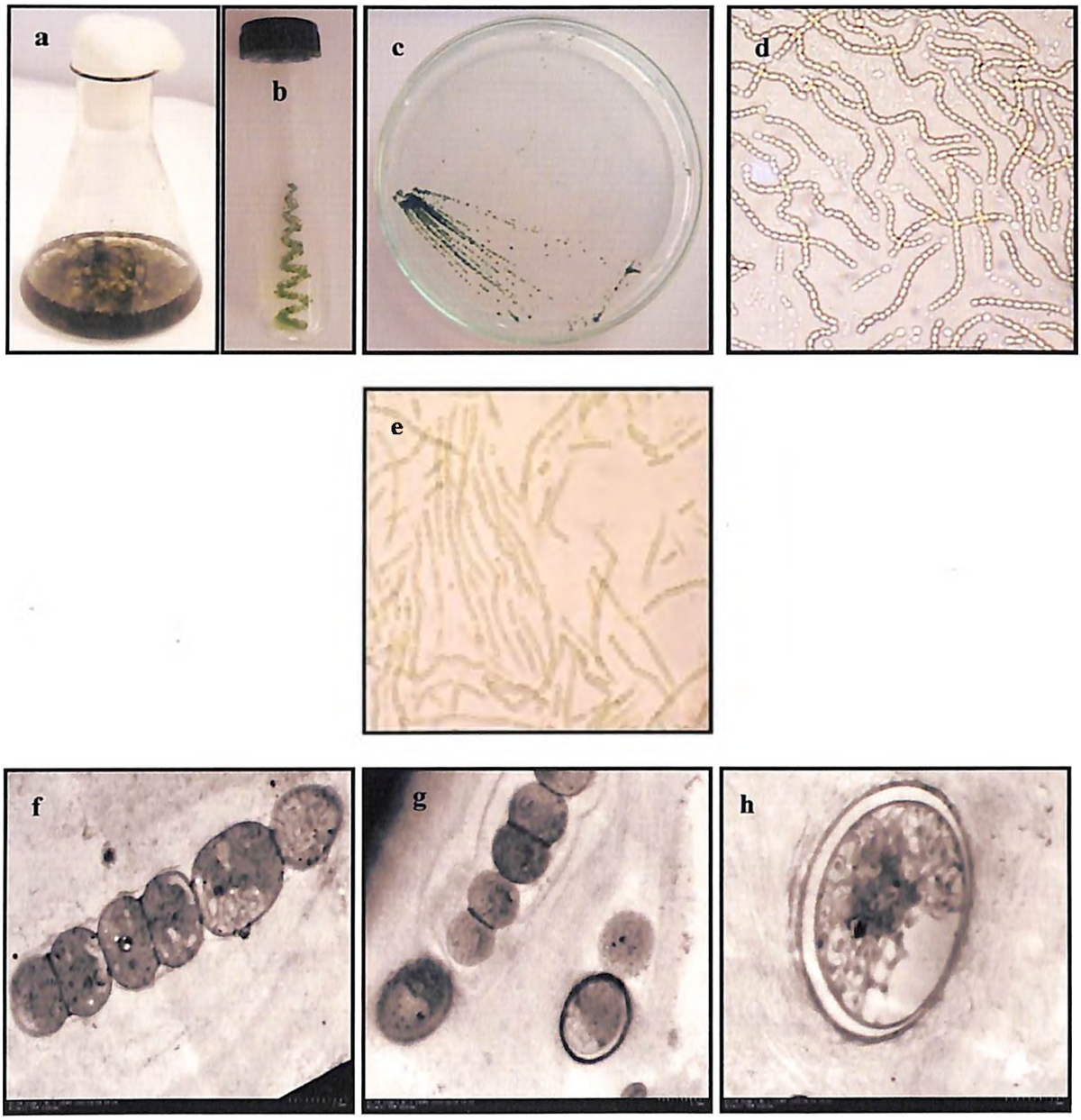


Fig. 16 Morphology of *Anabaena doliolum* isolated from site-2 (a) Chu-10 broth (b) Chu-10 slant (C) Chu-10 plate with unialgal single isolated colonies (c) Micrograph of *A. doliolum* under 10X magnification (e) Micrograph of *A. doliolum* under 40X magnification (f) Electron micrograph of *A. doliolum* showing cell division in equatorial plane (g) Electron micrograph showing barrel shaped spherical vegetative cells of *A. doliolum* (h) Double wall heterocyst of *A. doliolum*

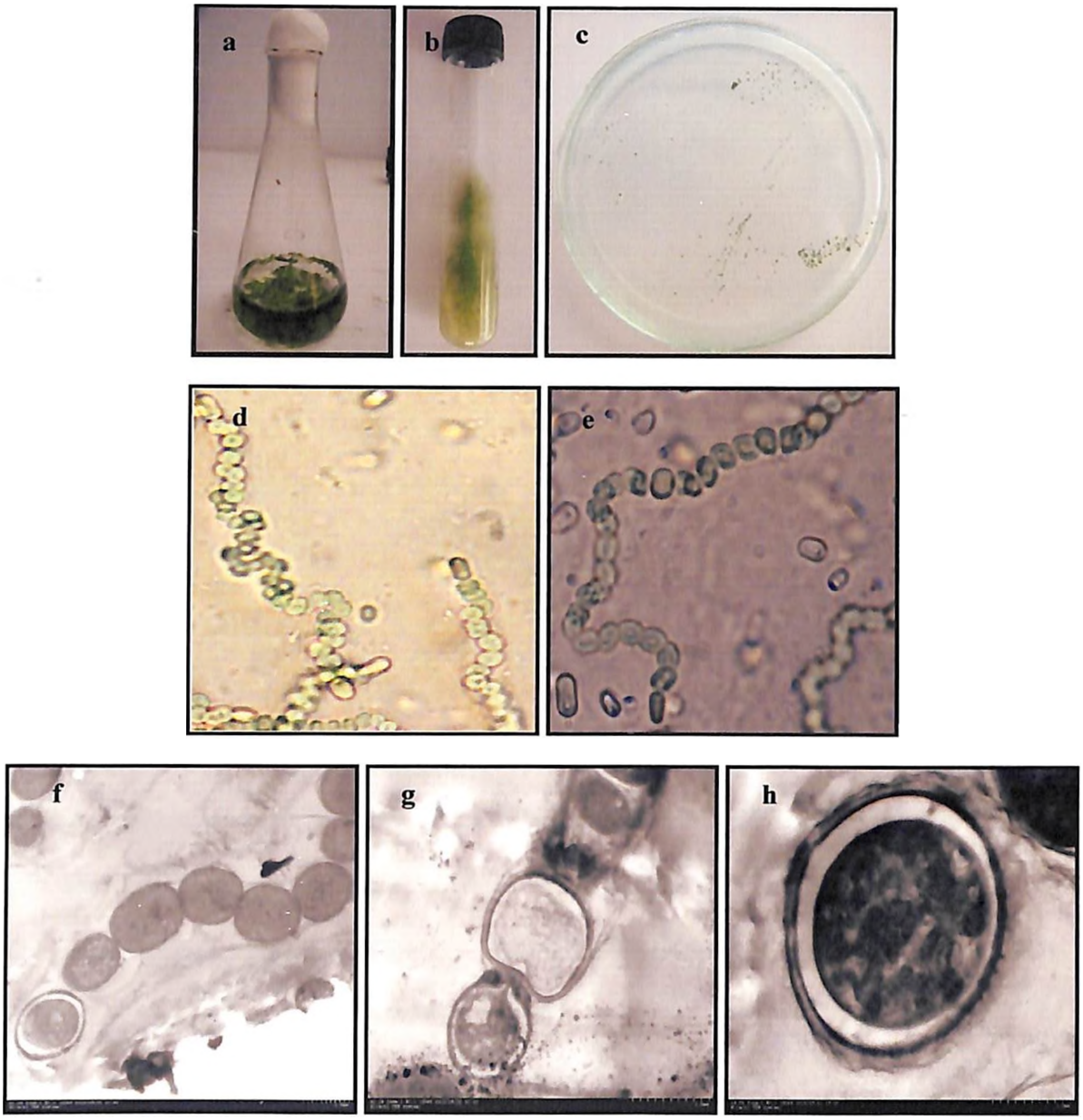


Fig. 17 Morphology of *Nostoc spongiaeforme* isolated from site-4 (a) Chu-10 broth (b) Chu-10 slant (C) Chu-10 plate with unialgal single isolated colonies (c) Micrograph of *N spongiaeforme* under 40X magnification (e) Micrograph of *N spongiaeforme* under 100X magnification (f) Electron micrograph showing barrel shaped cells with the end-cell having conical with round apex (g) Electron micrograph of akinetes. (h) Electron micrograph view of double wall heterocyst.

3.1.3. Media selection

The spectral analysis of methanolic extract of *A. doliolum* grown in different media for 17 days demonstrated that the peptide with absorbance at 200-300 nm was maximum in the cells grown in Chu-10 followed by BG_{11}, Fogg's and Allen and Arnon medium. The chlorophyll *a* content of the cells was reflected by the absorbance at 665nm. The highest peak at 665nm was obtained in case of methanolic extract of cells grown in BG_{11} followed by Chu-10, Fogg's and Allen and Arnon medium. The normal metabolic proteins in cyanobacteria are generally demonstrated by the absorbance peak of methanolic extract in the range of 300- 500nm. A comparison of peaks in this region showed the marginal supremacy of BG_{11} over Chu-10, whereas other media showed much lower peak (Fig. 18a). The studies with *N. spongiaeforme* grown in different media showed the similar pattern demonstrated by *A. doliolum* with minor variations in absolute values (Fig. 18b). The overall comparison of four media showed that Chu-10 was marginally less efficient in form of total chlorophyll *a* formation, but it showed highest concentration of biologically active peptide in both the organisms.

The suitability of different media for the formation of biologically active peptide in *A. doliolum* and *N. spongiaeforme* was further tested in animal model. The extract obtained from the *N. spongiaeforme* grown in Chu-10 media showed 3.5-fold higher malandialdehyde formation over BG_{11}, Fogg's and 7-fold higher formation over Allen and Arnon medium (Fig. 19). The crude extract obtained from *N. spongiaeforme* grown in the above four media also showed the supremacy of Chu-10 over other media in the formation of malandialdehyde in hepatocytes.

The spectral analysis and the crude extract toxicity studies demonstrated the efficiency of Chu-10 media in synthesizing biologically active, hepatotoxic peptide in *A. doliolum* and *N. spongiaeforme.*

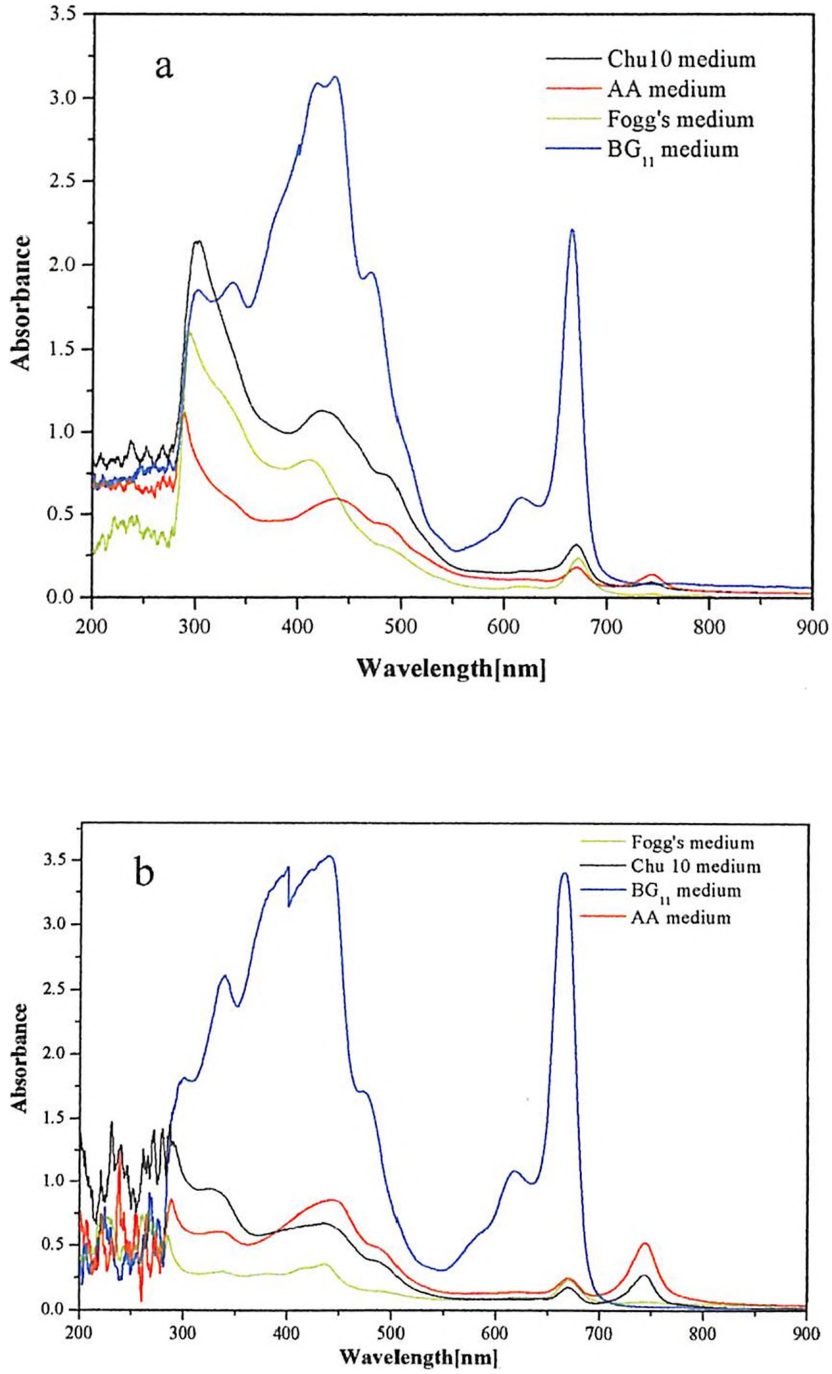


Fig. 18 Spectral analysis of methanolic extract of (a) *Anabaena doliolum* (b) *Nostoc spongiaeforme* grown in different culture media for 17 days under culture room conditions.

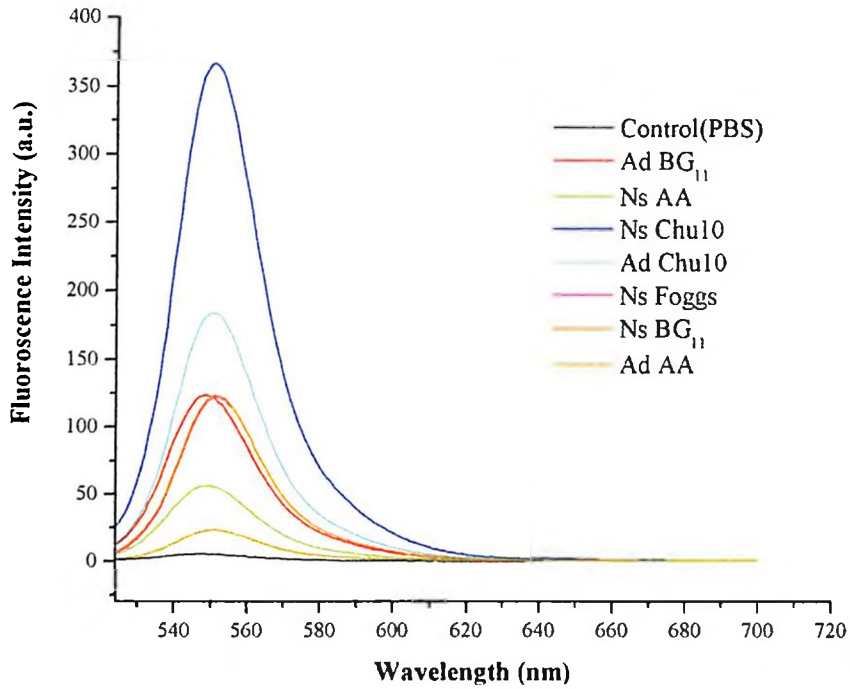


Fig. 19 Estimation of malandialdehyde level in hepatocytes of Swiss albino mice by TBARS assay to assess the toxicity of crude methanolic extracts prepared from *A. doliolum* and *N. spongiaeforme* grown in different media

3.2. Growth

3.2.1. Growth profile of *Anabaena doliolum*

The growth pattern of *A. doliolum* was monitored by total protein content as well as its chlorophyll *a* contents in Chu-10 medium. The growth rate with the initial inoculums of 50 µg protein ml^{-1} showed a lag phase (2 days) followed by an exponential phase extending till 13^{th} days, a slower late log phase (2 days) and a stationary phase (Fig. 20 a) with a generation time (g) of 30 hours. The chlorophyll *a* content also followed the similar pattern (Fig. 20 b) as demonstrated for protein.

3.2.2. Growth profile of *Nostoc spongiaeforme*

The growth pattern of *N spongiaeforme* was monitored by its protein as well as its chlorophyll *a* contents in Chu-10 medium (Fig. 21 a & b). The growth rate with the initial inoculums of 50 µg protein ml^{-1} and 0.1 µg ml^{-1} of chlorophyll *a* showed a typical sigmoidal pattern with a lag phase (1 day) followed by a exponential phase (14 days) and stationary phase corresponding to a final yield of 0.56mg protein ml^{-1} and 8.1 µg ml^{-1} chlorophyll *a*. The overall pattern showed a generation time (g) of 32 hours.

3.3. Biomass Production

Inorder to obtain desired quantity of toxins from *A. doliolum* and *N. spongiaeforme* for *in-vivo* studies, baby pool and plastic tubs containing Chu-10 media were used for cultivation (Fig. 22 a & b). Plastic tubs provided 100 g and 83g of dry weight of *A. doliolum* and *N. spongiaeforme* respectively in 13 days, whereas a quantity of 1kg and 0.84 kg of dry weight were obtained with the same strains and time period in baby pool method.

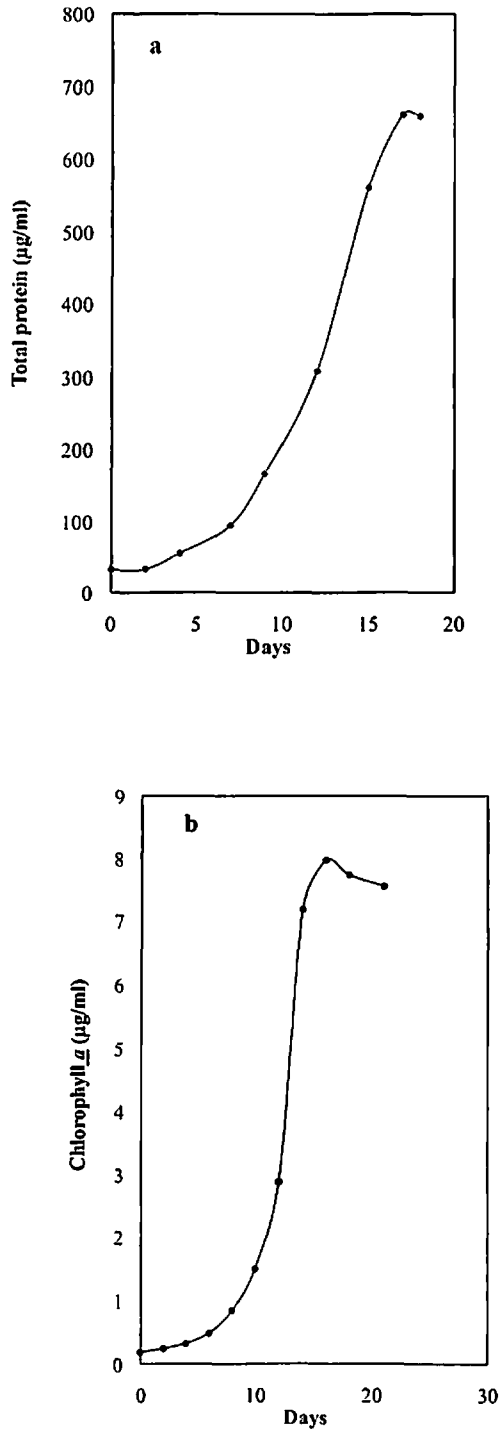


Fig. 20 Growth pattern of *Anabaena doliolum*. The growth was monitored as change in (a) Protein content and (b) Chlorophyll *a* content.

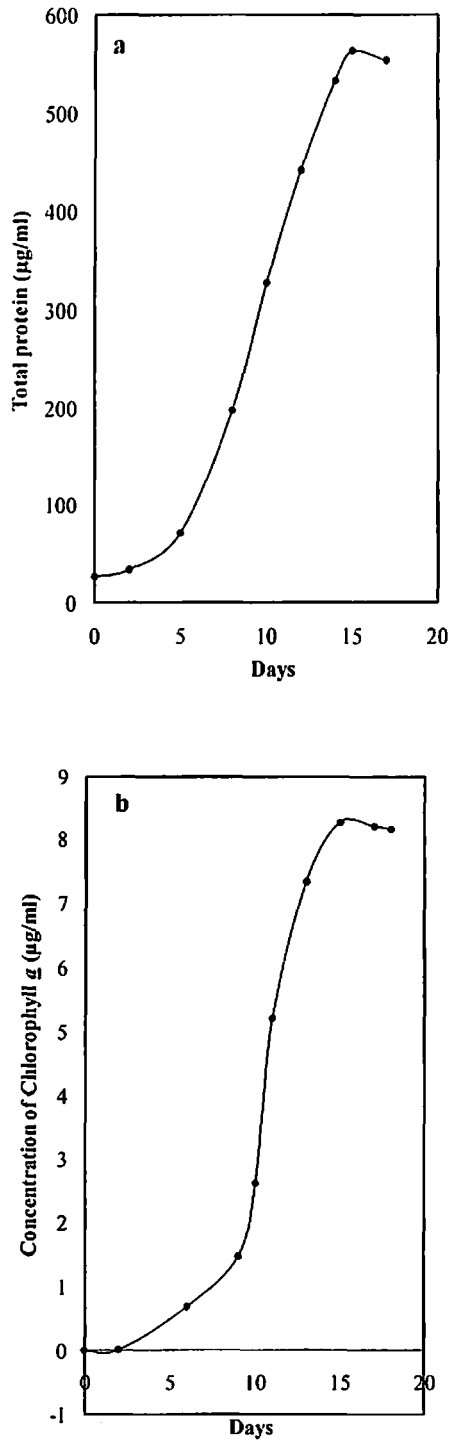
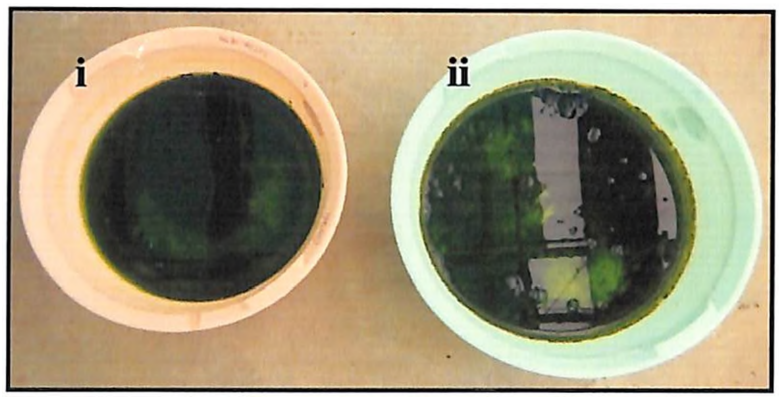
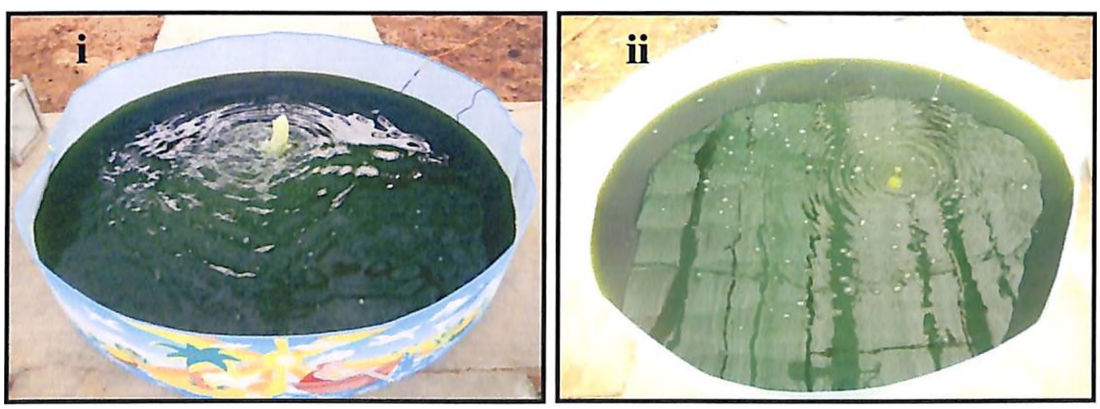


Fig. 21 Growth Pattern of *Nostoc spongiaeforme*. The growth was monitored as change in (a) Protein content and (b) Chlorophyll *a* content

Green house - biomass production site



(a) Plastic tub cultures (i) *Anabaena doliolum* (ii) *Nostoc spongiaeforme*



(b) Baby pool cultures (i) *Anabaena doliolum* (ii) *Nostoc spongiaeforme*

Fig. 22 Biomass production site of *Anabaena doliolum* and *Nostoc spongiaeforme*

3.4. Extraction of Microcystin variants from toxic cyanobacterial strains

The 22-days old cyanobacterial cultures were harvested for extraction of bioactive compounds as per the protocol described in section 2.4 of materials and methods. The different fractions were checked for total protein content by Lowry's method as described in section 2.2.1 of materials and methods and their *in-vivo* toxicity as described in section 3.11 of results.

3.5. Effect of nutrimental factors on Microcystin biosynthesis

3.5.1. Effect of culture condition on biomass production

The effect of macronutrients in Chu-10 medium on the biomass production of *A. doliolum* showed a drastic 9 fold reduction in the silicate deficient media and 8 fold with magnesium- and phosphate- deficient medium as compared to the control. Calcium deficiency showed 7 fold while carbonate showed 6 fold decrease in biomass production with respect to control. The nitrate- and iron deficiency in the media showed only 2 and 3 fold decrease in biomass production respectively. The absence of citric acid didn't affect the biomass production (Fig. 23).

The effect of macronutrients in Chu-10 on the biomass production of *N. spongiaeforme* showed a drastic reduction of 22 times in biomass with the media lacking phosphate and 21 times reduction with the media lacking magnesium as compared to the control. The effect of magnesium, silicate, iron, citric acid or carbonate deficiency in the media showed 2.5 fold decrease whereas the absence of calcium and nitrate did not show any significant decrease in biomass production (Fig. 24).

3.5.2. Effect of macronutrients on total microcystin synthesis

The total microcystin content of *A.doliolum* and *N. spongiaeforme* was estimated in the cells grown in Chu-10 medium deficient of magnesium, silicate, iron, nitrate, phosphate, calcium,

iron, citric acid or carbonate ions. The above nutrient deficiency does not seem to affect the total microcystin production in both the strains, as the total microcystin content in all the cases were found to be similar (Fig. 25 and 26).

3.5(1) Effect of light on synthesis of microcystin variants

The effect of light on the synthesis of microcystin variants in *A.doliolum* showed that the high amount of microcystin -LR was synthesized under high intensity of light of around 400 to 700 lux for the strains grown in Chu-10, whereas, synthesis of less toxic variants such as [D-Asp^3]microcystin-WR and dihydromicrocystin-LR was higher under low intensity of light (Fig. 27). At 700 lux, there was significant decrease in both [D-Asp^3]microcystin-WR and dihydromicrocystin-LR forms. At very low intensity (50 lux) there was 6 to 7 times decrease in concentration of microcystin-LR as compared to [D-Asp]microcystin-WR and dihydromicrocystin-LR. A similar trend of [D-Asp^3]microcystin-WR was seen in the strains grown in Allen and Arnon medium upto 100 lux. An 18% decrease in concentration of [D-Asp^3]microcystin-WR was observed in the strains grown in Allen and Arnon medium as compared to the strains grown in Chu-10. A difference of 90% in dihydromicrocystin-LR concentration was observed between the strains grown in Allen and Arnon medium and Chu-10 medium at light intensity of 700lux (Fig. 27).

N. spongiaeforme also followed the similar trend with microcystin-LR and [D-Asp^3]microcystin-HtyR synthesis when grown in Chu-10 and Allen and Arnon medium. The concentration of most toxic variants such as microcystin-LR and microcystin-RR increases with increase in intensity of light and shift to less toxic forms [D-Asp^3]microcystin-HtyR and microcystin-FR at low intensity of 50 to 100 lux (Fig. 28).

3.5.2. Effect of nitrate on synthesis of microcystin variants

The synthesis of microcystin variants in the *A.doliolum* grown in Chu-10 shows a high amount of microcystin-LR (326 µg/liter) at 0.5 mM of nitrate and 3fold (100 µg/liter) reduction in microcystin-LR at nitrate deficient medium. The strains grown in Allen and Arnon medium did not show the synthesis of microcystin-LR. A higher amount of [D-Asp^3]microcystin-WR (224µg/liter) and dihydromicrocystin-LR (273 µg/liter) was synthesized in nitrate deficient Chu-10 and Allen and Arnon medium. At lower concentration of nitrate the organism tend to synthesize less toxic variant such as [D-Asp^3]microcystin-WR and dihydromicrocystin-LR (Fig. 29).

In case of *N. spongiaeforme* an increase in synthesis of microcystin-LR was observed with increasing concentration of nitrate in Chu-10 medium. High concentration of [D-Asp^3]microcystin-HtyR was observed at 0.2mM of nitrate and it tends to decrease with higher nitrate level in Chu-10 medium (0.3mM, 0.4mM, 0.5mM) (Fig. 30). The strains grown in Allen and Arnon medium did not show the synthesis of microcystin-LR and [D-Asp^3]microcystin-HtyR. A gradual decrease in microcystin-FR synthesis was seen with increasing nitrate concentration in Allen and Arnon medium whereas a opposite effect was seen with microcystin-RR.

3.5.3. Effect of phosphate on synthesis of microcystin variants

A.doliolum showed less synthesis (71µg/liter) of highly toxic variant microcystin-LR in the Chu-10 medium lacking phosphate and tend to increase gradually with increasing phosphate concentration. An exact opposite trend was observed with other two variants [D-Asp^3]microcystin-WR and dihydromicrocystin-LR. Microcystin-LR was not synthesized by the strains grown in Allen and Arnon medium whereas the other two variants ([D-

Asp^3]microcystin-WR and dihydromicrocystin-LR) followed the similar pattern as compared to the strains grown in Chu-10 (Fig. 31).

N. spongiaeforme grown in Chu-10 showed high concentration of [D-Asp^3] microcystin-HtyR in 0.1mM phosphate that tend to decrease with the increase or decrease in phosphate concentration. On the other hand, microcystin-LR synthesis increased steadily with increase in phosphate concentration. A similar pattern was observed between microcystin-RR and microcystin-FR obtained from *N. spongiaeforme* grown in Allen and Arnon culture medium, where the microcystin-RR tend to increase with increasing phosphate concentration. Microcystin-FR attained a peak concentration of 0.05mM phosphate which decreased at increasing or decreasing the phosphate levels (Fig. 32).

3.5.4. Effect of temperature on synthesis of microcystin variants

The effect of another environmental factor i.e. temperature on synthesis of microcystin variants was studied by growing *A.doliolum* and *N. spongiaeforme* at various temperature (15°C- 30°C) for 17days in Chu-10 and Allen and Arnon medium. 25°C was found to be the optimal temperature for synthesis of other variants like of [D-Asp^3]microcystin-WR and dihydromicrocystin-LR. The synthesis of these variants decreased if we increase or decrease the incubation temperature from 25°C (Fig. 33). A temperature of 30°C significantly reduced the overall production of microcystin synthesis in *A.doliolum* grown in Chu-10 and Allen and Arnon medium. The synthesis of microcystin-LR was found to be increase with decreasing temperature in *A.doliolum* grown in Chu-10 in a temperature range of 15°C- 30°C and an opposite trend was observed for other variant ([D-Asp^3]microcystin-WR and dihydromicrocystin-LR) up to the temperature of 25°C (Fig. 33).

In case of *N. spongiaeforme*, lower temperatures of less than 18°C induced the production of highly toxic variant such as microcystin-LR and microcystin-RR grown in Chu-10 and Allen

and Arnon medium respectively. A favorable temperature of 25°C was observed for the synthesis of all variants such as microcystin-FR, microcystin-RR, [D-Asp^3]microcystin-HtyR and microcystin-LR from *N. spongiaeforme*. At higher temperature of around 30°C the overall synthesis of microcystins is drastically reduced (Fig. 34).

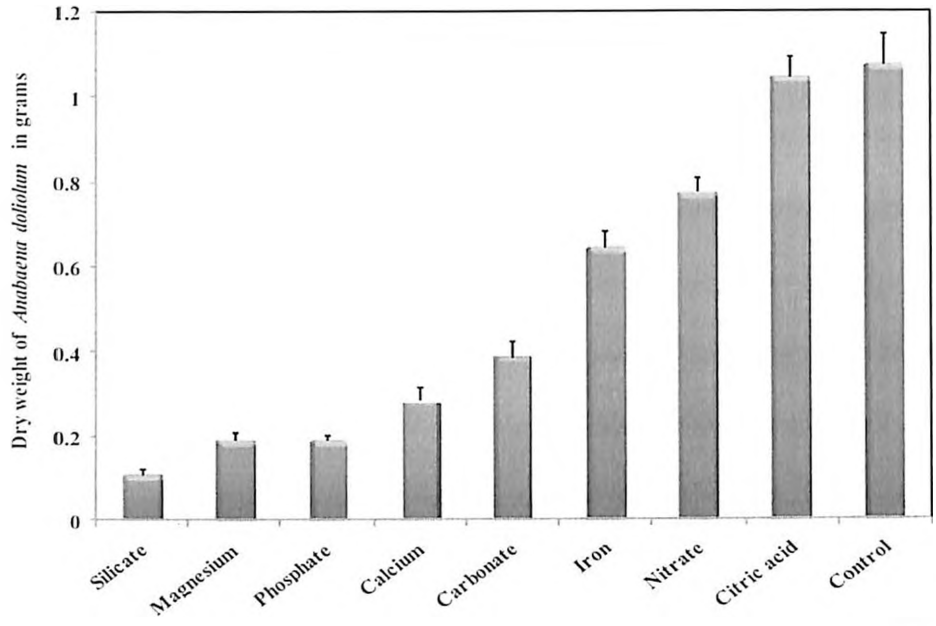


Fig. 23 Dry weight of *A. doliolum* obtained from 1liter Chu-10 media lacking in macronutrients

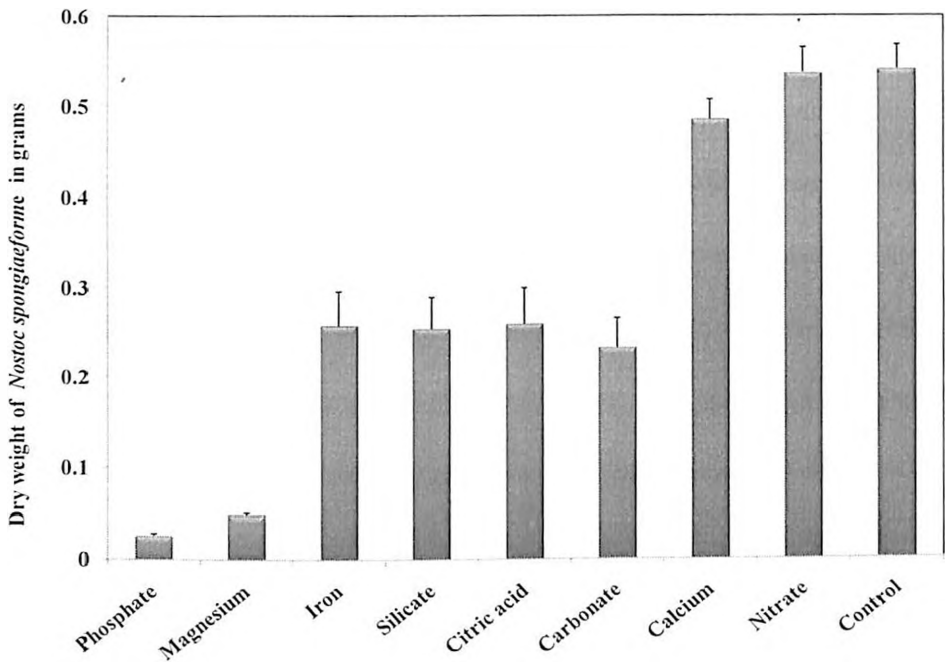


Fig. 24 Dry weight of *N. spongiaeforme* obtained from 1liter of Chu-10 media lacking in macronutrients.

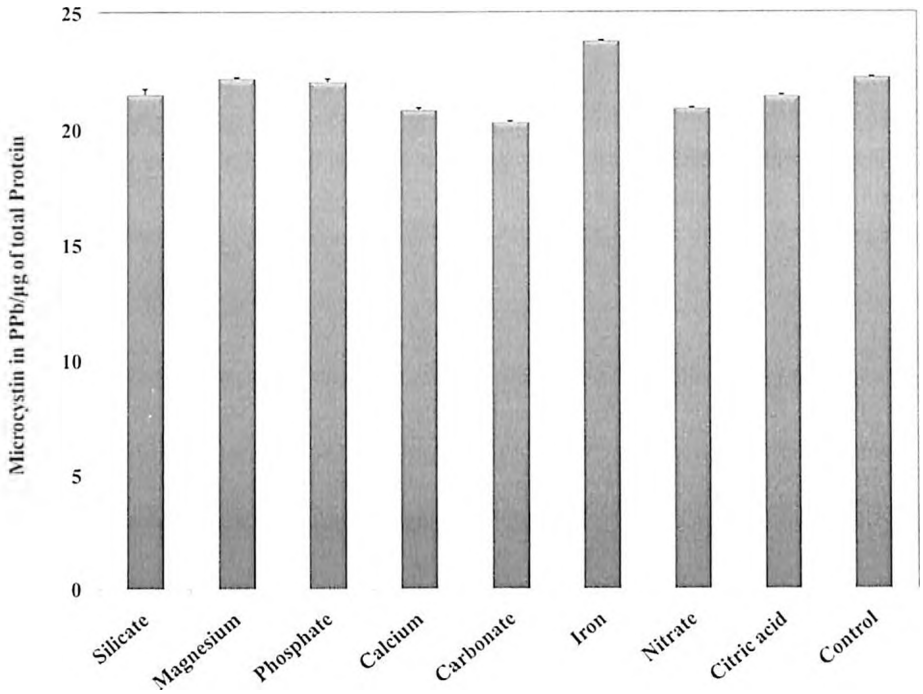


Fig. 25 Effects of macronutrients on the synthesis of microcystin in *A. doliolum*

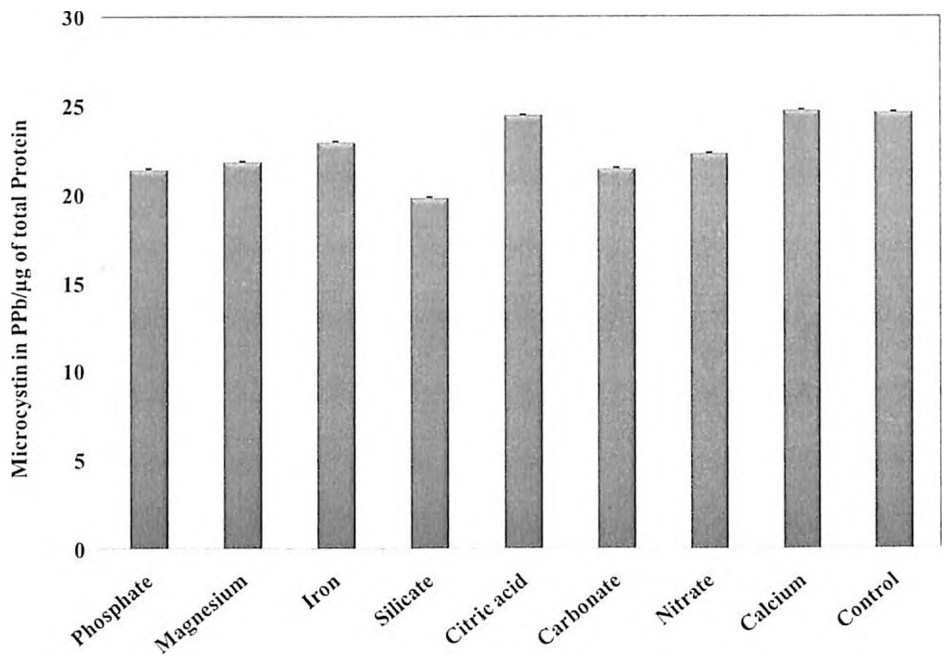


Fig. 26 Effects of macronutrients on the synthesis of microcystin in *N. spongiaeforme*

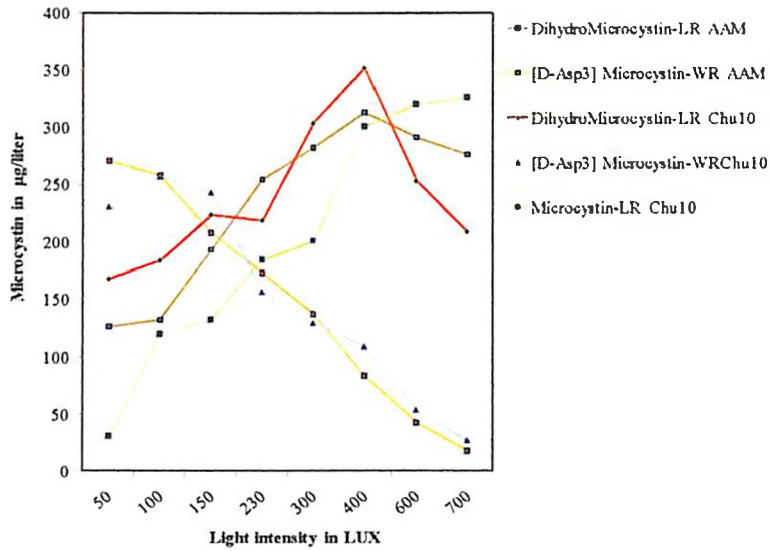


Fig. 27 Effect of light on the synthesis of microcystin variants by *A. doliolum* grown in Chu-10, Allen and Arnon culture medium.

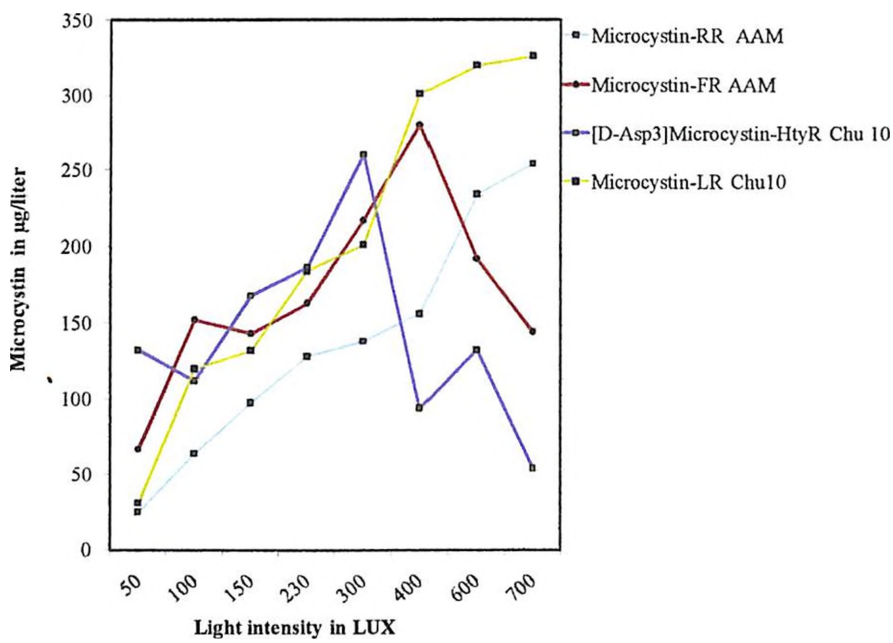


Fig. 28 Effect of light on the synthesis of microcystin variants by *N. spongiaeforme* grown in Chu-10, Allen and Arnon culture medium.

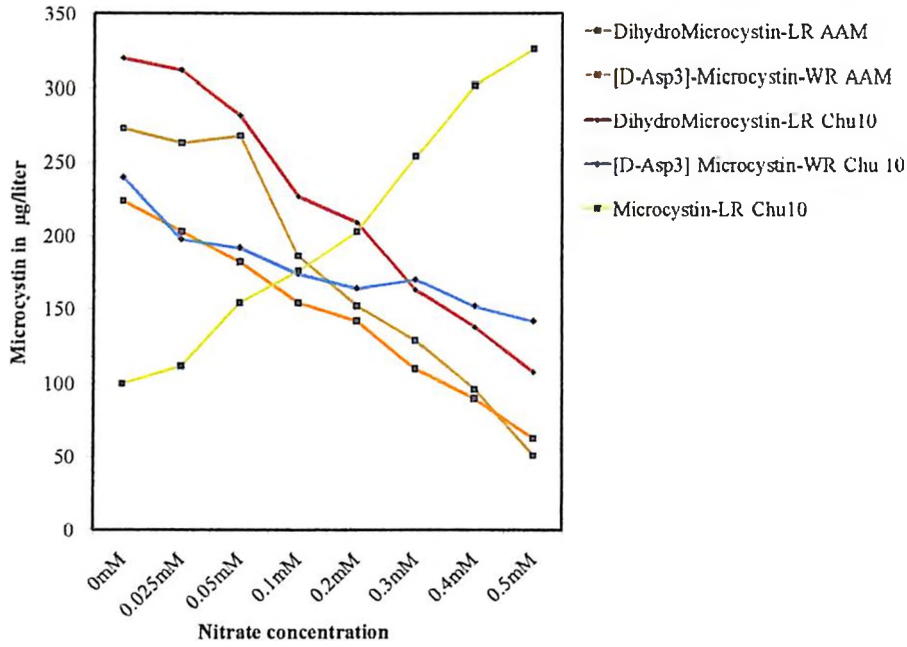


Fig. 29 Effect of nitrate on the synthesis of microcystin variants by *A. doliolum* grown in Chu-10, Allen and Arnon culture medium.

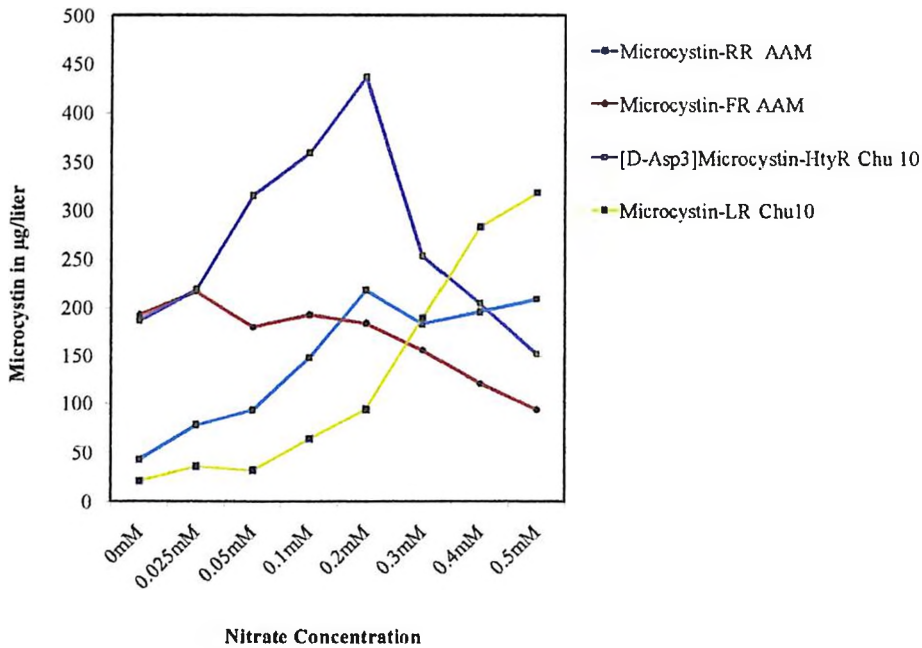


Fig. 30 Effect of nitrate on the synthesis of microcystin variants by *N. spongiaeforme* grown in Chu-10, Allen and Arnon culture medium.

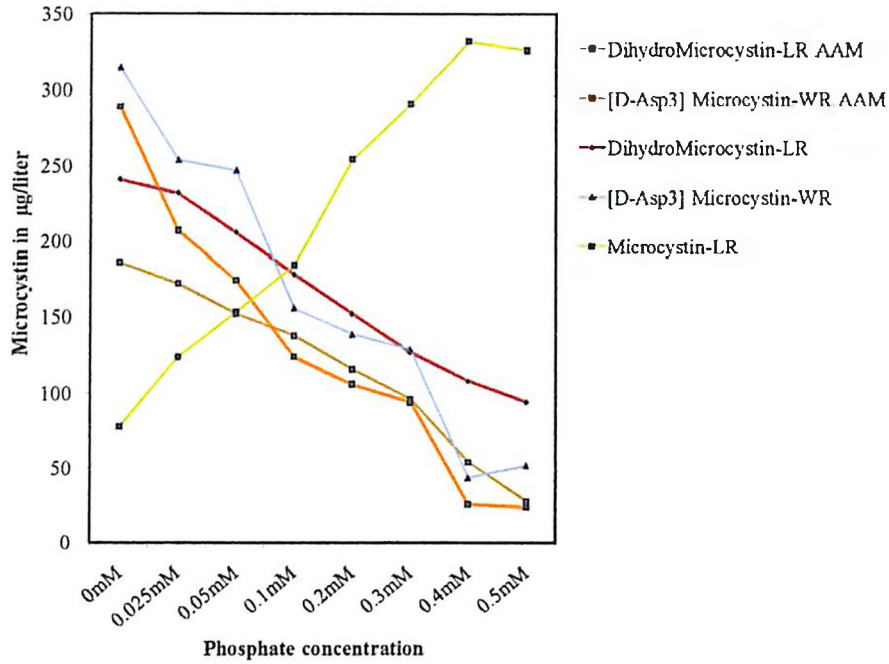


Fig. 31 Effect of phosphate on the synthesis of microcystin variants by *A .doliolum* grown in Chu-10, Allen and Arnon culture medium.

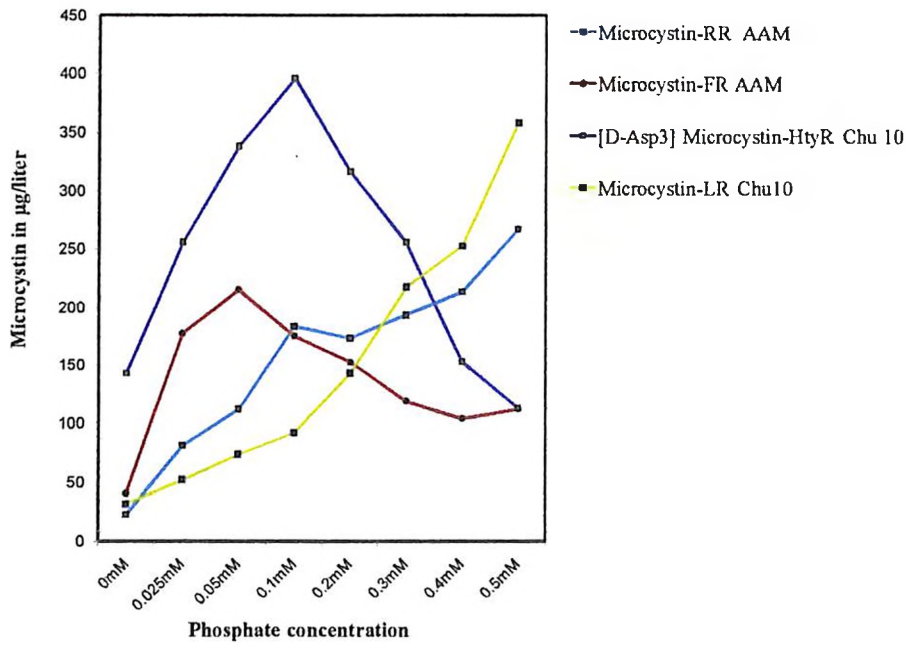


Fig. 32 Effect of phosphate on the synthesis of microcystin variants by *N. spongiaeforme* grown in Chu-10, Allen and Arnon culture medium.

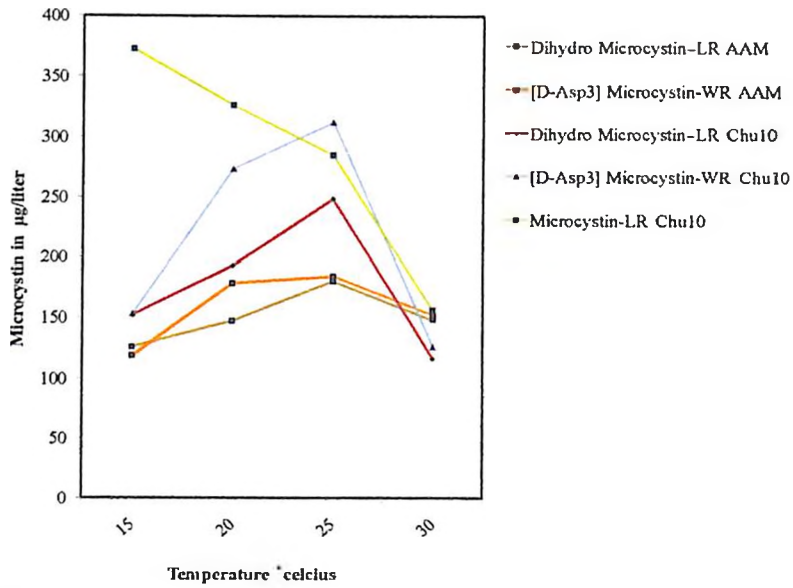


Fig. 33 Effect of temperature on the synthesis of microcystin variants by *A .doliolum* grown in Chu-10, Allen and Arnon culture medium.

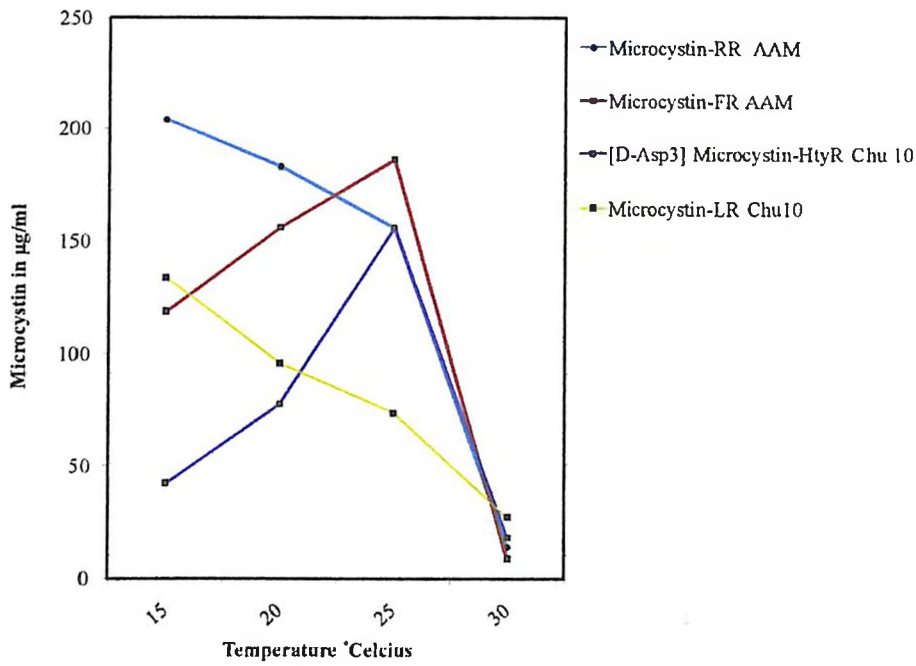


Fig. 34 Effect of temperature on the synthesis of microcystin variants by *N. spongiaeforme* grown in Chu-10, Allen and Arnon culture medium.

3.6. Isolation of peptides by chromatographic techniques

Partial separation of Microcystins by TLC

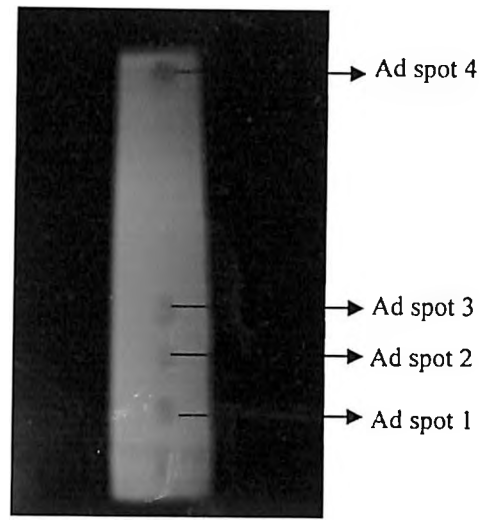
Fractioned extracts of *A. doliolum* and *N. spongiaeforme* grown in Chu-10 showed four spots in thin layer of silica gel G60 using Chloroform: Methanol: Water (60:24:16) as mobile phase. The spots were labeled after their isolated organisms as Ad spot 1, Ad spot 2, Ad spot 3, Ad spot 4 for *A.doliolum* (Fig. 35 a) and Ns spot 1, Ns spot 2, Ns spot 3, Ns spot 4 for *N. spongiaeforme* (Fig. 35 b). First two spots in each case i.e. Ns spot1 and spot 2 as well as Ad Spots 1 and 2 showed lower R_f values indicating the presence of highly polar compounds, with higher affinity toward the stationary phase whereas the Ad spots 4 and Ns spot 4 showed higher R_f values indicating higher affinity towards the mobile phase and hence more amount of non-polar compounds (lower fatty acids). Spots 3 of *A. doliolum* and *N. spongiaeforme* showing an R_f values closer to standard microcystin-LR (Table 4), were subjected to spectral UV analysis using HPTLC detector as described in materials and method section 2.6.1. The HPTLC analysis showed a characteristic peak of microcystin at 238 nm in (Fig. 36) in Ad spot 3 and Ns spot 3. A similar type of results was obtained for the strains grown in Allen and Arnon culture medium also (data not shown).

3.7. Studies with MALDI-TOF/MS

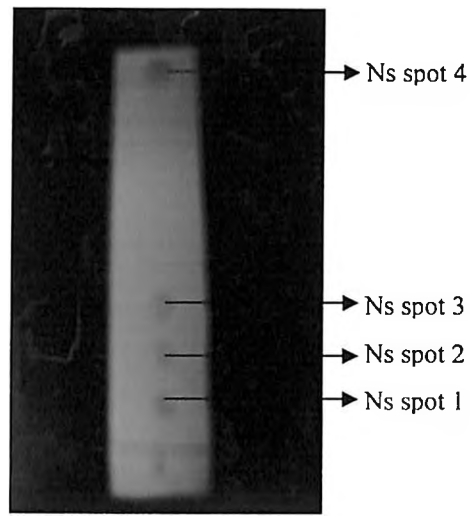
MALDI TOF MS of Ad spot-3 purified from *A.doliolum* grown at 25°C showed the presence of two prominent molecular ions Dihydromicrocystin-LR (m/z=996.8219), [D-Asp3]-microcystin-WR (m/z=1051.954) from microcystin family along with some other peptides such as Phoephorbide (m/z=5999.444), Aeruginosin (m/z=656.372, 713.532, 768.673), Oscillamide (m/z=882.752), Anabaenopetin K (m/z=939.796) (Fig. 37). The *A.doliolum*

cultures grown at 15°C showed prominent molecular ions Microcystin-LR (m/z=995.638) along with less amount of Oscillamide (m/z= 880.491) (Fig. 38)

The MALDI TOF Ms analysis of Ns spot 3 partially purified from *N. spongiaeforme* grown in Chu-10 showed two molecular ions Microcystin-LR (m/z=995.472) and [D-Asp3]microcystin-HtyR (m/z=1043.756) from microcystin family along with other peptides such as Oscillarin (m/z=617.157), Aeruginosin (m/z=656.151,691.1932, 705.624) and Oscillamide (m/z=861,237) (Fig. 39). The Ns spot 3 partially purified from *N. spongiaeforme* grown in Allen and Arnon culture medium showed the presence of Microcystin-FR (m/z=1029.521), Microcystin-RR (m/z=1037.970) from microcystin family along with Aeruginosin (m/z= 672.646, 765.289 (Fig. 40)



(a) Compounds from *A.doliolum*



(b) Compounds from *N. spongiaeforme*

Fig. 35 Compounds separated by TLC using silica gel G60 as stationary phase and Chloroform: Methanol: Water (60:24:16) as mobile phase. Spots were visualized by Iodine chamber method

Table 4 R_f values of TLC separated spots of *A. doliolum* and *N. spongiaeforme*

Sl. No	**Toxic Strains**	**Spots separated by TLC**	**R_f values**
1	*Anabaena doliolum*	Ad spot 1	0.27
		Ad spot 2	0.36
		Ad spot 3	0.49
		Ad spot 4	0.72
2	*Nostoc spongiaeforme*	Ns spot 1	0.32
		Ns spot 2	0.42
		Ns spot 3	0.54
		Ns spot 4	0.79
3	Standard Microcystin	Single spot	0.52

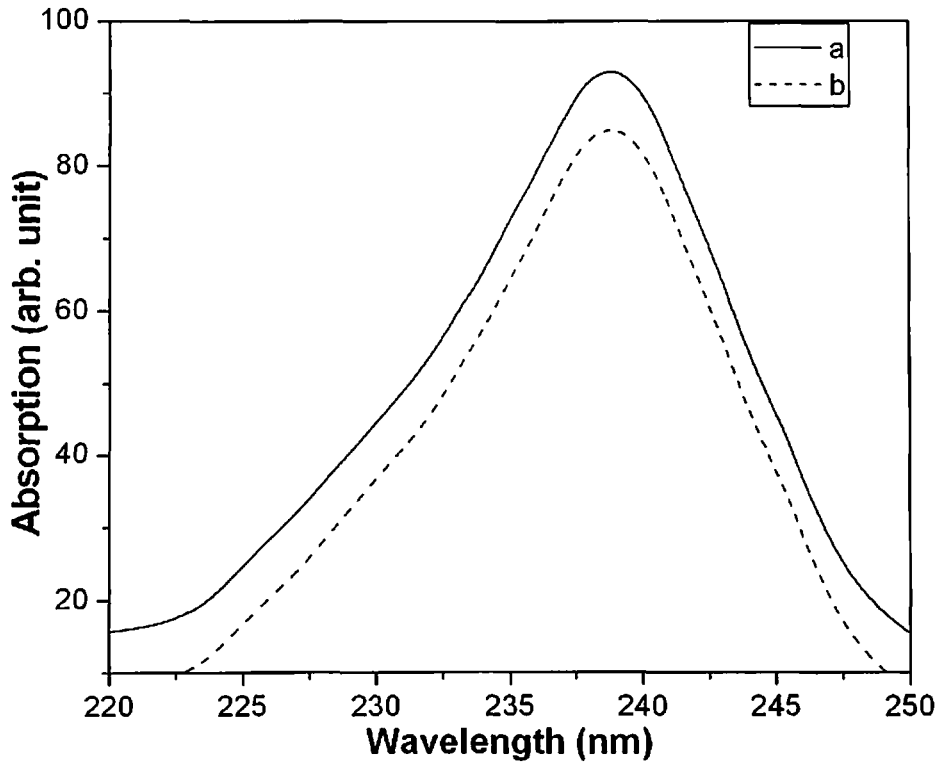


Fig. 36 HPTLC Spectrum analysis of (a) Ad spot 3 (b) Ns spot 3

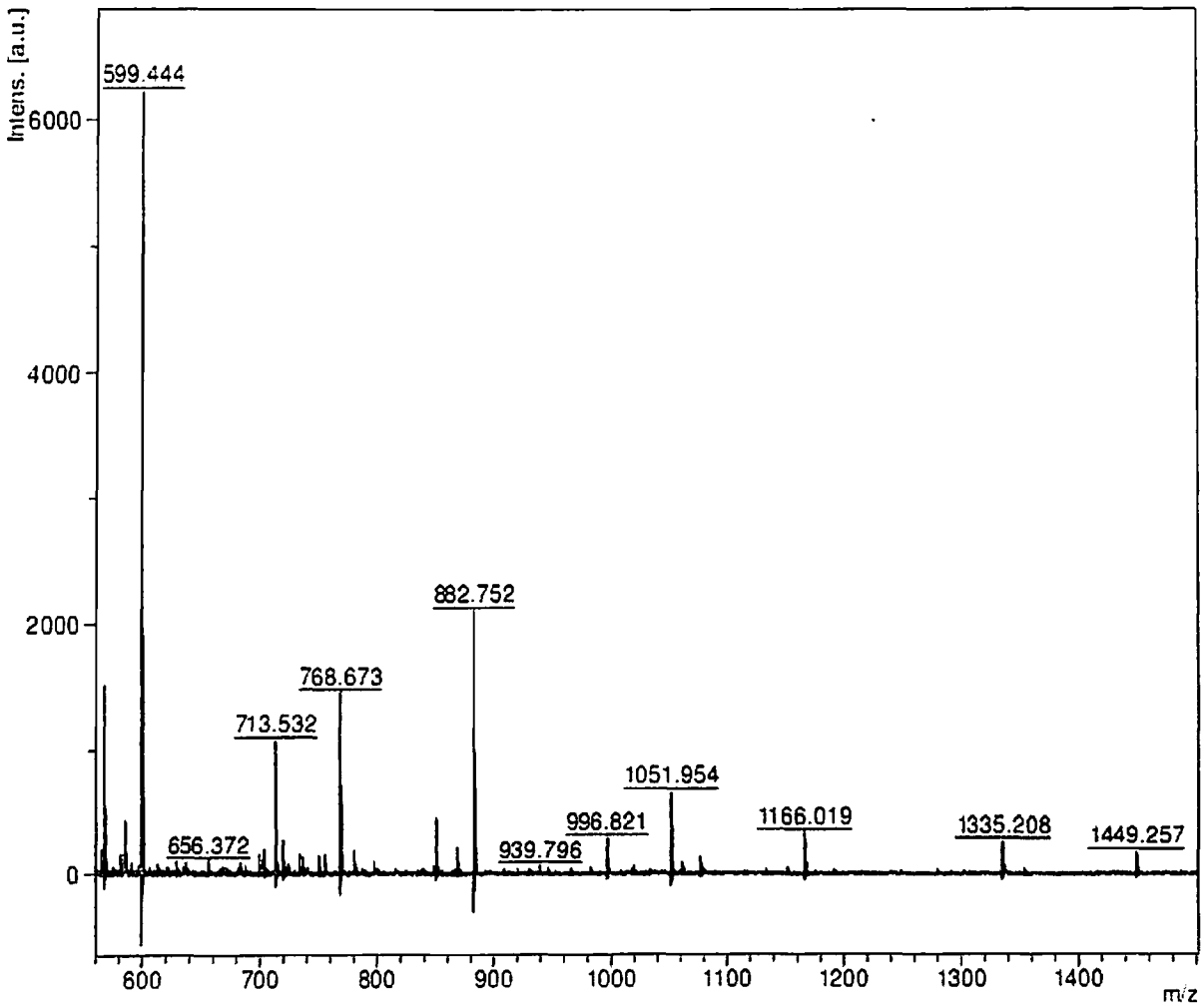


Fig. 37 MALDI TOF MS of partially purified Ad spot 3 from *A. doliolum* grown at 25°C showing the presence of dihydromicrocystin-LR (m/z=996.821), [D-Asp3]microcystin-WR (m/z=1051.954).

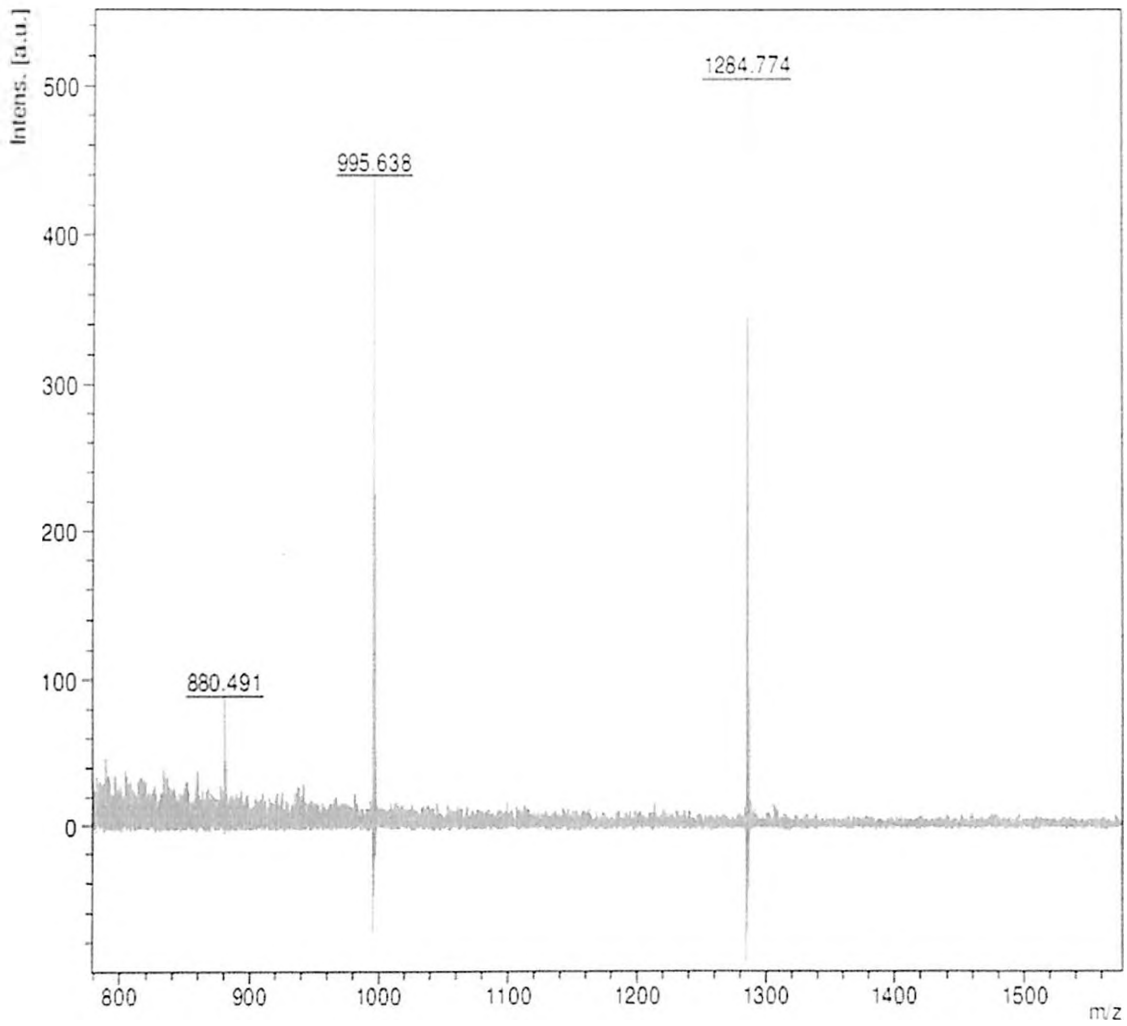


Fig. 38 MALDI TOF MS of partially Ad spot 3 purified from *A. doliolum* grown at 15°C showing the presence of microcystin-LR (m/z=995.638)

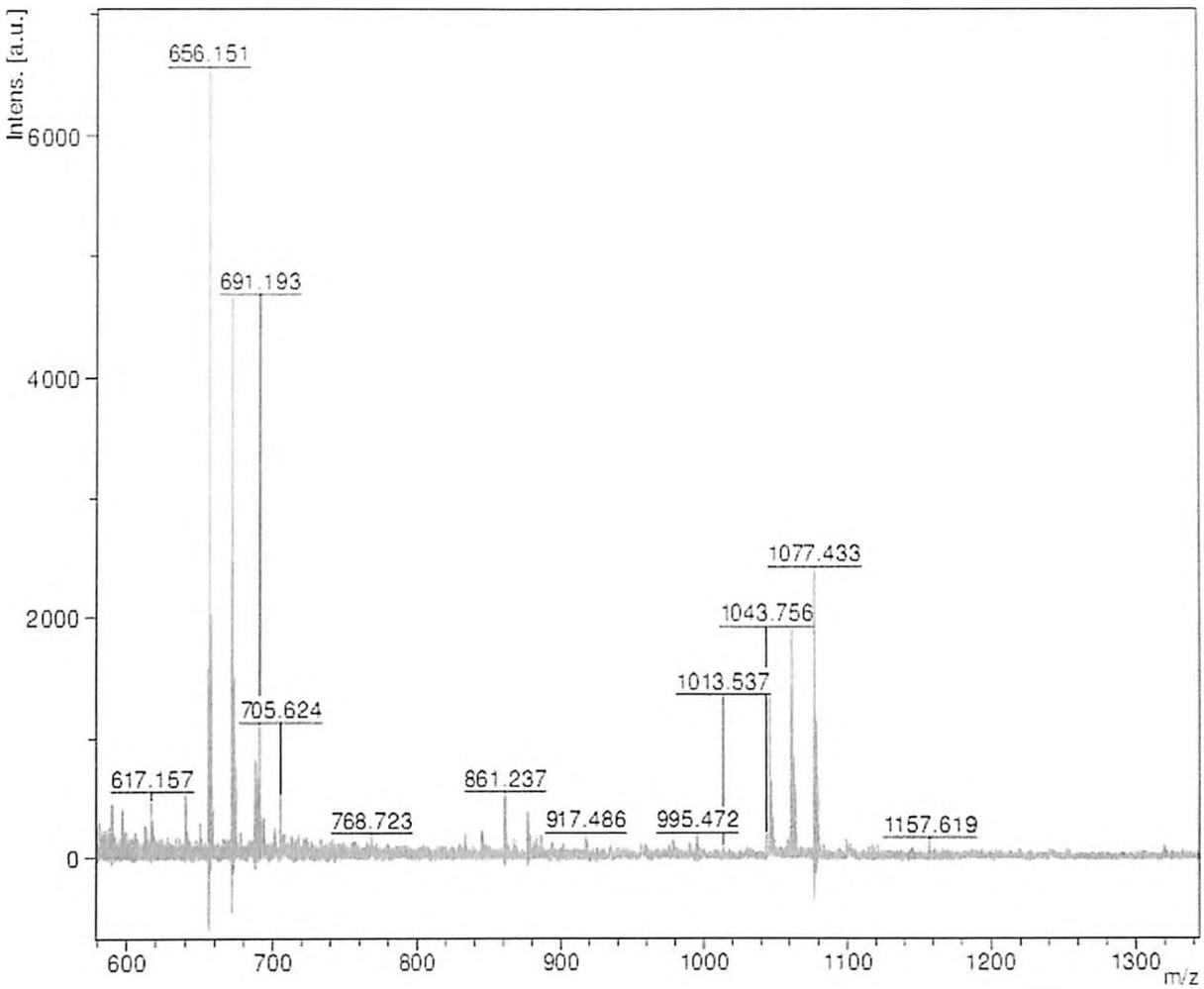


Fig. 39 MALDI TOF MS of partially purified Ns spot 3 from *N. spongiaeforme* grown in Chu-10 shows the presence of microcystin-LR (m/z=995.472), [D-Asp3]microcystin-HtyR (m/z=1043.756).

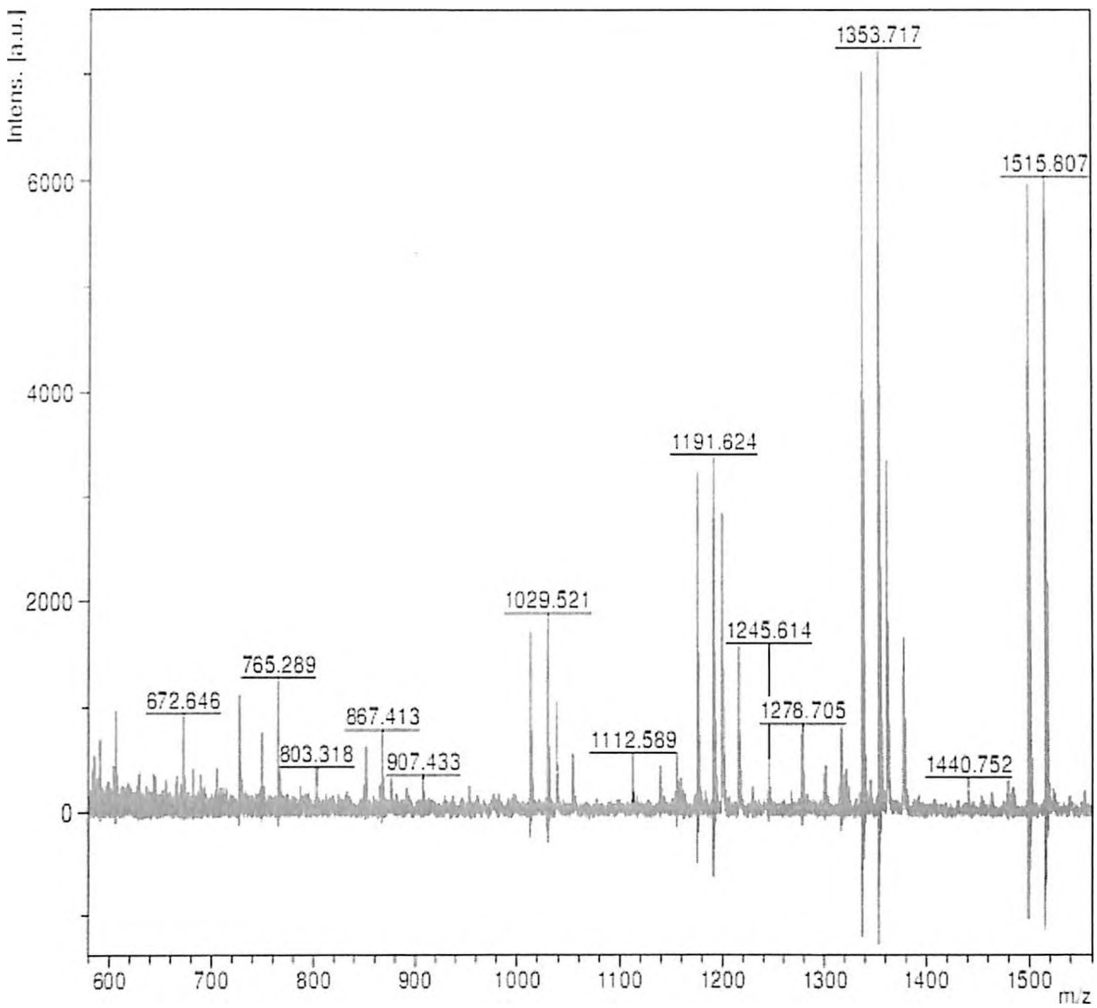


Fig. 40 MALDI TOF MS of partially purified Ns spot 3 from *N. spongiaeforme* grown in Allen and Arnon culture medium shows the presence of microcystin-FR (m/z=1029.521), microcystin-RR (m/z=1037.970).

3.8. Quantification and Purification of Microcystins by HPLC

HPLC analysis of Ad spot3 from *A.doliolum* showed three major peaks with the R_t values of 3.7 mins, 4.3 mins and 8.4 mins corresponding to dihydromicrocystin-LR, [D-Asp3]microcystin–WR and microcystin-LR respectively (Fig. 41 a). The pure microcystin standard showed an R_t values of 8.2mins (Fig. 41 b) with the same solvent system (0.01M ammonium acetate: acetonitrile in the ratio of 64: 36 (v/v)) as used for the elution of Ad spot 3 compounds. The lower R_t values of dihydromicrocystin-LR indicate a higher polarity of the compound as compared to [D-Asp3]microcystin–WR and microcystin-LR with respect to the mobile phase.

The Ns spot 3 obtained from the *N. spongiaeforme* grown in Chu-10 showed the presence of two peaks with the R_t values of 9.9 mins and 12.6 mins corresponding to microcystin-LR and [D-Asp3]microcystin-HtyR respectively using the same solvent system (0.01M ammonium acetate: acetonitrile) of different ratio of 72: 38 (v/v) (Fig. 42 a). The standard microcystin showed a characteristic peak at 9.9 mins (Fig. 42 b). Similarly, Ns spot3 of *N. spongiaeforme* grown in Allen and Arnon culture medium showed peaks with the R_t values of 7.9 mins and 12.1 mins corresponding to microcystin-FR and microcystin-RR in 0.01M ammonium acetate: methanol in the ratio of 50: 50 (v/v) (Fig. 43 a). The standard microcystin-LR showed an R_t values of 8.5 mins (Fig. 43 b). The lower R_t values of microcystin-LR and microcystin-FR infer the affinity of the compound towards respective mobile phase.

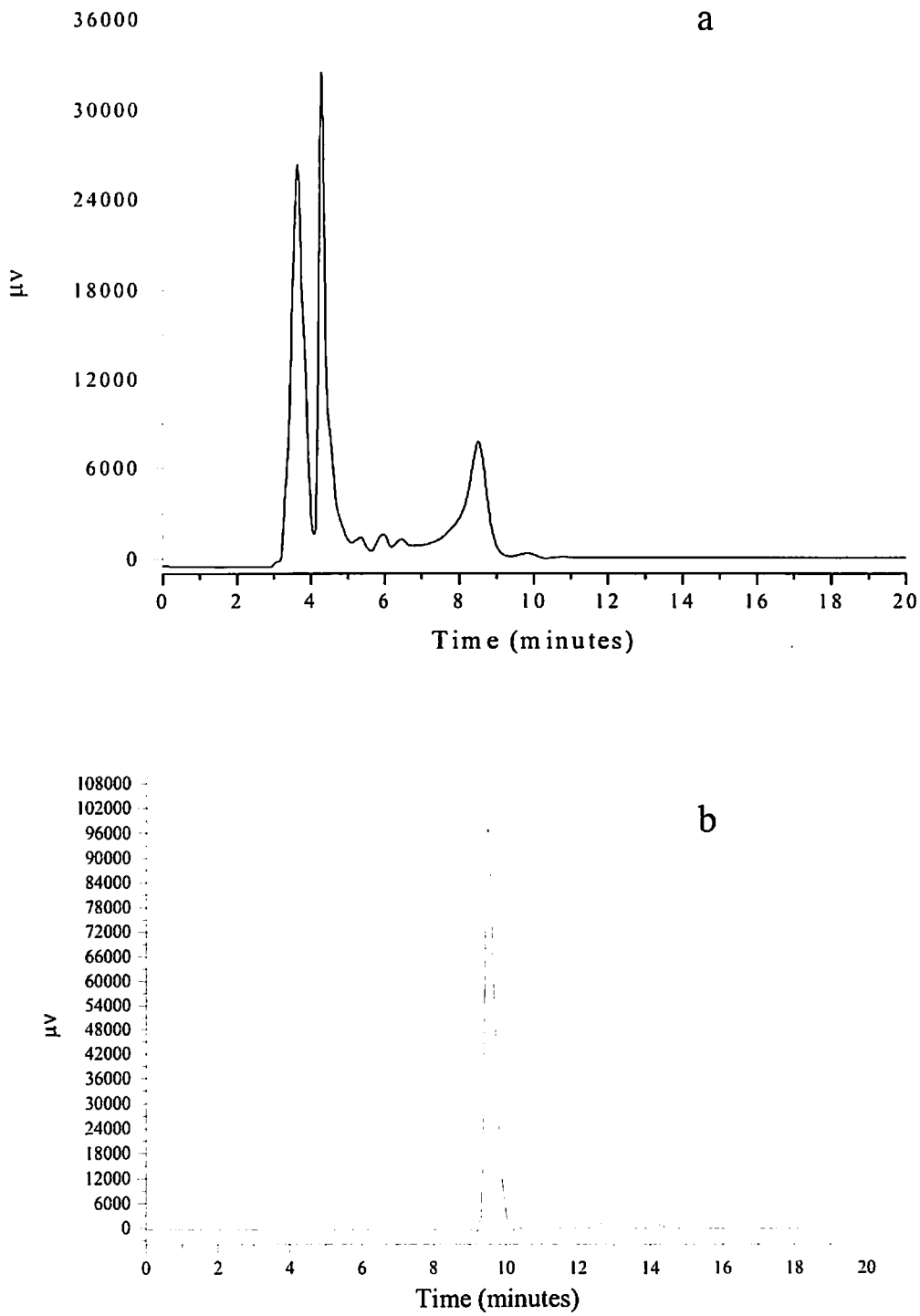


Fig. 41 RP-HPLC elution profiles of (a) Ad spot 3 isolated from the *A. doliolum* showing two peaks and (b) Standard microcystin-LR showing single peaks.

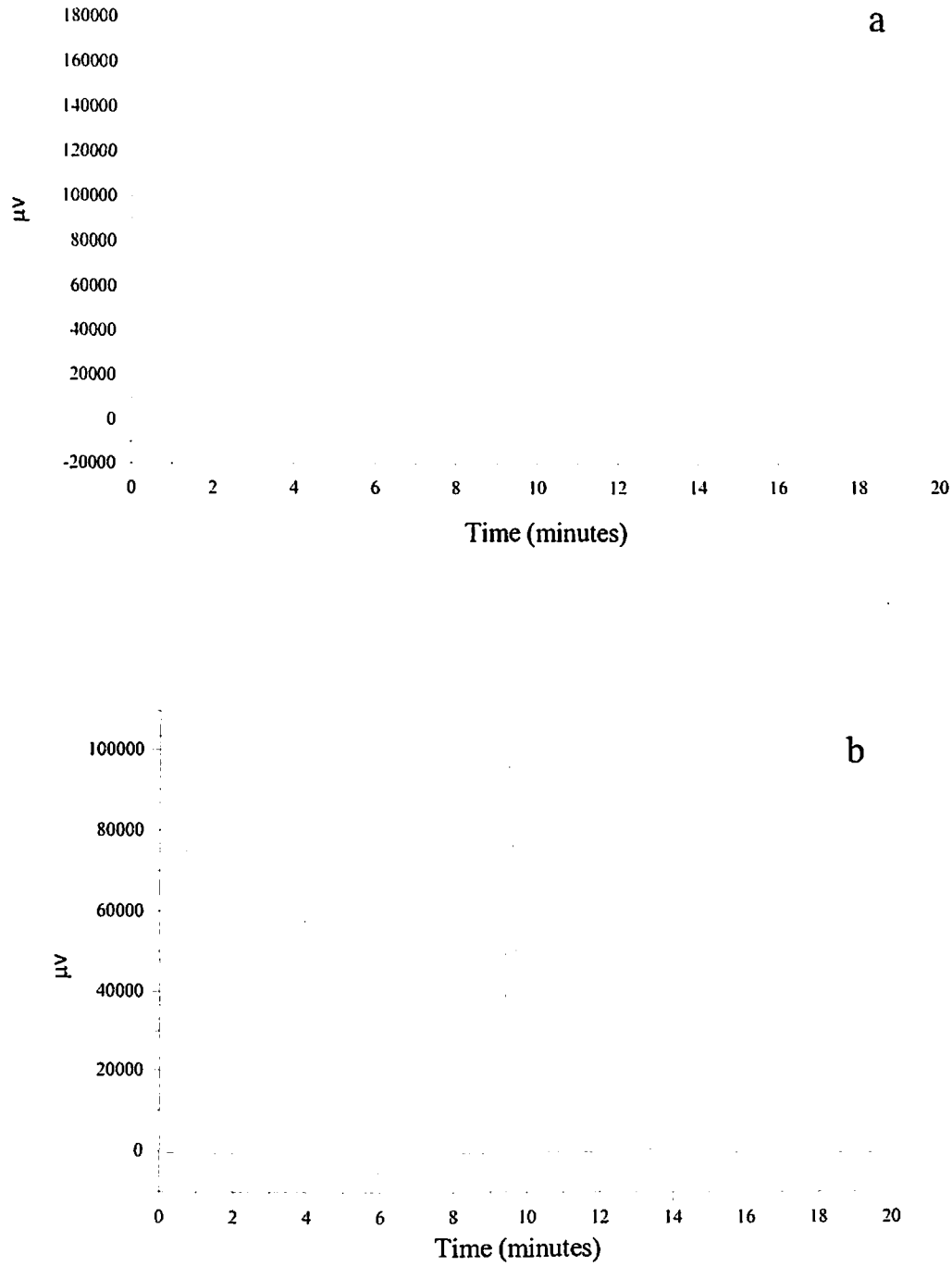


Fig. 42 RP-HPLC elution profiles of (a) Ns spot 3 isolated from the *N. spongiaeforme* grown in Chu-10 medium showing two peaks and (b) Standard microcystin-LR showing single peaks.

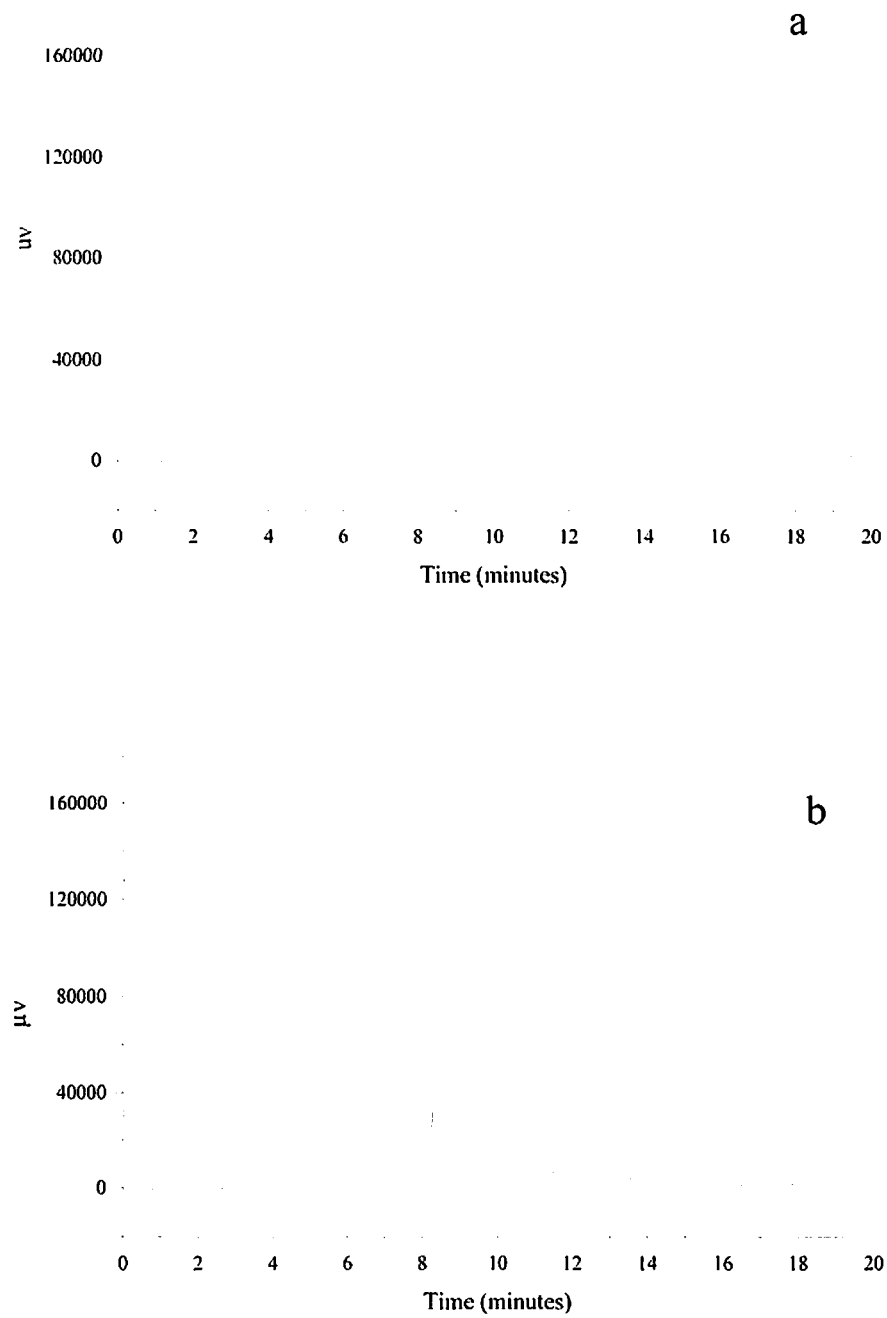


Fig. 43 RP-HPLC elution profiles of (a) Ns spot 3 isolated from the *N. spongiaeforme* grown in Allen and Arnon medium showing two peaks and (b) Standard microcystin-LR showing single peaks.

3.9. Studies with FTIR

FT-IR (KBr, cm^{-1}) analysis of microcystin varients purifed by HPLC showed various peaks corresponding to the funtional groups of microcystins. The peak at 3466 in microcystin-LR infers the presence of O-H and N-H streching whereas presence of single or double peaks at 2918, 2920, 2848, 2850 wavelength in dihydromicrocystin-LR, microcystin-LR, [D-Asp3]microcystin-HtyR, microcystin-FR, microcystin-RR, [D-Asp3]microcystin-WR, [D-showed the presence of aliphatic C-H streching. The peak corresponds to 2358 of [D-Asp3]microcystin-WR, dihydromicrocystin-LR, microcystin-LR, [D-Asp3]microcystin-HtyR, microcystin-FR and microcystin-RR, 2337 of microcystin-LR, 2339 of [D-Asp3]microcystin-WR, dihydromicrocystin-LR , [D-Asp3]microcystin-HtyR and 2341 of microcystin-RR infers NH_2^+, asymmetry and symmetry streching. The peaks at 1745 of microcystin-LR, 1747 of [D-Asp3]microcystin-HtyR and dihydromicrocystin-LR infers the presence of C=O streching in COOH. The peak at 1680 of microcystin-LR infers C=O and C=C streching and 1566 of microcystin-LR infers N-H bend. The peak corresponds to 1456 of [D-Asp3]microcystin-WR, dihydromicrocystin-LR and microcystin-RR infers CH_2 bend and 1346 of microcystin-LR, 1338 of [D-Asp3]microcystin-HtyR infers CH_3 bending. The1265 peak of microcystin-FR, 1263 of dihydromicrocystin-LR, microcystin-LR and [D-Asp3]microcystin-HtyR, 1149 of microcystin-LR infers the presence of C-O streching. The peak at 790 wavelenth of [D-Asp3]microcystin-WR, dihydromicrocystin-LR, microcystin-LR, microcystin-FR and microcystin-RR & peak at 667 of microcystin-LR infers C-H group is out of plan bending and has mono substituted ring. Slight shift between peaks representing same functional groups is due difference in the molecular weight of the compound and the position in the structure (Fig. 44 - 49)

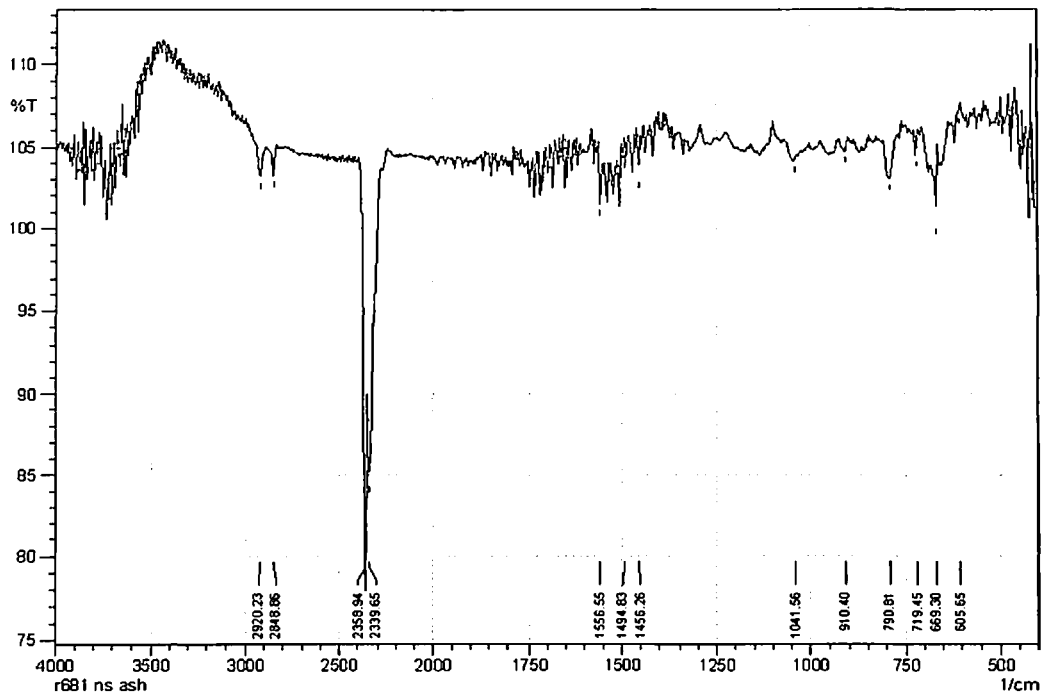


Fig. 44 FTIR spectrum of [D-Asp3]microcystin-WR

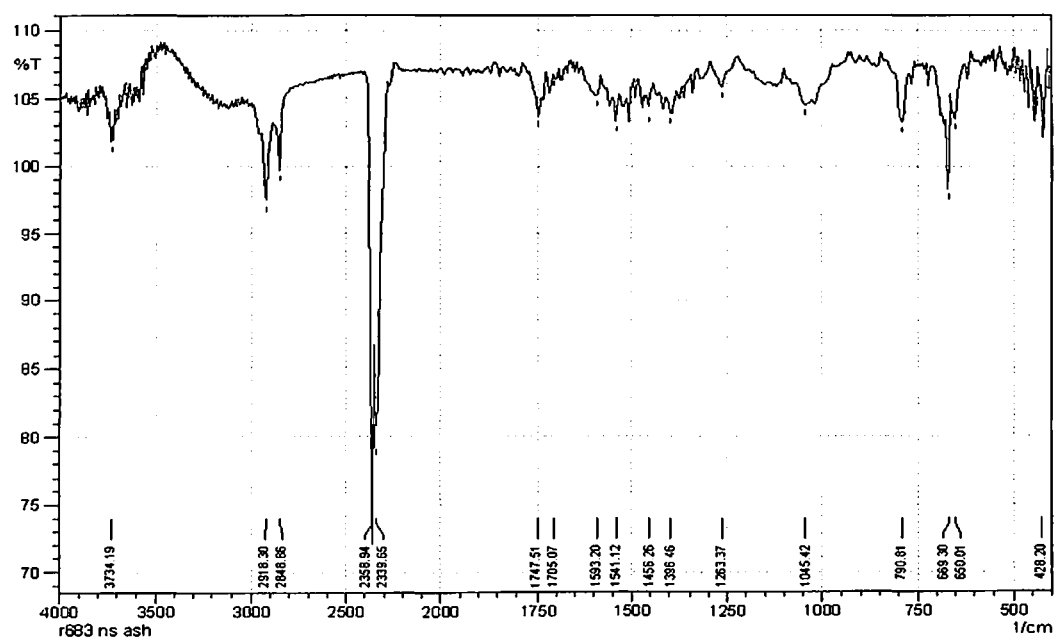


Fig. 45 FTIR spectrum of dihydromicrocystin-LR

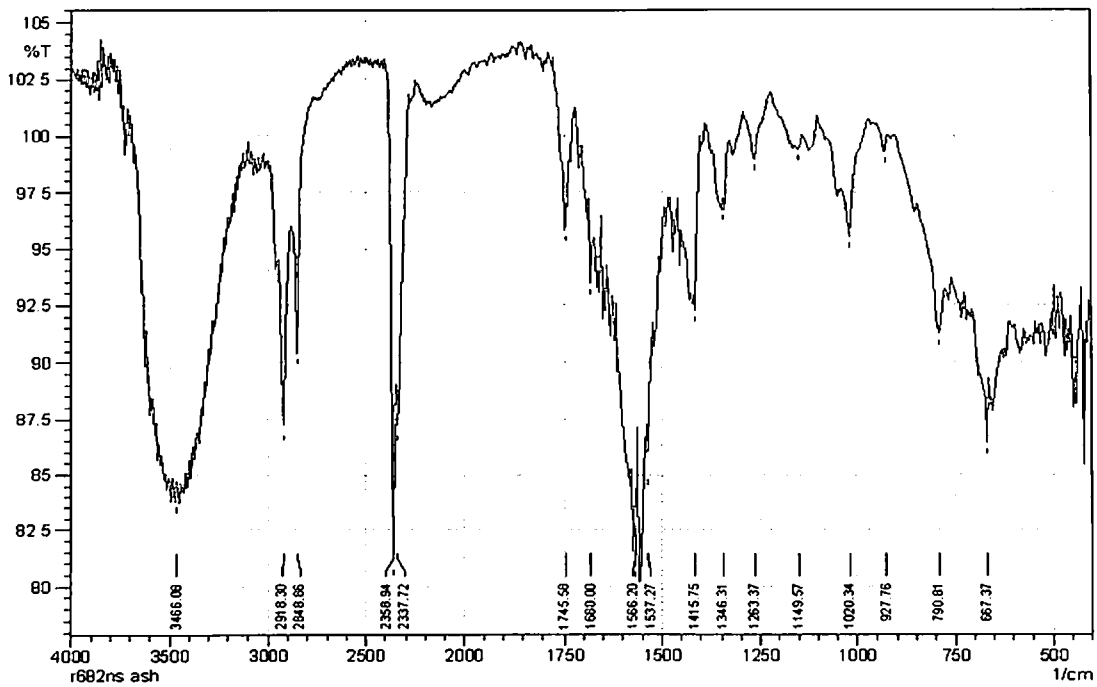


Fig. 46 FTIR spectrum of microcystin-LR

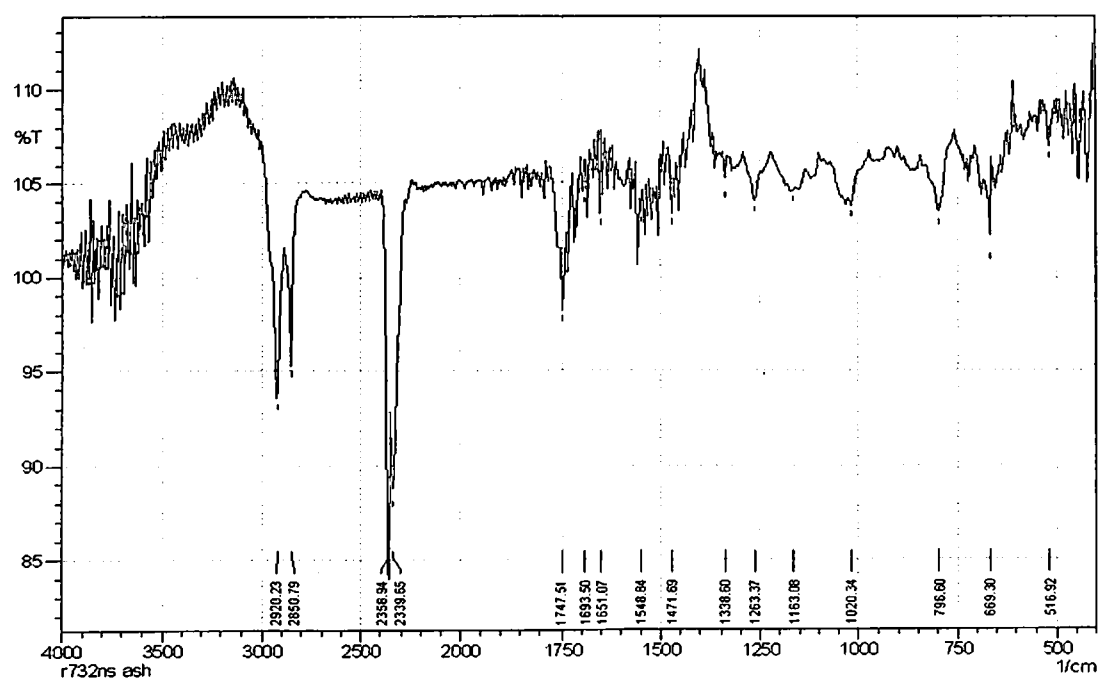


Fig. 47 FTIR spectrum of [D-Asp3]microcystin-HtyR

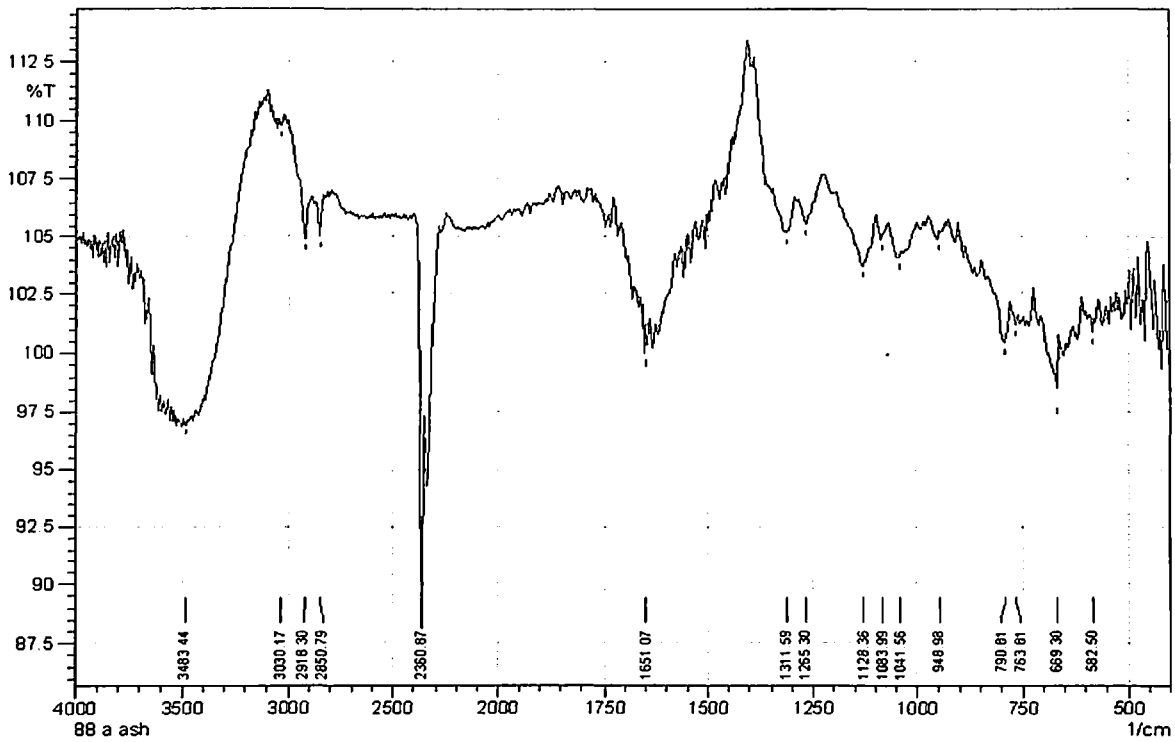


Fig. 48 FTIR spectrum of microcystin-FR

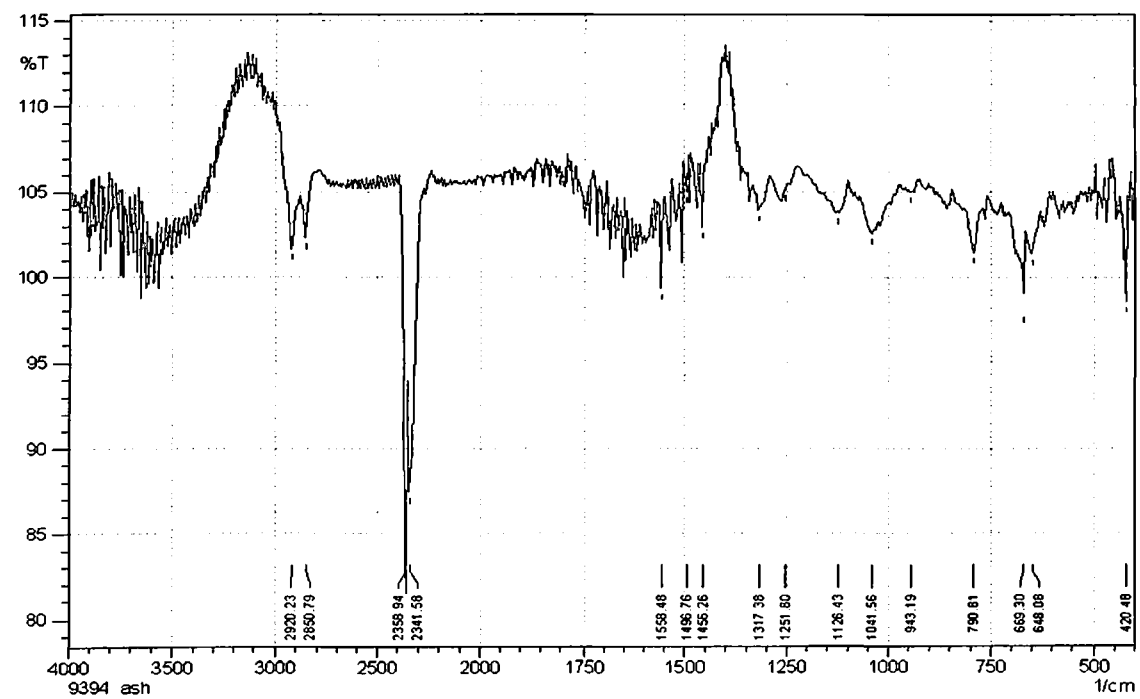
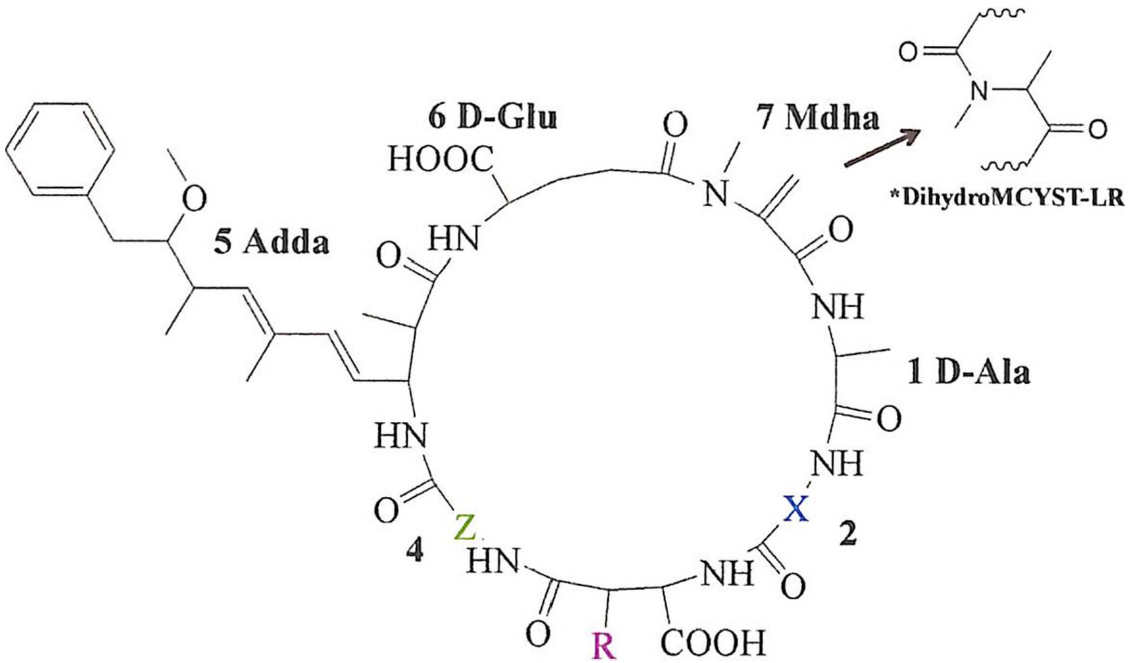


Fig. 49 FTIR spectrum of microcystin-RR

3.10. Peptide structure prediction

The identification of purified peptides were carried out by comparing the molecular ions of isolated cyclic peptides [Dihydromicrocystin-LR (m/z=996.8219), [D-Asp3]microcystin-WR (m/z=1051.954), Microcystin-LR (m/z=995.472 & 995.638), [D-Asp3]microcystin-HtyR (m/z=1043.756), Microcystin-FR (m/z=1029.521), Microcystin-RR (1037.970)] with the previously reported microcystin variants such as [D-Asp3]microcystin-HtyR (m/z=1045.5358) by Song *et al.,* 1998, Dihydromicrocystin-LR (m/z=996) by Namikoshi *et al.,* 1993, [D-Asp3]microcystin-WR (m/z= 1053.5975) and Microcystin-LR (m/z=995) by Namikoshi *et al.,* 1992, Microcystin-FR (m/z=1029), Microcystin-RR (m/z=1038) by Lee & Chou 2000. The difference in elution profile between the isolated microcystin variants as compared with standard microcystin-LR and presence of similar functional groups in FTIR spectra confirms the identity of the isolated compounds. Therefore, the structures of compounds were predicted by substituting the variable amino acids reported by Namikoshi *et al.,* 1992 & 1993, Song *et al.,* 1998 and Lee & Chou 2000 in the general structure (Fig. 6) of microcystin (Sivonen *et al.,* 1990). The predicted structures of the compounds are shown in Fig. 50.



Microcystin	X	Z	R
MCYST-LR	Leu	Arg	CH_3
*DihydroMCYST-LR	Leu	Arg	CH_3
MCYST-FR	Phe	Arg	CH_3
[D-Asp^3]MCYST-HtyR	Tyr	Arg	H
[D-Asp^3]MCYST-WR	Trp	Arg	H
MCYST-RR	Arg	Arg	CH_3

Fig. 50 Structures of microcystin variants isolated from *A. doliolum* and *N. spongiaeforme*

3.11. Screening of extracts/compounds for its *in vivo* toxicity

3.11.1. Acute toxicity of *A. doliolum* and *N. spongiaeforme*

The crude extracts prepared from different isolates were screened for their toxicity. The acute toxicity studied involving varying concentration (0.1mg-1mg/kg body weight) of *A. doliolum* showed that the animals dosed with concentration less than 100 µg protein/kg body weight survived for more than 10 days. The acute toxicity symptoms caused by crude extracts from *A. doliolum* with a dose 100 µg protein/kg include restlessness, fast breathing, slow movement, loss of co-ordination leading to death within 5.40 hour of i.p administration. Similarly a dose of 120 µg protein/kg crude extracts of *N. spongiaeforme* caused spasmodic leaping, restlessness and loss of co-ordination leading to death within 4 hour of i.p administration. Histopathological examination of animal's hepatocytes treated with crude extracts of *A. doliolum*, *N. spongiaeforme* or one of the spot from partially purified extracts (Ad spot 3 and Ns spot3) revealed a swollen livers and centrilobular to pan-lobular hemorrhagic necrosis. There were gross changes in normal architecture with engorged blood. The liver histology showed signs of hepatotoxicity such as swollen liver with degeneration and vaculolation of hepatic parenchyma and congestion (Fig. 51). A similar sign of toxicity were observed with 3 µg/kg body weight of purified [D-Asp^3]microcystin-WR and microcystin-RR, 2 µg/kg body weight of microcystin-LR, 3.5 µg/kg body weight of [D-Asp^3]microcystin-HtyR , 4 µg/kg body weight of microcystin-FR.

3.11.2. Sub acute toxicity of *A. doliolum* and *N. spongiaeforme*

The sub acute toxicity of partially purified extracts of *N. spongiaeforme* was tested by injecting varying concentration for 18 days. A dose of 25 µg/kg body weight showed a prominent prostrate tumour (Fig. 52). The same concentration of *A. doliolum* extract did not produce

tumour. The autopsy showed a swollen livers and centrilobular to pan-lobular hemorrhagic necrosis in the animals dosed with partially purified extracts of *N. spongiaeforme* and *A. doliolum*. HPLC purified compounds of *N. spongiaeforme* when injected individually or co-administed (100 ng each/kg body weight) did not show prostrate tumour. All animals showed restlessness, fast breathing, slow movement, loss of co-ordination from 4th day onwords.

3.11.3. Chronic toxicity of *A. doliolum* and *N. spongiaeforme*

The chronic toxicity of crude extracts of *A. doliolum* and *N. spongiaeforme* were tested by injecting sub-lethal dose (46 µg protein/kg) i.p to Swiss albino mice for 28 days. The extract of *A. doliolum* caused prominent colon tumor (Fig. 53) whereas, the extract of *N. spongiaeforme* caused intestinal tumor (Fig. 54). The toxicity of extracts was also estimated biochemically by measuring the level of serum ALT, AST, ALP, LDH and MDA in the hepatocytes during the 28 days of study against the control. The crude extracts from *A. doliolum* and *N. spongiaeforme* showed increased level of serum ALT, AST, ALP, LDH ($p<0.01$) and MDL ($p<0.01$) level against control (Table 5). The liver histology showed signs of hepatotoxicity such as swollen liver with degeneration and vaculolation of hepatic parenchyma and congestion (Fig. 55).

The crude methanolic extract of *A. doliolum* and *N. spongiaeforme* were fractionated using various organic solvents of increasing polarity as mentioned in materials and methods. The organic fractions (Hexane, chloroform and ether) did not show considerable increase in serum ALT, AST, ALP, LDH ($p>0.05$), and MDA level ($p>0.05$), whereas the methylene chloride extracts of both *A. doliolum* and *N. spongiaeforme* showed almost six fold increases in serum ALT, AST, ALP level ($p<0.01$) and five-fold increase in serum LDH level ($p<0.01$) against the control. Almost similar trend was observed for MDA level ($p<0.01$) (Table 6).

Chronic toxicity assay was carried out for all TLC spots of *A. doliolum* and *N. spongiaeforme* using crude heat inactivated methylene chloride fractions as negative controls and Microcystin–LR as positive control (Table 7). The Ad spot 1, Ad spot 4, Ns spot 1, Ns spot 2 and Ns spot 4 showed no increase in MDA level as compared to control ($p>0.05$) whereas Ad spot 2, Ad spot 3, Ns spot 3 showed significant ($p<0.01$) increase in MDA level (Table 7). Although Ad spot 2 showed increase in MDA level ($p<0.01$) but it was unable to generate tumor (data not shown). Ad spot 3 of *A. doliolum* induced colon tumors, similar to that of the crude extract (refer Fig. 53) also showed with increase in serum ALT, AST, ALP, LDH level ($p<0.01$). Similarly, Ns spot -3 of *N. spongiaeforme* showed increase in serum ALT, AST, ALP, LDH level ($p<0.01$). The Ad spot 3 and Ns spot 3 demonstrated almost similar level of toxicity in terms of serum ALT, AST, ALP, LDH ($p<0.01$) and MDL level ($p<0.01$) in hepatocytes. Toxicity was not observed with other spots of *A. doliolum* and *N. spongiaeforme* as their serum ALT, AST, ALP, LDH and MDL level ($p>0.05$) of hepatocytes did not show significant difference with that of controls (Table 7). The body weight of the test animal has been used as preliminary indicator of tumor development. Average body weight of the test animals treated with partially purified Ad spot 3 and Ns spot 3 decreased gradually due to the induction of tumor as compared to control during the course of toxicity assay (Fig. 56). To analyze the cause of reduction on body weight, the animals were dissected after 28 days of toxin administration. The physical examination of livers exposed to both Ad spot 3 and Ns spot 3 showed a high level of toxicity. Further histopathological examination revealed a swollen livers and centrilobular to pan-lobular hemorrhagic necrosis. There were gross changes in normal architecture with engorged blood and showed 2 fold increase in weight of the liver as compared to both the control. The livers of animals exposed to Ad spot-3 showed a deep red

coloration which was not observed in control animals (compared Fig. 57 a & b). Although Ns spot 3 did not show significant difference in liver's colour as compared to the control, a marked congestion of livers was observed in both the cases. In addition, the autopsy also showed the formation of rudimentary appendix in case of Ns spot 3, weighing about 0.9568gm and 1.9cm in length (Fig. 58 a). A lower dose of Ns spot 3 (10µg/kg body weight) for 28 days showed the formation of intestinal cyst (Fig. 58 b).

HPLC purified compounds when administered individually with a dose of 0.80 µg/kg in Swiss albino mice did not show colon or intestinal tumour formation. The prominent colon tumors were observed when two compounds of Ad spot 3 (dihydromicrocystin-LR and [D-Asp^3]microcystin-WR) were co-administered with a dose of 0.35 µg/kg each in Swiss albino mice. Similar types of results were obtained in the induction of prominent intestinal tumors when two compounds of Ns spot 3 ([D-Asp^3]microcystin-HtyR and microcystin-LR) were co-administered with a dose of 0.5 µg/kg each. Microcystin-FR and microcystin-RR did not show any tumour formation (Table 8). All variants of microcystins except microcystin FR increase the liver weight by 2 fold as compared to control. Microcystin-FR showed least 12.5% less accumulation of toxin which is reflected in the weight of hepatocytes as compared to other variants of microcystins (Fig. 59). The microcystin-FR, therefore was selected for further studies on its antidepressive, anxiolytic, analgesic and spontaneous locomotive activities in subsequent experiments.

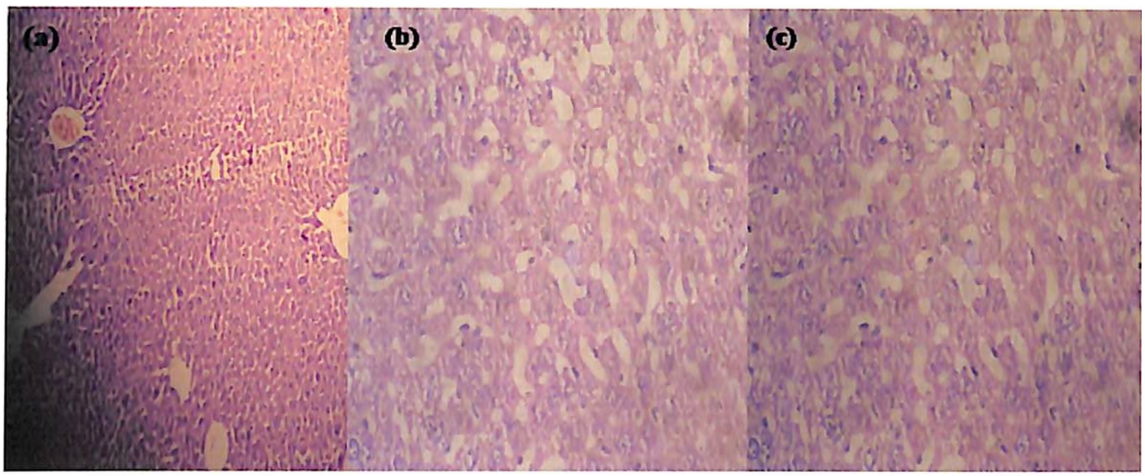


Fig. 51 Histomicrographs of perfused liver of Swiss albino mice injected via i.p (a) PBS without algal extract (b) Crude methanolic extract of *A. doliolum* (100 µg/kg body weight) (c) Crude methanolic extract of *N. spongiaeforme* (120 µg/kg body weight).

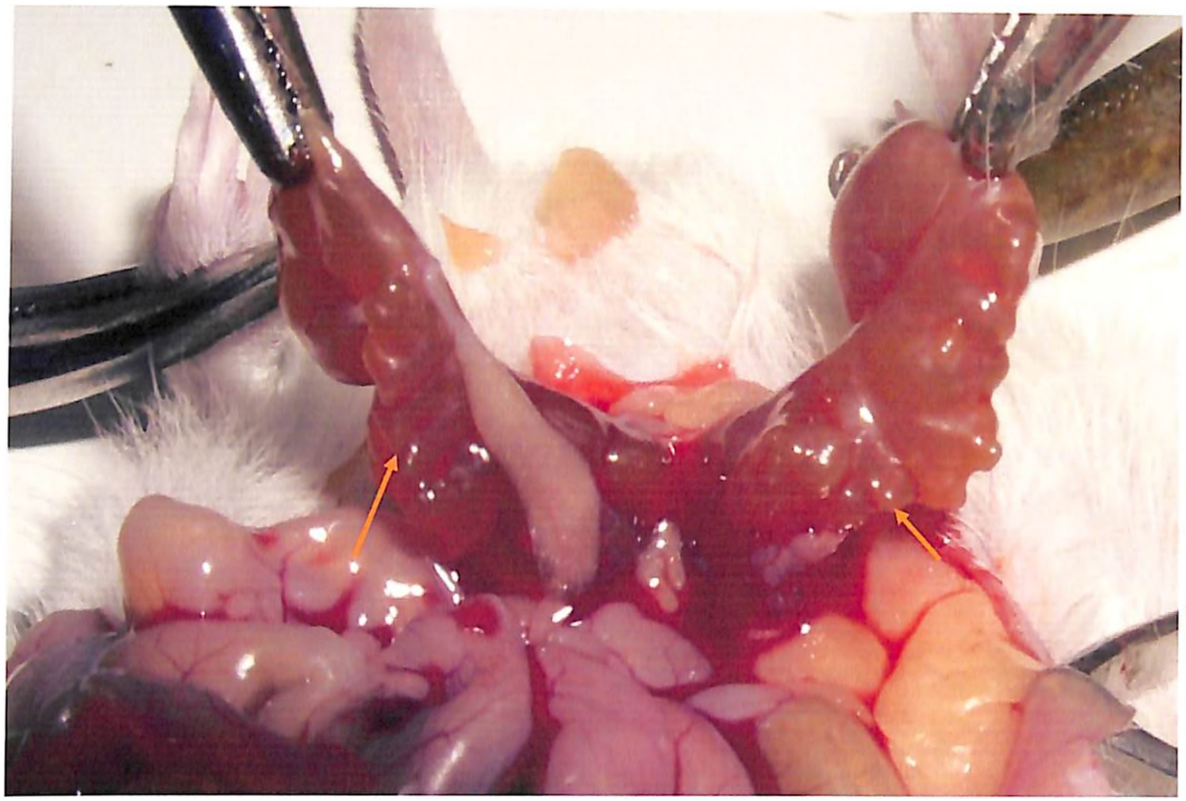


Fig. 52 Prostrate tumor induced by partially purified extracts of *N. spongiaeforme* at sub acute dose of 25 µg/kg body weight

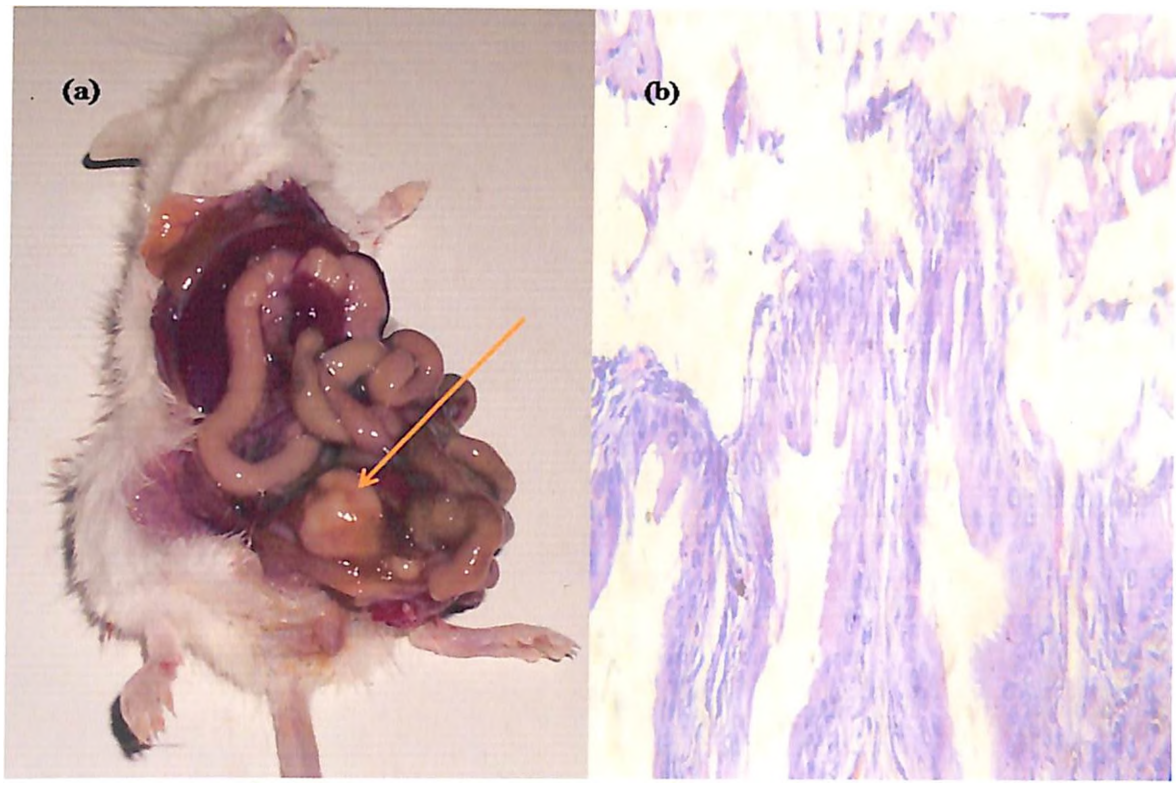


Fig. 53 (a) Colon tumor induced by crude methanolic extract of *A. doliolum* injected (i.p) to Swiss albino mice at the sub lethal dose (46 µg/kg body weight). (b) Histomicrographs of colon tumor.

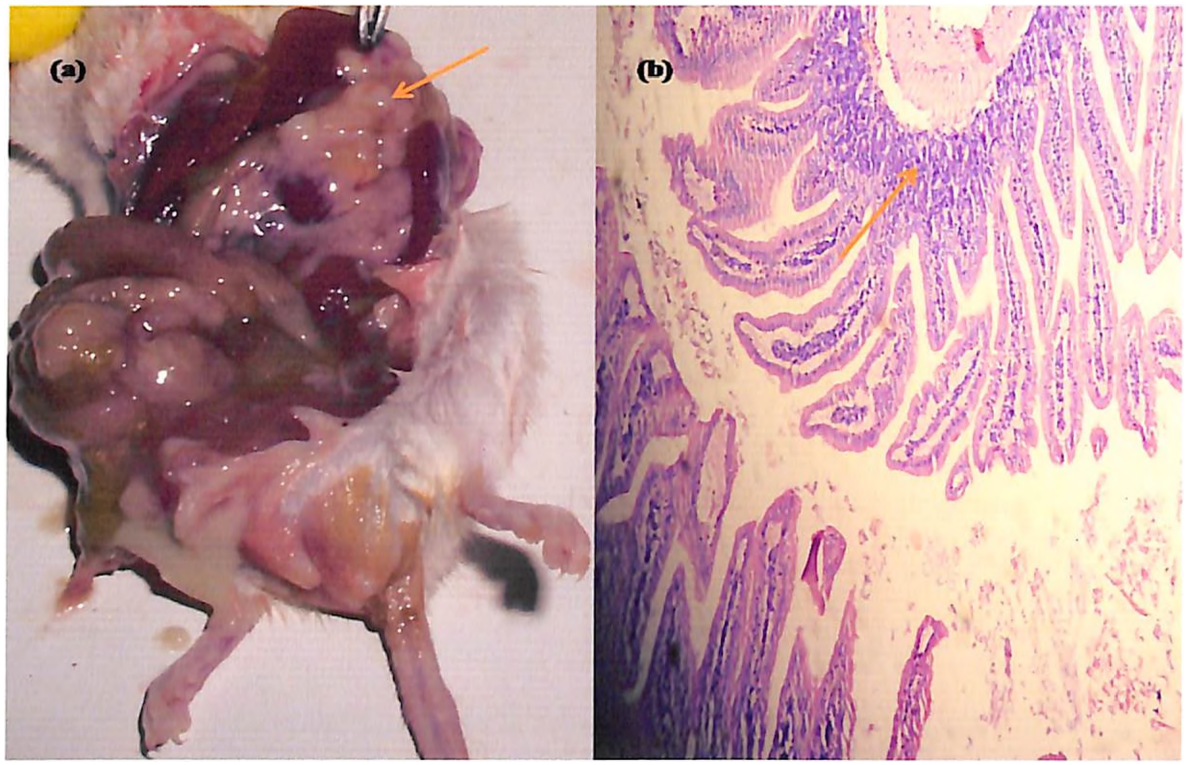


Fig. 54 (a) Intestinal tumor induced by crude methanolic extract of *N. spongiaeforme* injected (i.p) to Swiss albino mice at the sub lethal dose (46 µg/kg body weight). (b) Histomicrographs of intestinal tumor.

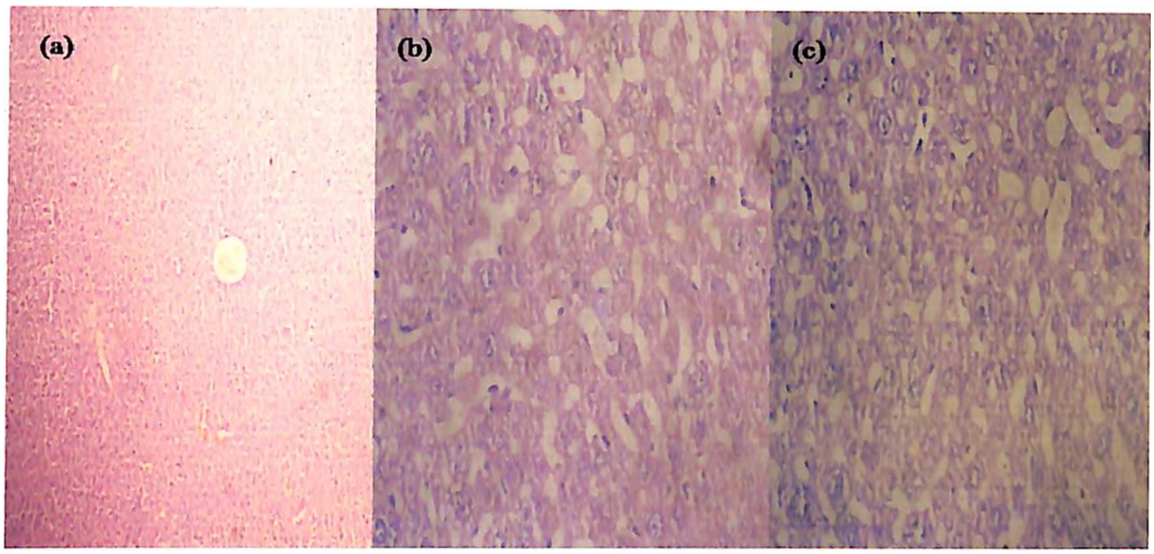


Fig. 55 Histomicrographs of perfused liver of Swiss albino mice injected via i.p (a) PBS without algal extract (b) Crude methanolic extract of *A. doliolum* (46 µg/kg body weight) (c) Crude methanolic extract of *N. spongiaeforme* (46 µg/kg body weight).

Table 5 Toxicity of crude extract obtained from *A. doliolum* and *N. spongiaeforme*: Serum ALT, AST, ALP, LDH and MDA level was measured after 28 days with a dose of 46 µg/kg body weight (i.p) of Swiss albino mice

Strains isolated	Toxicity of crude Methanolic extract				
	Serum ALT in U/L Mean±S.E.	Serum AST in U/L Mean±S.E.	Serum ALP in U/L Mean±S.E.	Serum LDH in U/L Mean±S.E.	MDA level in flouresence intensity Mean±S.E.
Anabaena doliolum	15.559±0.3274	36.59±0.4623	17.76±0.8450	68.39±2.025	240.5±3.704
Nostoc spongiaeforme	12.367±0.2972	39.17±0.710	16.80±0.8308	79.84±2.542	173.166±3.219
Control (Phosphate buffered saline)	4.255±0.1772	6.428±0.3572	4.131±0.6033	16.92±1.336	26.6±1.7

Table 6 Toxicity analysis of various organic fractions: Values of serum ALT, AST, ALP, LDH and MDA level was measured after 28 days with a dose of 38 µg/kg body weight (i.p) of Swiss albino mice.

Fractions prepared from Methanolic extract	Toxicity *of A. doliolum* in mice bioassay					Toxicity *of N. spongiaeforme* in mice bioassay				
	Serum ALT in U/L Mean±S.E.	Seru AST in U/L Mean±S.E.	Serum ALP in U/L Mean±S.E.	Serum LDH in U/L Mean±S.E.	MDA level in flouresence intensity Mean±S.E.	Serum ALT in U/L Mean±S.E.	Serum AST in U/L Mean±S.E.	Serum ALP in U/L Mean±S.E.	Serum LDH in U/L Mean±S.E.	MDA level in flouresence intensity Mean±S.E.
Hexane	3.50±0.121	9.315± 0.9942	6.081± 1.158	18.05 ± 1.813	24.783± 0.4301	3.646± 0.2198	9.842± 1.249	6.045 ± 1.107	17.45 ± 0.456	25.113±0 0.2795
Ether	3.688± 0.1279	8.152± 0.6734	6.009± 1.531	19.34 ± 1.430	24.786± 0.3254	3.764± 0.2759	9.127± 1.054	5.034 ± 1.058	17.50 ±1.603	26.535±0 .3373
Chloroform	3.751± 0.1669	9.966± 0.9704	6.716± 1.115	18.00 ± 1.898	24.4± 0.3679	3.348± 0.1957	8.557± 0.9619	4.978 ± 0.9921	15.80 ± 0.9951	25.06± 0.1414
Methylene chloride	24.991±0.4544	46.41±2.099	33.61±1.708	85.86 ± 1.914	240.668±2. 92	24.373±0. 3917	50.70±2.6 80	36.57 ± 1.601	87.64 ± 1.471	251.833± 2.272
Control (Phosphate buffered saline)	3.723±0.1266	7.365±0.3942	5.775 ±1.117	17.90 ± 1.792	23.488±0.3 098	3.675±0.2 198	7.365 ±0.3942	5.775 ± 1.117	17.90 ± 1.792	26.71±0. 195

Table 7 Toxicity analysis of the compounds separated by TLC: Serum ALT, AST, ALP, LDH and MDA level was estimated after 28 days with a dose of 20 µg/kg body weight (i.p) of Swiss albino mice daily.

Toxic Strains	Spots separated by TLC	MDA Level of hepatocytes in flouresence intensity Mean±S.E.	Serum ALT Level in U/L Mean±S.E.	Serum AST in U/L Mean±S.E.	Serum ALP in U/L Mean±S.E.	Serum LDH in U/L Mean±S.E.	Weight of hepatocytes in gms Mean	Mean survival in days
Anabaena doliolum	Ad spot 1	24.926±0.354	3.416±0.1264	10.22±0.9906	7.083±1.136	18.96±1.450	1.532	28 days
	Ad spot 2	75.599±0.329	3.443±0.1876	9.440±1.219	8.273±1.038	25.19±1.802	2.004	28 days
	Ad spot 3	366.17±0.3331	24.908±0.2355	44.77±2.975	48.78±2.695	66.74±2.594	3.600	28 days
	Ad spot 4	26.281±0.3981	4.073±0.07984	7.342±1.126	8.691±0.9299	18.70±1.663	1.383	28 days
Nostoc spongiaeforme	Ns spot 1	27.036±0.3233	4.300±0.1309	8.519±1.497	8.341±1.098	24.62±2.105	1.355	28 days
	Ns spot 2	26.286±0.3937	4.047±0.1138	8.470±1.682	8.585±0.9461	23.81±2.097	1.427	28 days
	Ns spot 3	315.16±0.2144	27.080±0.1301	38.21±2.892	41.52±2.308	65.00±1.777	4.350	28 days
	Ns spot 4	29.005±0.3164	4.583±0.105	10.49±2.320	8.209±0.7580	19.24±1.367	1.529	28 days
Control -1(vehicle)	-	28.47±0.1265	4.225±0.1362	6.261±0.9757	8.116±1.020	17.59 ± 1.528	1.342	28 days
Control-2 (heat inactivated fraction of *Anabaena doliolum*)	-	26.845±0.3006	4.035±0.2176	6.773±1.117	7.459±1.361	16.74±1.438	1.366	28 days
Control-3 (heat inactivated fraction of *Nostoc spongiaeform*)	-	27.271±0.2867	4.007±0.1714	6.140±1.079	7.503±0.9432	15.12±1.706	1.574	28 days
Standard Microcystin (0.14µg/kg of body weight for animal studies)	Single spot	365.035±0.127	19.321±0.2328	54.45±3.545	56.10±3.225	53.21±2.267	4.035	28 days

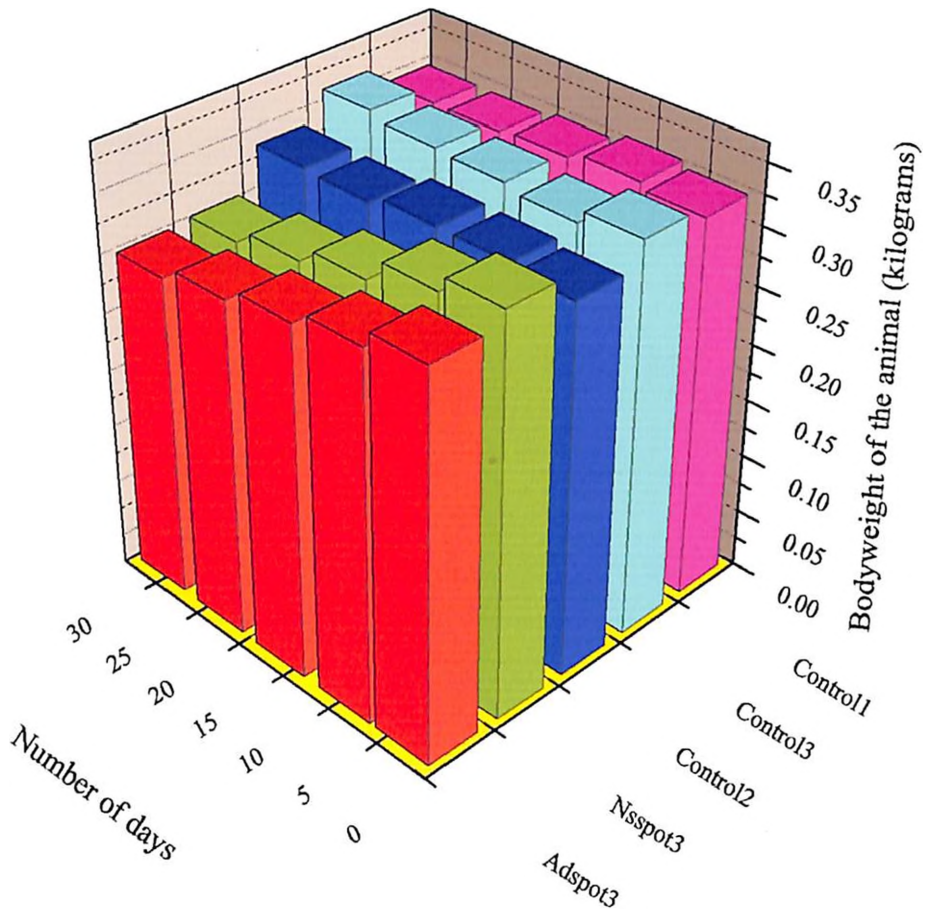


Fig. 56 Average body weight analysis of Swiss albino mice injected with Ad spot 3 and Ns spot 3 (20 µg/kg body weight) via i.p for 28days against the control animals.

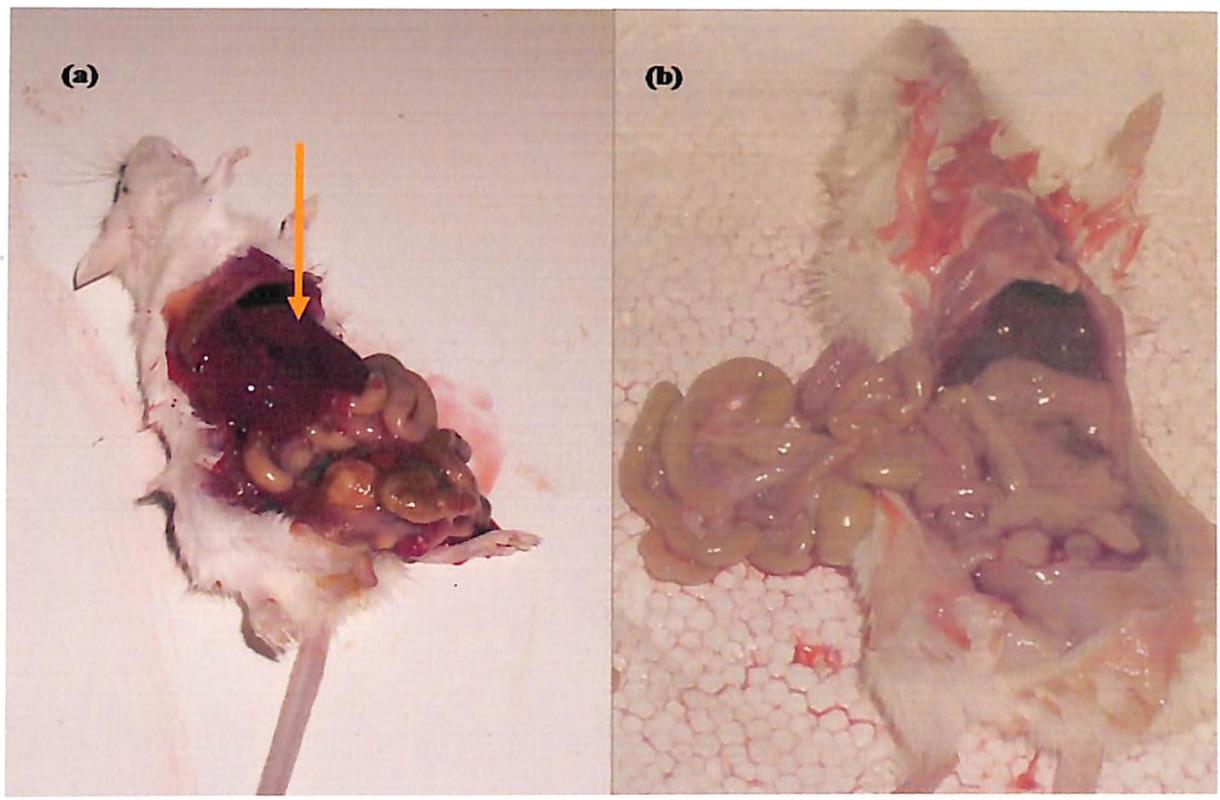


Fig. 57 (a) Enlargement of liver in Swiss albino mice (20 µg /kg body weight) due to i.p administration of Ad spots 3 for 28 days showing hemorrhage and blood pooling in hepatocytes (b) Control

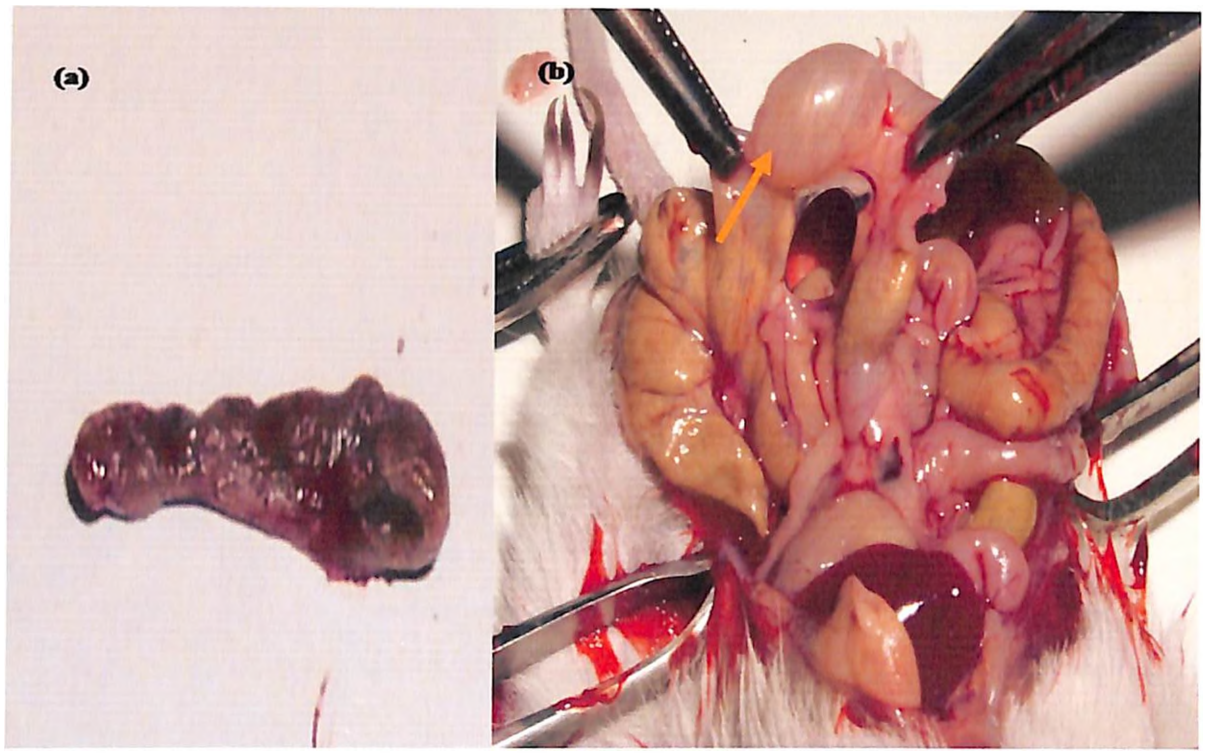


Fig. 58 (a) Formation of rudimentary appendix in Swiss albino mice after i.p administration of Ns spots 3 (20 µg/kg body weight) for 28 days. (b) Formation of intestinal cyst in Swiss albino mice after i.p administration of Ns spots 3 (10 µg/kg body weight) for 28 days.

Table 8 Tumour formations in Swiss albino mice treated with HPLC purified compounds.

Strains	HPLC purified compounds	Tumour Formation
A. doliolum	DihydroMicrocystin–LR	-
	[D-Asp3] Microcystin-WR	-
	Microcystin-LR	-
	DihydroMicrocystin–LR + [D-Asp3] Microcystin-WR	+
	DihydroMicrocystin–LR + Microcystin-LR	-
	[D-Asp3] Microcystin-WR+ Microcystin-LR	-
N. spongiaeforme	Microcystin-LR	-
	[D-Asp3]Microcystin-HtyR	-
	Microcystin-RR	-
	Microcystin FR	-
	Microcystin-LR + [D-Asp3]Microcystin-HtyR	+
	Microcystin-LR+ Microcystin-RR	-
	Microcystin-LR + Microcystin FR	-
	[D-Asp3]Microcystin-HtyR + Microcystin-RR	-
	[D-Asp3]Microcystin-HtyR + Microcystin-FR	-
	Microcystin-RR + Microcystin FR	-

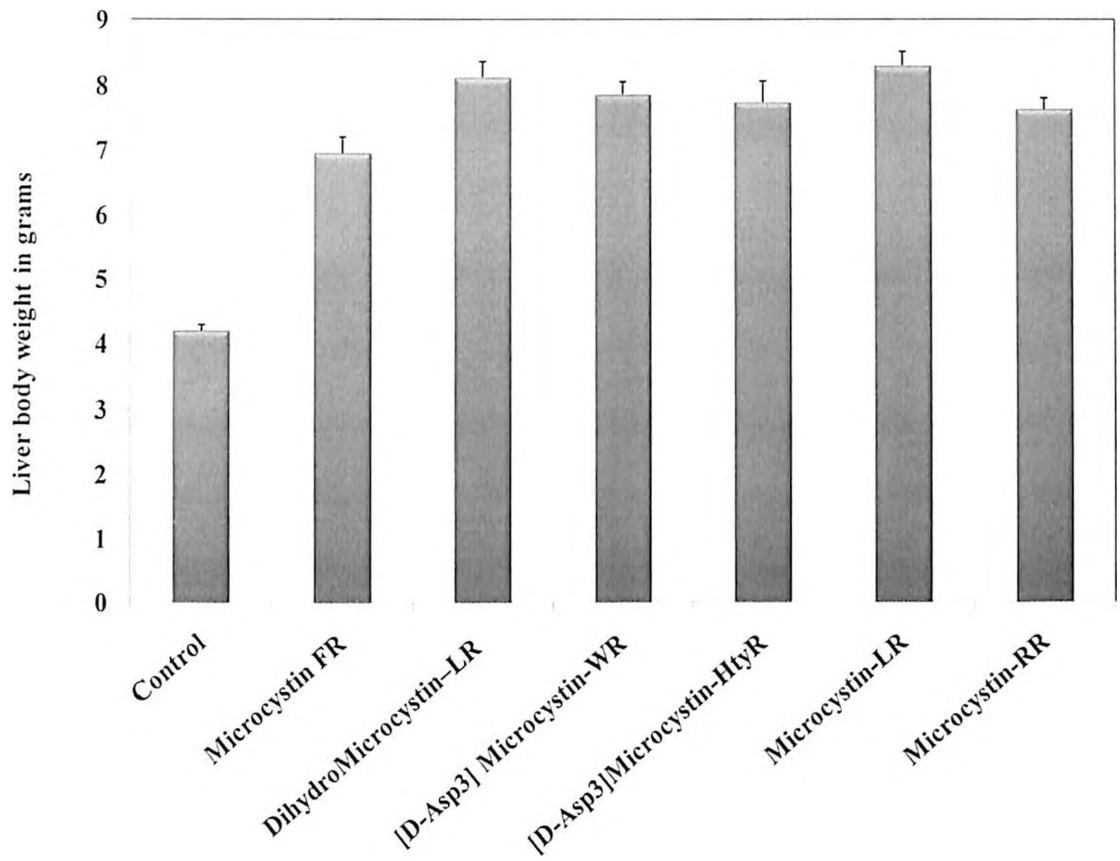


Fig. 59 Liver body weight analysis in Swiss albino mice treated with microcystin variants of 0.5 µg/kg body weight. One way ANOVA and Dunnett's multiple comparison test was carried to find the mean, SEM and significant level.

3.12. Screening of microcystin -FR for Antidepressive activity

3.12.1. Hepatotoxicity of microcystin-FR along with Cyclosporin-A

The animals treated with 0.8 µg/kg microcystin-FR (McyFR) alone showed 3.0 fold increase in serum LDH and 8.0 fold increase in AST level. The addition of 8mg/kg body weight of CsA showed only 3.0 fold and 2.5 fold increase in LDH and AST levels respectively (Fig. 60) suggesting a definite role of CsA in protecting McyFR induced hepatotoxicity.

3.12.2. Histopathology

Animals treated with 0.8 µg/kg body weight McyFR alone showed swollen livers and changes in normal architecture of hepatocytes (Fig. 61 a). Addition of 8mg/kg body weight CsA to McyFR treated animals showed no significant change in liver morphology (Fig. 61 b) when compared to the controls (Fig. 61 c & d). The liver body weight analysis also showed no change in liver weight of the animal receiving a mixture of microcystin-FR and cyclosporin-A as compared to the control, whereas McyFR alone showed significant increase in liver weight due to accumulation of toxin (Fig. 62).

Therefore, microcystin-FR (0.8 µg/kg body weight) along with cyclosporin-A (8 mg/kg body weight) was selected for further studies on its antidepressive, anxiolytic, analgesic and spontaneous locomotive activities in subsequent experiments

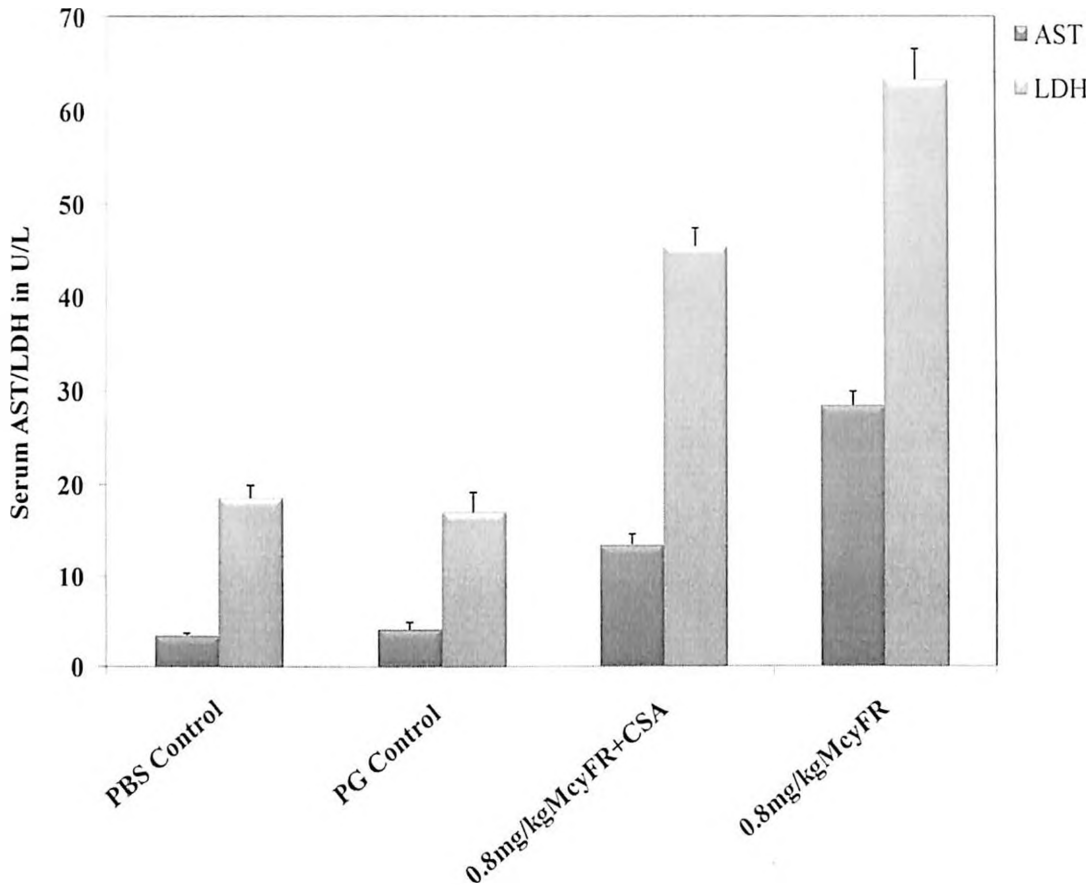


Fig. 60 Chemoprotective efficiency of CsA against the hepatotoxic effect of microcystin-FR on liver enzymes AST & LDH levels. One way ANOVA and Tukey's multiple comparison test was carried to find the significance level

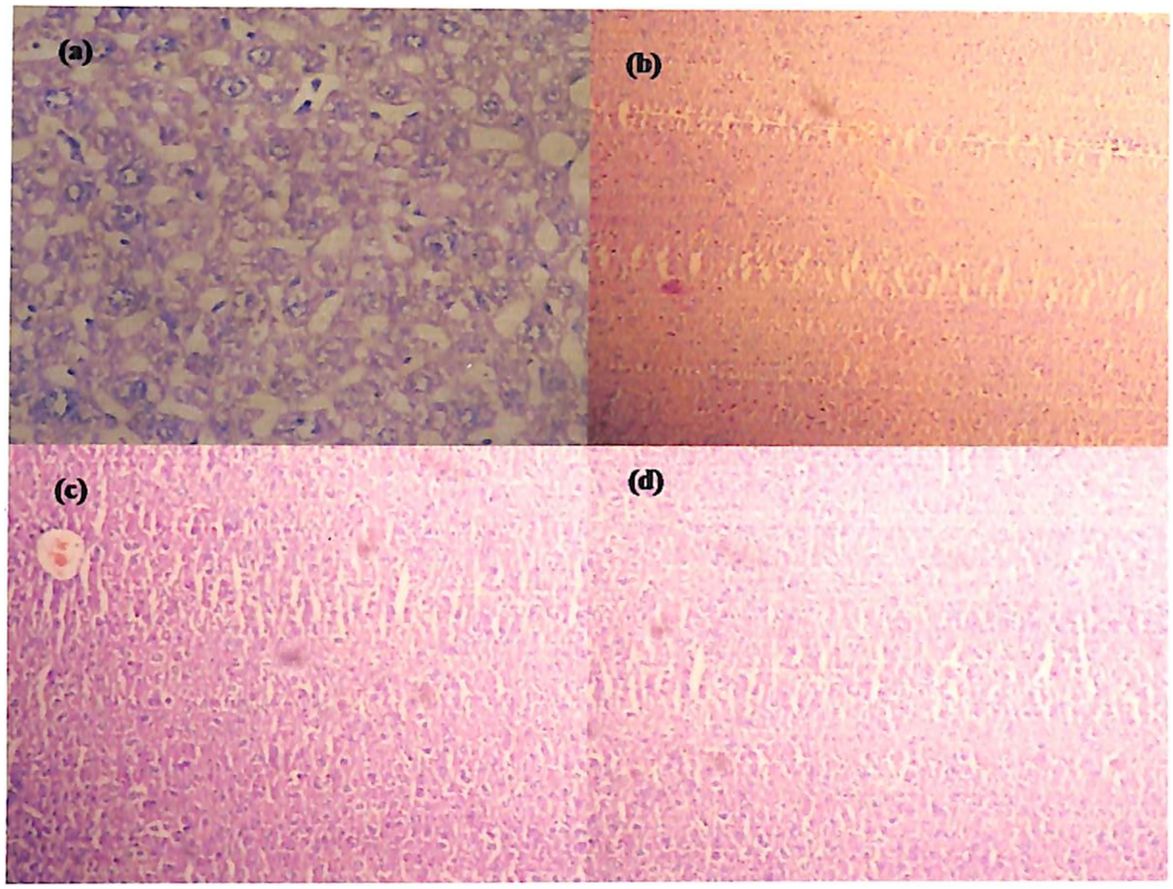


Fig. 61 Histomicrographs of perfused liver of Swiss albino mice injected with (a) 0.8 µg/kgMcyFR alone (b) 0.8 µg/kgMcyFR+8 mg/kg CsA (c) PBS Control (d) PG Control (PG)

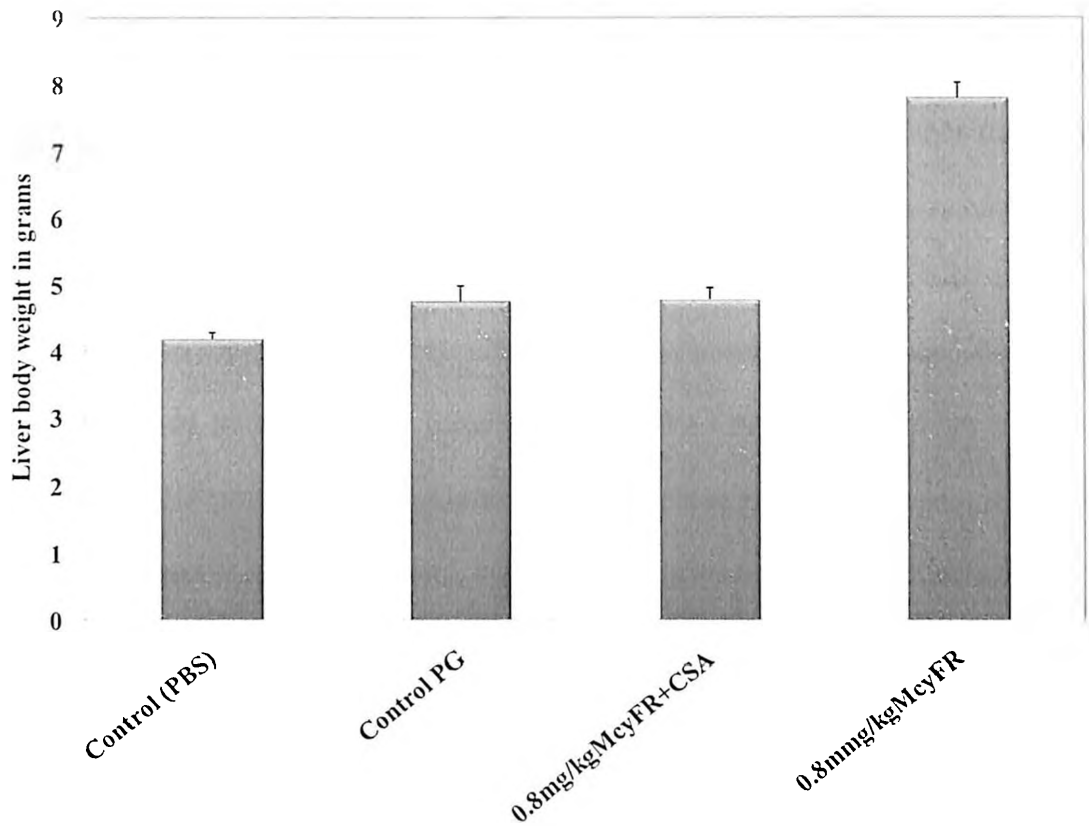


Fig. 62 Liver body weight analysis in Swiss albino mice treated with: 0.8 µg/kg McyFR (P<0.01) alone and in combination with 8mg/kg CsA body weight (P>0.05), PBS Control and PG Control. One way ANOVA and Dunnett's multiple comparison Test was carried to find the mean, SEM and significant level.

3.12.3. Force swim test

A 45% decrease in immobility of Swiss albino mice was observed in force swim test with the animals treated with 0.8 µg/kg microcystin-FR (McyFR) and 8 mg/kg CsA ($P>0.05$) with respect to the positive controls (Bupropion & Desvenlafaxine). CsA treated group showed similar immobility to that of negative controls (PBS and PG), suggesting that CsA alone did not cause any antidepressive activity. (Fig. 63)

3.12.4. Tail suspension test

A 40% decrease in immobility of Swiss albino mice was observed in tail suspension test with the animals treated with 0.8 µg/kg McyFR and 8mg/kg CsA ($P>0.05$) with respect to the positive controls (Bupropion & Desvenlafaxine). CsA treated group showed similar immobility to that of negative controls (PBS and PG), suggesting that CsA alone did not cause any antidepressive activity (Fig. 64)

3.12.5. Elevated Plus Maze Test

The time spent in open arm by the animals treated with 0.8 µg/kg McyFR + 8mg/kg CsA was 89% of the time spent by positive control animals (Fig. 65). The animals treated with CsA alone (8 mg/kg McyFR) showed similar effect as that of negative control group suggesting that CsA has no prominent role in anxiolytic activity.

3.12.6. Spontaneous locomotive activity

In order to understand the microcystin induced locomotive disorder, the spontaneous locomotive activity test was conducted. As shown in Table 9, the locomotive score was almost same for the mice treated with CsA alone or in the combination with 0.8 µg/kg McyFR compared to the negative controls. Therefore, microcystin-FR alone or in combination of CsA did not induce locomotive disorder.

3.12.7. Analgesic activity

3.12.7.1. Acetic acid induced writhing test:

In order to understand the microcystin induced analgesic effect, acetic acid induced writhing test was conducted. The number of writhing induced by acetic acid in the mice treated with CsA or in the combination with 0.8 μg/kg McyFR or in control, was almost same (Fig. 66). Therefore, microcystin-FR alone or in combination of CsA did not show any analgesic activity.

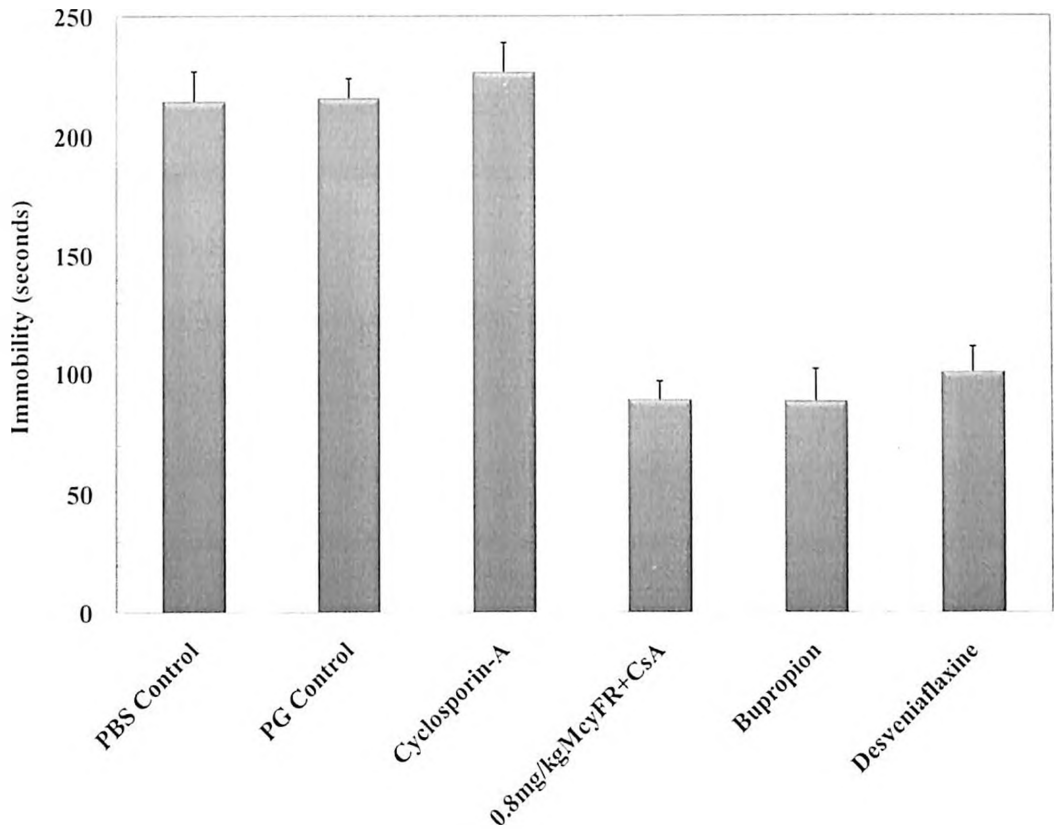


Fig. 63 Antidepressive activity of microcystin-FR along with cyclosporin-A in Force swim test (FST). One way ANOVA and Bonferroni's multiple comparison Test was employed to determine the significance level.

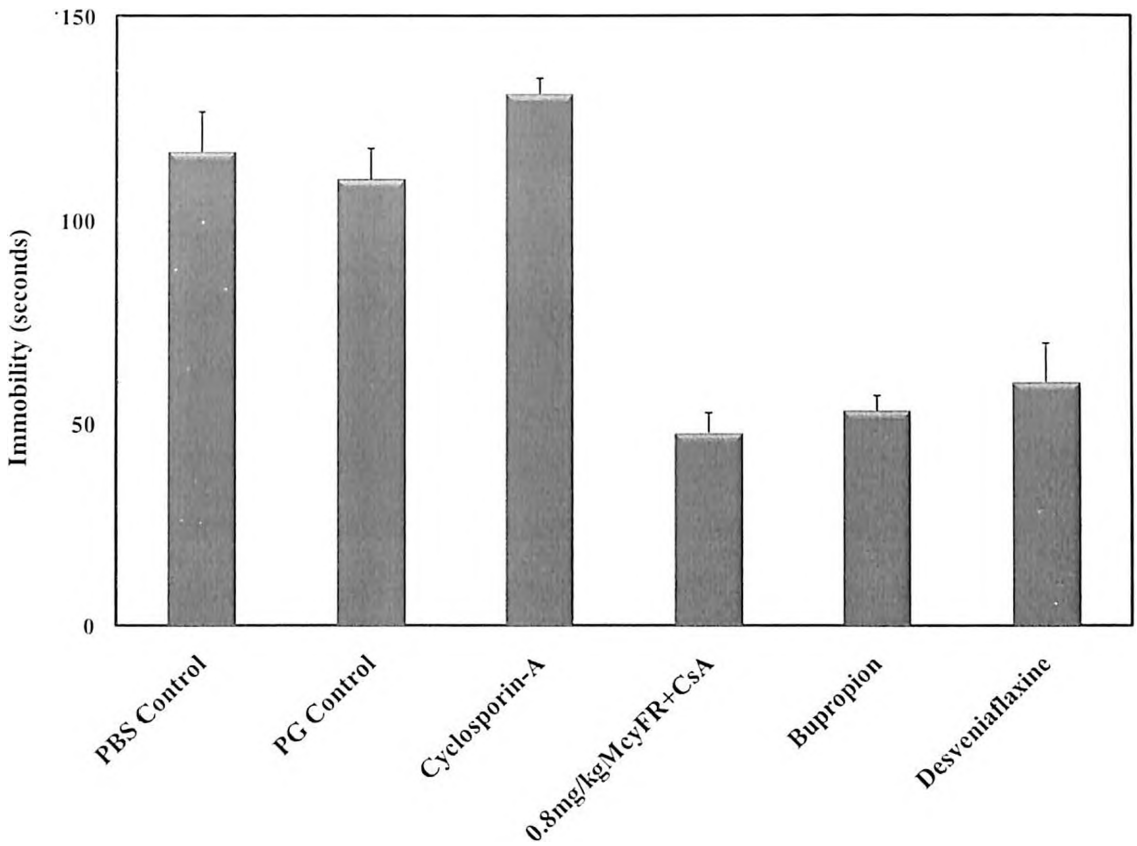


Fig. 64 Antidepressive activity of microcystin-FR along with cyclosporin-A in Tail suspension test (TST). One way ANOVA and Bonferroni's multiple comparison test was employed to determine the significance level.

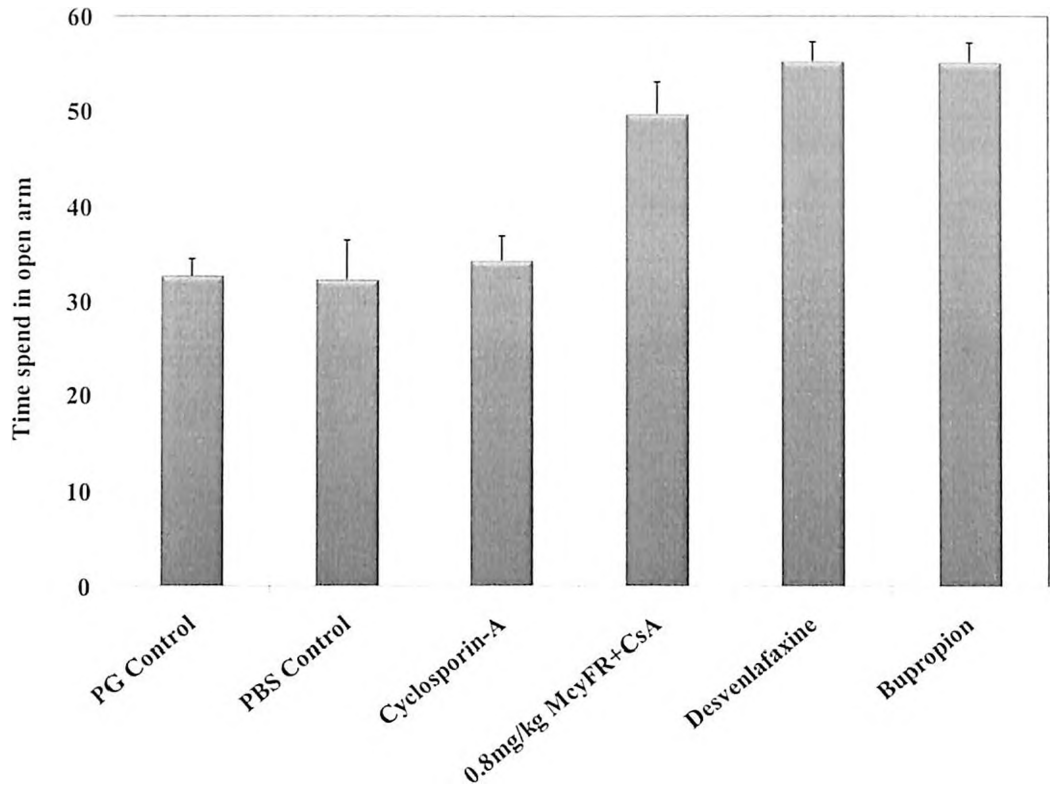


Fig. 65 Anxiolytic effect of microcystin-FR along with cyclosporin-A in Elevated plus maze test. One way ANOVA and Bonferroni's multiple comparison test was employed to determine the significance level.

Table 9 Effect of microcystin-FR and cyclosporin-A on Spontaneous locomotive activity. One way ANOVA and Dunnett's multiple comparison test was conducted to find the significance level.

Sl.no	Treatment	Locomotor Score Mean±S.E.
1	PBS Control	291.8 ± 5.307
2	PG Control	287.3 ± 4.529
3	CsA 8mg/kg	300.7 ± 6.712
5	Microcystin FR (0.8 µg/kg + CsA 8 mg/kg)	300.3 ± 7.329

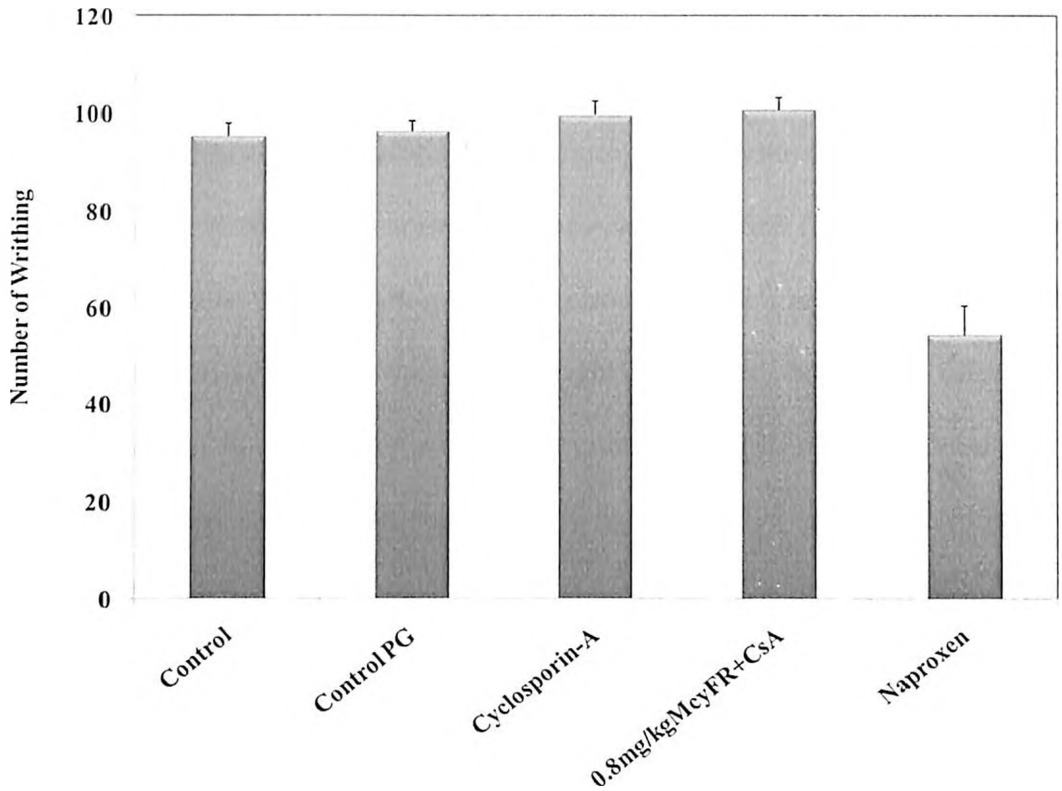


Fig. 66 Analgesic effect of microcystin-FR along with cyclosporin-A in acetic acid induced Writing test. One way ANOVA and Dunnett's multiple comparison test was conducted to find the significance level.

3.13. Docking of microcystin variants with PP_2A receptor:

In order to investigate the reason behind the difference in toxicity of microcystin variants, docking studies were performed and the results were analyzed to find out the binding pattern of microcystin variants to PP_2A receptor. The study infers that the binding energy pattern of methylated variants of microcystin (dihydromicrocystin, microcystin-FR and microcystin-RR) to PP_2A is closely correlated with the orientation of ADDA position (Table 10). Among above three methylated variants, the orientation of dihydromicrocystin-LR was closer to microcystin-LR as compared to other variants. The slight variation found in orientation of dihydromicrocystin-LR in relationship with microcystin-LR may be due to the benzene ring in ADDA position forming a nonbonding interaction with Proline 190 of receptor which leads to the movement of fatty acid chain towards the anticlockwise direction (Fig. 67). The other methylated variants (microcystin-FR and microcystin-RR) show almost similar bending orientation. The benzene ring in the ADDA position of two variants (microcystin-FR and microcystin-RR) not only inclines towards left side but also moved toward anticlockwise direction. This change in the orientation was due to the nonbonding interaction of benzene ring in ADDA position with valine 189 & proline 190 of PP_2A in microcystin-RR (Fig. 68). In case of microcystin-FR, the presence of bulky phenylalanine at Z position (Fig. 68) not only forms a nonbonding interaction with cystine-269 but also makes the benzene ring of ADDA moiety to rotate in anticlockwise direction to make nonbonding interactions with valine 189, proline 190 and His 191 of PP_2A receptor.

The docking energy of demethylated derivatives microcystin-HtyR, and microcystin-WR decreased to 36 and 30% respectively (Table 1) as compared to microcystin-RR. In microcystin-HtyR the hydroxyl group of tyrosine at Z position (Fig. 69) forms a non bonding

interaction with arginine 89 of the receptor, which leads to higher bending of ADDA chain more towards anticlockwise as compared to microcystin-FR. In case of microcystin-WR the presence of tryptophan at Z position (Fig. 70) makes it much heavier. The presence of bulky imidazole ring at the tail position present at the tip of receptor cavity not only reduces the binging energy, but also changes the total binding orientation of the ADDA moiety which might lead to decrease in activity (Fig. 70). The lower docking energy of demethylated derivative may be correlated to lesser time interaction with PP_2A leading to lower level of *in-vivo* toxicity as compared to methylated derivatives.

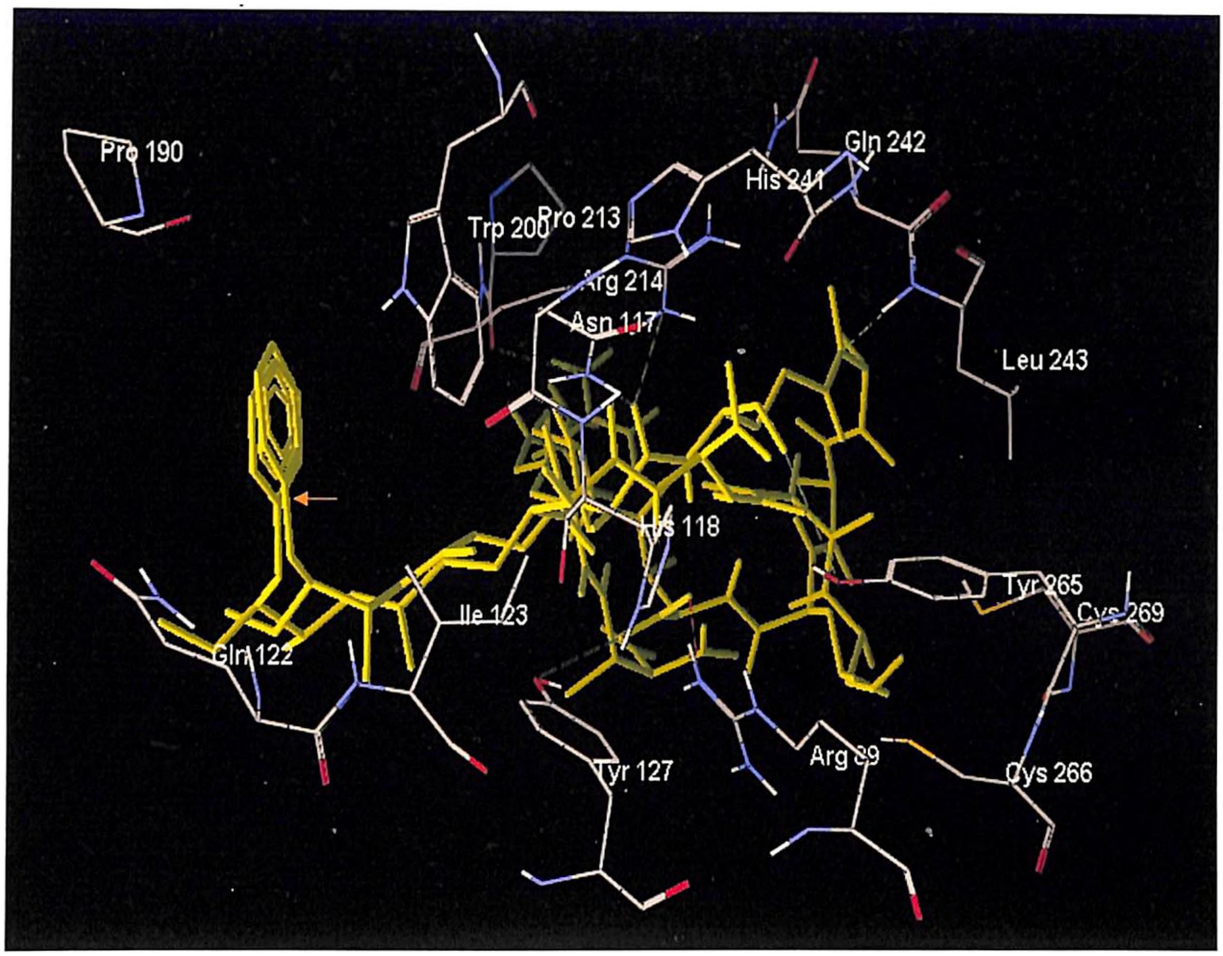


Fig. 67 Comparison of docked structure of dihydromicrocystin-LR (indicated by arrow) along with standard microcystin-LR in PP_2A receptor. The residues of PP_2A receptor which were involved in interaction with microcystin are represented as wire frame where as ligand (microcystin) are represented as stick (greenish yellow).

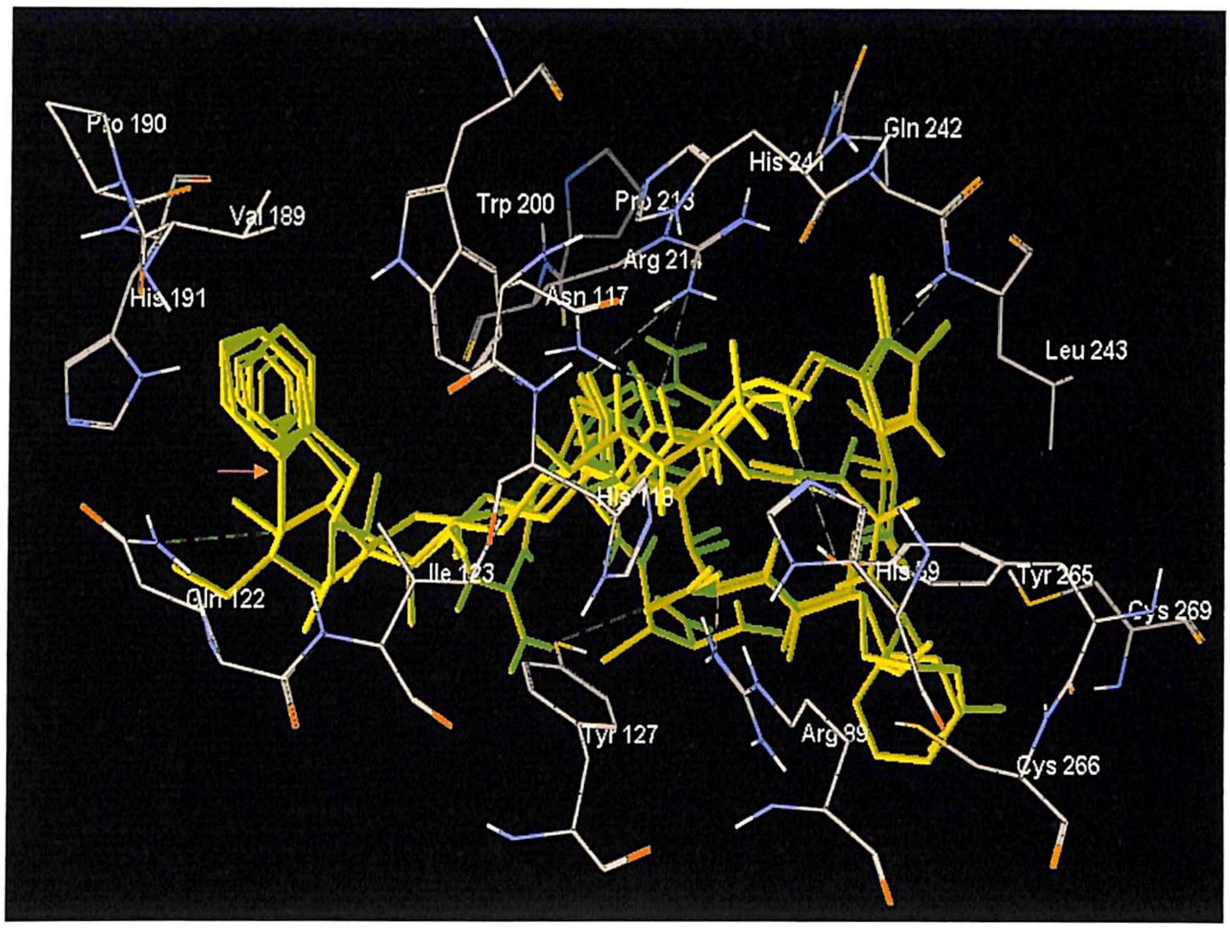


Fig. 68 Comparison of docked structure of microcystin-FR, microcystin-RR, along with standard microcystin-LR (indicated by arrow)in PP_2A receptor. The residues of PP_2A receptor which were involved in interaction with microcystin are represented as wire frame where as ligand (microcystin) are represented as stick.

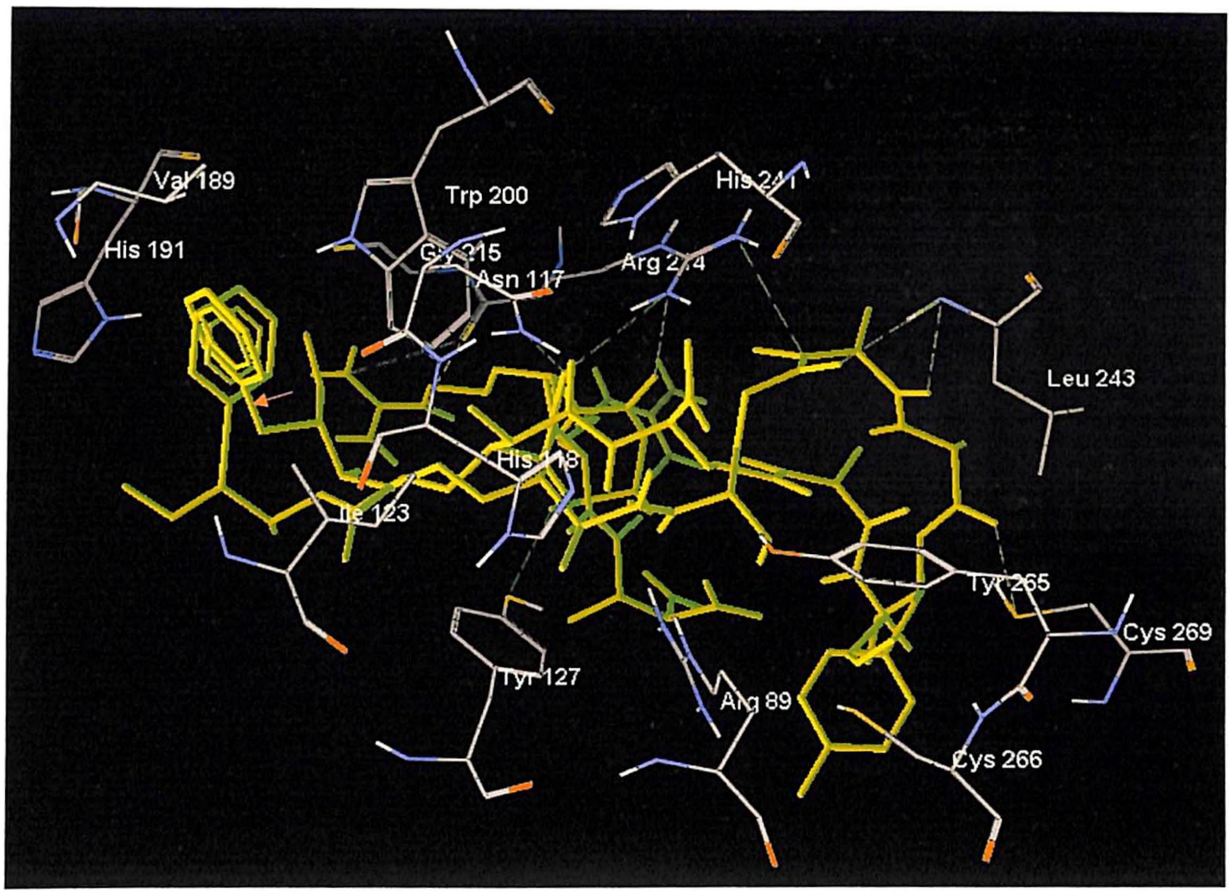


Fig. 69 Comparison of docked structure of microcystin-HtyR (indicated by arrow) along with standard microcystin-LR in PP_2A receptor. The residues of PP_2A receptor which were involved in interaction with microcystin are represented as wire frame where as ligand (microcystin) are represented as stick.

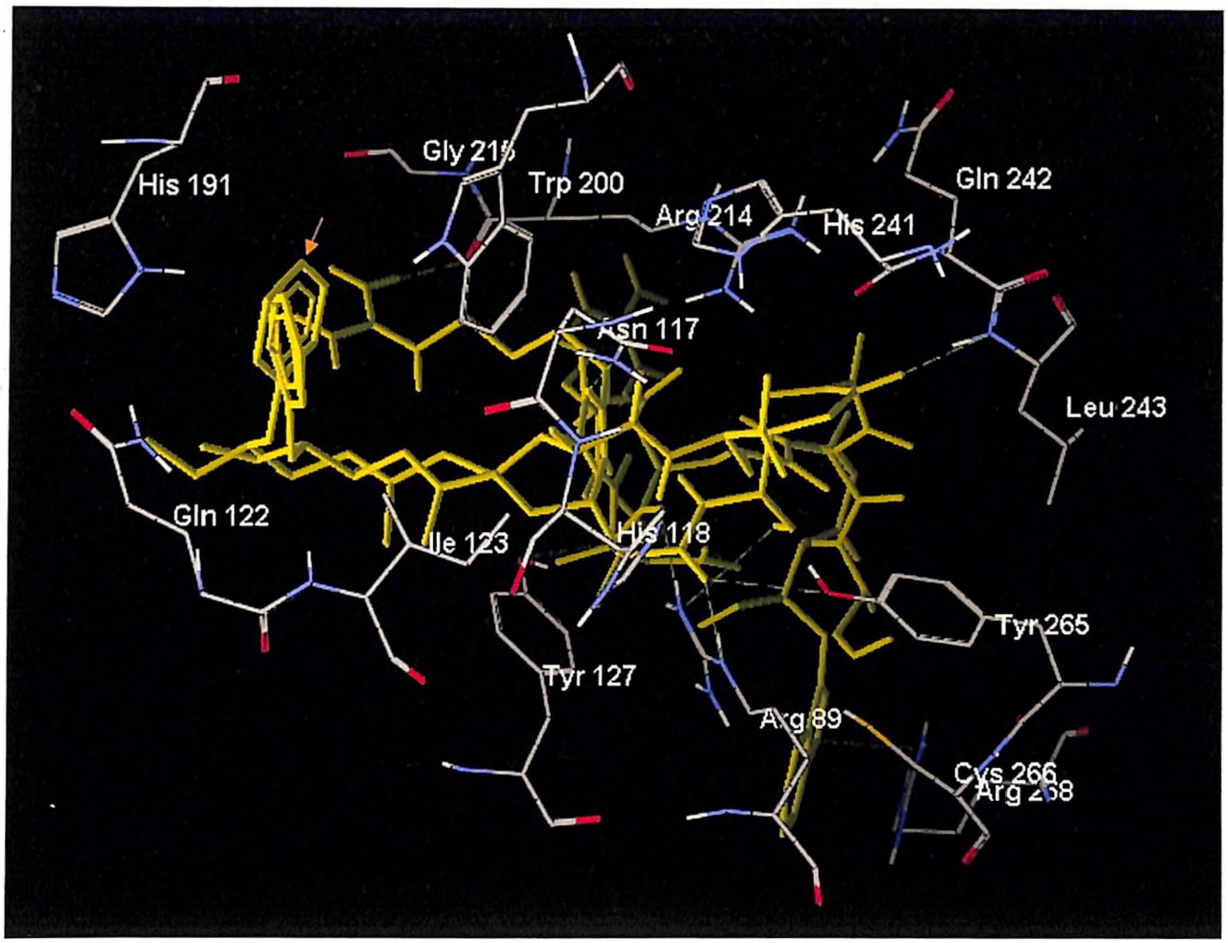


Fig. 70 Comparison of docked structure of microcystin-WR indicated by arrow along with standard microcystin-LR in PP_2A. The residues of PP_2A receptor which were involved in interaction with microcystin are represented as wire frame where as ligand (microcystin) are represented as stick.

Table 10 Docking analysis of isolated microcystin variants from *A.doliolum* and *N. spongiaeforme*

Sl.No	Name of microcystin Variants	Docking energy	Number of Hydrogen bond interaction	Residues of PP2A receptors involved in interaction	Number of non bond interaction
1	Standard Microcystin -LR	-173.944	12	His 59, Asp 85, Arg 89, Asn 117, His 118, Gln 122, Ile 123, Tyr 127, His 191, Trp 200, Pro 213, Arg 214, His 241, Tyr 265, Cys 266, Cys 269	3 (His 59, His 191, Cys 269)
2	Dihydromicrocystin-LR	-182.438	7	Arg 89, Asn 117, His 118, Gln 122, Ile 123, Tyr 127, **Pro 190**, Trp,200, Pro 213, Arg 214, His 241, **Gln 242**, **Leu 243**, Tyr 265, Cys 266, Cys 269	2 (Pro 190, Cys 269)
3	Microcystin-RR	-199.775	13	Arg 89, Asn 117, His 118, Ile 123, Tyr 127, **Val 189**, **Pro 190**, Trp 200, Arg 214, His 241, **Gln 242**, **Leu 243**, Tyr 265, Cys 266, **Arg 268**, Cys 269	3 (Pro 190, Val 189, Trp 200)
4	Microcystin-FR	-186.153	8	His 59, Arg 89, Asn 117, His 118, Gln 122, Ile 123, Tyr 127, **Val 189**, **Pro 190**, His 191, Trp 200, Pro 213, Arg 214, His 241, **Gln 242**, **Leu 243**, Tyr 265, Cys 266, Cys 269	4 (His 191, Val 189, Pro 190, Cys 269)
5	Microcystin-WR	-140.269	9	Arg 89, Asn 117, His 118, Gln 122, Ile 123, Tyr 127, His 191, Trp 200, Arg 214, Gly 215, His 241, **Gln 242**, **Leu 243**, Tyr 265, Cys 266, **Arg 268**	1 (His 191)
6	Microcystin-HtyR	-122.828	10	Arg 89, Asn 117, His 118, Ile 123, Tyr 127, **Val 189**, His 191, Trp 200, Arg 214, **Gly 215**, His 241, **Leu 243**, Tyr 265, Cys 266, Cys 269	4 (Arg 89, Ile 123, Val 189, His 191)

The bold letters indicates the difference in the residues of PP_2A involved in interaction with specific microcystin variants as compared to standard microcystin-LR.

Chapter IV

Discussion

A survey of four eutrophicated ponds (in 2006) situated in Shekhawati region of Western India, revealed profuge algal bloom during the period of February to April and September to November. This may be attributed to favorable temperature and light conditions present in this region (Batheja *et al.,* 2008, Zagajewski *et al.,* 2009). Toxic cyanobacterial blooms are frequently reported from water bodies in India, mainly comprising of *Microcystis sp.* (Agrawal *et al.,* 2006, Tyagi *et al.,* 2006, Rao *et al.,* 2006). However, in the present study the major component of algal bloom contains isolated strains of *Anabaena doliolum* and *Nostoc spongiaeforme* similar to the one described in the monograph Cyanophyta by Desikachari (1959). The influence of different culture media over chlorophyll *a* synthesis and total protein content of *A. doliolum* and *N. spongiaeforme* revealed that the BG_{11} is more suitable for the normal growth and culture maintenance. A similar type of results were reported with the strains of *Microcoleus lacustris, Scytonema schmidtii, Calothrix javanica, Tolypothrix fragilis, Westilliopsis prolifica and phormidium fragile* (Janardhan 2010). The choice of Chu10 over BG_{11} in all experiments is to increase the synthesis of toxic peptide with lesser chlorophyll *a*.

The acute toxicity of cyanobacterial cyclic peptides is attributed mainly to their hepatotoxic effect on mice models (Ito *et al.,* 1997). The characteristic symptoms of Microcystin (cyclic peptide) administration i.e. restlessness, fast breathing, slow movement and loss in co-ordination leading to death within 5.4 and 4 hours with crude extracts of *A. doliolum* and *N. spongiaeforme* suggest that the overall toxicity of these strains were similar to *Microcystis* as reported earlier (Falconer *et al.,* 1983, Runnegar *et al.,* 1991, Theiss *et al.,* 1988). The sub acute toxicity studies induces the formation of prostrate tumour with the administration of 25 µg/kg body of *N. spongiaeforme* along with the symptoms of restlessness, fast breathing, slow movement with both the extracts, indicates the presence of microcystin like peptides. The long term (28 days: 46

μg/kg body weight) exposure to the extracts from *N. spongiaeforme* leads to the formation of intestinal tumor, whereas the extracts from *A. doliolum* showed prominent colon tumor under similar conditions. The formation of tumours is similar to the hepatic tumours of microcystin-LR, YR and RR (Yoshizawa *et al.*, 1990). The tumour-promoting activity of microcystin is based on its ability to inhibit Ser/Thr residues of PP_1 and PP_2A which regulates several mitogen-activated protein kinases (MAPK) in cell cycle. Inhibition of protein phosphatase disturbs the balance between phosphorylation and dephosphorylation of proteins involved in cell cycle (Nishiwaki *et al.*, 1992). The specific dephosphorylation of Ser/Thr residues plays an essential role in control of the cell cycle, with more than 97% of protein phosphatase occurring at Ser/Thr residues which suggest that the toxic effects of microcystins results in activation of protein kinases. Both PP_1 and PP_2A covalently bind microcystin-LR at cysteine-273 for PP_1 and cysteine-266 for PP_2A. The inhibition of PP_1 and PP_2A leads to affect variety of molecular targets (Fig. 7), results in cellular phosphorylation imbalance and causes hyperphosphorylation of variety of cytoskeletal proteins (actin, talin, and a-actin) (Eriksson *et al.*, 1990 a, Falconer & Yeung 1992, Runnegar *et al.*, 1993) which plays a significant role in cell growth.

Previous report on the effect of microcystin-LR over hepatocytes revealed that the transport of toxin is due to preferential uptake of the toxin across the ileum and into the hepatocytes via bile acid carriers (Eriksson *et al.*, 1990 b). The formation of intestinal tumour by extracts of *N. spongiaeforme* can be correlated with the uptake of toxin across the ileum as the histology shows loss of basic structure with greater disorientation of villi along with edema formation. The liver histology of the animals treated with extracts/partially purified compounds (Ad spot 3 and Ns spot 3) of *A. doliolum* and *N. spongiaeforme* showed gross changes in normal architecture with engorged blood swollen liver showing a deep red colouration with degeneration and vaculolation

of hepatic parenchyma. The hepatoxicity of extracts resulted in increased lipid per-oxidation, an indicator of oxidative stress by increasing the serum ALT, AST, ALP, LDH and MDA level, in primary hepatocytes. The increase in lipid per-oxidation indicates generation of reactive oxygen species (ROS) which is determined by changes in H_2O_2 and O_2^- concentrations in hepatocytes. ROS are reported to react with many intracellular molecules, especially lipids with unsaturated fatty acids present in the membranes, proteins, carbohydrates and nucleic acids (Blokhina *et al.*, 2003, Timofeyev *et al.*, 2006, Lopez-Ongil *et al.*, 2000). The increase in ROS induces the production of arachidonic acid metabolites such as prostacyclin (6-Keto F1 alpha) and thrombaxane (TXB2) by stimulation of the cyclooxygenase pathway (Naseem *et al.*, 1990, Nobre *et al.*, 2001). The animals treated with crude extracts/partially purified compounds (Ad spot 3 and Ns spot 3) of *A. doliolum* and *N. spongiaeforme* shows alteration in hepatocytes leading to an aggregation of the microfilaments near the center of the cell, causing the loss of cellular support which lead to widening of intercellular junction and the hepatocytes becoming round. These results can be explained on the basis of destruction of the sinusoid endothelium as reported in the case of microcystin-LR on rat hepatocytes (Nishiwaki *et al.*, 1992). Thus it can be concluded that the observed structural disorientation of villi and degeneration of hepatocytes may be due the oxidative stress created by the family of microcystin variants.

The formation of tumor similar to crude extracts by Ad spot 3 and Ns spot 3, suggest that the active components are present in these fractions. A comparison of R_f values of these spots (Ad spot 3 and Ns spot 3) with standard Microcystin-LR revealed a close relationship between Ad spot 3, Ns spot 3 and Microcystin-LR. A further study involving UV spectra of Ad spot 3, Ns spot 3 showed a maximum absorption (λ_{max}) at 238 nm, which infers that the Ad spot 3 and Ns spot 3 has ADDA-like structure as ADDA of microcystin-LR provides the molecule with a

characteristic wavelength absorbance at 238 nm (Rinehart *et al.,* 1988). These results clearly suggest that the toxicity of Ad spot 3 and Ns spot 3 is similar to the one reported for Microcystin family (Falconer *et al.,* 1983, Runnegar *et al.,* 1991, Theiss *et al.,* 1988).

The components of TLC purified spots were also identified on the basis of protonated molecular ion $(M+H)^+$ in MALDI TOF MS analysis as Dihydromicrocystin-LR (m/z=996.821), [D-Asp3] Microcystin-WR (m/z=1051.954), Microcystin-LR (m/z=995.472), [D-Asp3] Microcystin-HtyR (m/z=1043.756), Microcystin-FR (1029.521 m/z) and Microcystin-RR (1037.970 m/z). These compounds corresponds with the earlier compounds isolated from *M. aeruginosa, A.flos-aquae, M.viridis, Nostac sp.* strain 152 respectively (Agrawal *et al.,* 2006, Gupta *et al.,* 2003, Lee & Chou 2000, Sivonen 1990, Sivonen *et al.,* 1990, Krishnamurthy *et al.,* 1986). Apart from the family of microcystin, Ad spot 3 and Ns spot 3 also revealed the presence of other linear peptides, presumably Oscillarin (m/z=617.157), Aeruginosins (m/z=656.372, 656.151, 672.133, 672.646, 691.1932, 705.624, 765.289), Oscillamide (m/z=882.752), Anabaenopetin K (m/z=939.796), Oscillamide (m/z= 861,237, 880.491) similar to the one reported in *M. aeruginosa* (Agrawal *et al.,* 2006, Czarnecki *et al.,* 2006, Fujii *et al.,* 2000).

The inference of spectral analysis of TLC spots and MALDI TOF MS analysis of Ad spot 3 and Ns spot 3 were supported by the microcystin variants obtained by HPLC and FTIR analysis. HPLC purification of Ad spot 3 gave three variants of microcystins (dihydromicrocystin-LR, [D-Asp3]microcystin-WR and microcystin-LR) with varying R_t values whereas Ns spot 3 spots obtained from the strain grown in two different media (Chu10 and Allen and Arnon) gave four variants (microcystin-LR, [D-Asp3]microcystin–HtyR and microcystin-FR, microcystin-RR respectively). FTIR analysis showed characteristic peak for microcystins in all isolated variants

by comparing with standard microcystin-LR. The presences of different variants of microcystin were reflected from their molecular mass and their respective R_t values.

The synthesis of different variants of microcystins by *A. doliolum* and *N. spongiaeforme* is found to be dependent on various factors like phosphate and nitrate as well as pH of the media and the physical factors such as incubation temperature and light intensity. Previous reports with non-nitrogen fixing *Oscillatoria agrdhii*, *M. aeruginosa* showed marginal decrease in total microcystin content with decrease in concentration of phosphate (Sivonen 1990, Sivonen *et al.*, 1990) but removal of phosphate did not affects the total synthesis of microcystin in *M. aeruginosa* (Codd & Poon 1988). The results with *Anabaena sp.* strain 90 and 202A1 showed increase in intercellular microcystin concentration with increase in phosphate concentration in the medium (Rapala *et al.*, 1997). The present study showed decrease in total protein synthesis in *A. doliolum* and *N. spongiaeforme* with decreasing concentration of phosphate leading to slower growth. The decrease in growth under phosphate starvation does not influence the microcystin content present total protein fraction of the cell and its toxicity. The decreasing concentration of phosphate increases the synthesis of less toxic variants in both the strains ([D-Asp^3]microcystin-WR and dihydromicrocystin-LR in *A. doliolum* and [D-Asp^3]microcystin-HtyR or microcystin FR in *N. spongiaeforme*) whereas the synthesis of highly toxic variants (microcystin-LR in *A. doliolum* and microcystin-LR or microcystin-RR in *N. spongiaeforme*) increased with increasing concentration of phosphate.

The role of nitrate is reported to follow a different pattern of microcystin synthesis in *M. aeruginosa* and *Oscillatoria sp.* showing increased level of microcystins at higher concentration of nitrate in the medium (Watanabe & Oishi 1985, Sivonen 1996) whereas Rapala *et al.*, (1997) reported an opposite effect in nitrogen fixing *Anabaena sp.* strain 90 and 202A1. Present study

showed that the nitrate do not have any effects on *N. spongiaeforme*. The lack of nitrate in the medium is overcome by the nitrogen fixation as indicated by the development of heterocyst in the filaments. The increasing concentration of nitrate did not show major difference in total microcystin synthesis in *A. doliolum* and *N. spongiaeforme* whereas the concentration of individual variants varies with different concentrations of nitrate. The synthesis of less toxic variants such as [D-Asp^3]microcystin-WR and dihydromicrocystin-LR from *A. doliolum* and microcystin-FR from *N. spongiaeforme* decreases with increasing concentration of nitrate. An opposite effect is observed with microcystin-LR of *A. doliolum* and microcystin-LR or microcystin-RR of *N. spongiaeforme.*

The present study showed no significant change in toxicity and total microcystin content with increase in light intensity, with more concentration of less toxic variants such as [D-Asp^3]microcystin-WR and dihydromicrocystin-LR in *A. doliolum* and [D-Asp^3]microcystin-HtyR or microcystin-FR in *N. spongiaeforme* at low intensity of light. The earlier studies showed high microcystin synthesis in low intensity of light at 25°C in *M. viridis* (Song *et al.,* 1998). Studies on *M. aeruginosa* have been reported to decrease the microcystin synthesis at low intensity of light (Utkilen & Gjolme 1992, Watanabe & Oishi 1985).

These results are in accordance with the earlier report that microcystin synthesis in *M. aeruginosa* and *Oscillatoria agardhii* prefer 25°C temperatures and the synthesis was decreased with the change in temperature (Van der Westhuizen & Eloff 1985, Van der Westhuizen *et al.,* 1986, Sivonen 1990). In the present study, the temperature of growth media was studied as an environmental factor for the synthesis of microcystins. Both the strains tend to synthesize more toxic variants (microcystin-LR and microcystin-RR) at lower temperature (15°C) and gradually shifted to demethylated derivatives or less toxic forms at 25°C. A higher temperature of 30°C and

above not only affects the synthesis of microcystin but also affects the normal growth and biomass. Synthesis of toxic variants at low temperature opens the avenue to explore their role in intra-specific signaling molecules in stress conditions (Dittmann *et al.*, 2001).

The exact role of toxins in cyanobacteria is still not clearly understood. Although the toxins are localized in all regions in the interior of cyanobacteria cells, it was suggested that most of cell's toxin complement is localized in the thylakoids, followed by the nucleoplasm and pyrophosphate bodies (Young *et al.*, 2005, Shi *et al.*, 1995). The association of Microcystin to the thylakoids might occur through the hydrophobic ADDA moiety inserted in the membrane and the polar cyclic structure interacting with cytoplasm. This preferential localization suggests that the molecule may play an important role in light harvesting and adaptation responses (Shi *et al.*, 1995, Pearson *et al.*, 2004). Microcystins have also been suggested to act as protective compounds against grazing by zooplankton (Carmichael 1992) or as intracellular chelators inactivating free cellular Fe^{2+} (Utkilen & Gjolme 1992) in addition to some specific cell regulatory function (Shi *et al.*, 1995). It was shown that protein phosphatase activity in a marine dinoflagellate *Prorocentrum lima*, appears to be exquisitely regulated by Okadaic acid, a well-known inhibitor of protein phosphatases produced by the organism (Boland *et al.*, 1993). Synthesis of different variants of microcystin by the strains isolated from the Shekhawati region of India (semi-desert region) where the temperature reaches to 25 to 30°C during the period of February-April and September-November with intense sunlight, may be attributed to the harsh environmental conditions as described for *M. aeruginosa* (Orr & Jones 1998, Rapala *et al.*, 1997). The effect of the toxins depends on the relative proportions of different variants and derivatives of microcystins. The presence of hepatotoxicity, without tumor formation by the compound purified from Ad spot 3 or Ns spot 3 alone could be attributed to the presence of large

amount of dihydromicrocystin-LR, [D-Asp3]microcystin-WR, [D-Asp3]microcystin-HtyR as compared to microcystin-LR. It has been reported that the demethylated derivatives are less toxic as compared to microcystin-LR (Gupta *et al.*, 2003). The formation of prominent colon tumor by co-administering dihydromicrocystin-LR and [D-Asp3]microcystin-WR (Purified from Ad spot - 3) indicating their synergetic effects. A similar synergetic effect was also observed with the case of microcystin-LR and [D-Asp3]microcystin-HtyR (Purified from Ns spot-3) for the formation of intestinal tumor. Individually, microcystin-LR has shown higher hepatotoxicity with hepatic tumor formation. The formation of tumors in different body parts of test animal may be attributed to different functional groups and varying long chains fatty acids present in different microcystin variants as compared to microcystin-LR. The binding of the microcystin-LR to the surface pocket of PP_2A is located above two manganese atoms and the active site of the enzyme. The binding is strengthened by hydrophobic interactions between the ADDA side chain of microcystin-LR and the residues Gln122, Ile123, His191, and Trp200 of the binding pocket, by van der Waals interactions with the hydrophobic portion of the toxin and the other residues Leu243, Tyr265, Cys266, Arg268, and Cys269 by a covalent linkage between the Sγ atom of Cys269 and the terminal carbon atom of the Mdha side chain (Xing *et al.*, 2006). The present *in-vivo* toxicity study with different variants of microcystin showed lower toxicity and lesser accumulation of microcystin-FR as compared to microcystin-LR in hepatocytes. The above results could be attributed to the presence of bulky phenylalanine in microcystin-FR which not only forms a nonbonding interaction with Cys-269 but also makes the benzene ring of ADDA moiety to rotate in anticlockwise direction to make nonbonding interaction with Valine 189, Proline 190 and His 191 of PP_2A receptor. This change in orientation might make the compound microcystin-FR less toxic as compared to the other two methylated variants. Among different

variants, the microcystin LR showed maximum toxicity followed by methylated and demethylated forms. Thus, the difference in the toxicity of microcystin variants ~~were~~ might be due to difference in binding orientation and binding energy with PP_2A receptors. The docking studies infer the orientation of benzene ring in ADDA position towards anticlockwise direction or inclination of benzene ring or bending of benzene ring as compared to microcystin-LR might decrease the toxicity. The lower docking energy of demethylated derivative might be correlated to lesser time interaction with PP_2A leading to lower level of *in-vivo* toxicity as compared to methylated derivatives. The hypothesis is also supported by previous report that demethylated derivatives are less toxic than methylated variants (Gupta et al 2003, Jaiswal *et al.*, 2006). Studies on 293-hSERT cell lines showed that microcystin-LR inhibits protein phosphatase 2A (PP_2A) activity (Torres 2006, Carneiro *et al.*, 2002, Ramamoorthy *et al.*, 1998). Since the role of PP_2A is well established in regulating the biogenic amine, which is indirectly linked to depression (Carmichael 1992, Harada *et al.*, 1990) it prompted us to investigate the role of microcystins in reducing the depression effect created by battery of established depression test models. The choice of microcystin-FR (methylated variants) for antidepressive activity over the other demethylated and methylated variants was due to the better interaction with PP_2A receptor which is reflected in the higher docking energy as compare to demethylated variants (microcystin-HtyR, and microcystin-WR) and lesser accumulation in liver as compared to other methylated variants (microcystin-LR, dihydromicrocystin-LR, microcystin-RR).

The psychological status of normal animals when subjected to a non-soluble aversive situation does alter between agitation and immobility (Pandey *et al.*, 2008, Ramamoorthy *et al.*, 2008). The antidepressive activity of microcystin-FR (Mcy-FR), in its pure form was evaluated in a battery of behavioural antidepressant assay in Swiss albino mice models. The forced swimming

test (FST) and the tail suspension test (TST) used in the present study to create temporary depression, are simple and sensitive behavioral models for screening of antidepression compounds based on the immobility response to inescapable aversive stimulation as reported earlier in understanding the role of specific monoamines and its receptors (Pandey *et al.*, 2008, Petit-Demouliere *et al.*, 2005, Cryan *et al.*, 2005, Thierry *et al.*, 1986). The reduction in FST and TST immobility showed definite role of microcystin-FR as antidepressive agent. A variant of microcystin (microcystin-LR) has been reported to inhibit PP_2A activity in 293-hSERT cell lines by inhibiting the expression of serotonin transporter (SERT), dopamine transporter (DAT) and norepinephrine transporter (NET) receptors on the cell surface which leads to the accumulation of serotonin (5-HT), dopamine (DA) and nonepinephrine (NE) in neuronal cleft (Torres 2006, Carneiro *et al.*, 2002, Ramamoorthy *et al.*, 1998, Bauman *et al.*, 2000). In order to study the antidepressive activity of microcystin-FR, it was necessary to use a potent anti-hepatotoxic agent, as microcystin-FR is known to cause severe hepatotoxicity in Swiss albino mice (Torres 2006). Studies on effective chemoprotectant over hepatotoxicity of microcystins have shown 100% protection in mice by Rifampin and Cyclosporin A (Rao *et al.*, 2004). Out of these, Cyclosporin A was chosen in the present study for its effectiveness in reducing microcystin uptake by hepatocytes. As cyclosporin-A has more affinity toward organic anion transporting polypeptides (Oatps), it competes with microcystin for its transports. Due to the high molecular weight and complexity of structure, microcystins cannot readily diffuse through plasma membrane. Ericksson et al. (1990 a & b) studied the cellular up-take of a radio labelled derivative of microcystin-LR in isolated rat hepatocytes, human hepatocarcinoma cell line $HepG_2$ and the mouse fibroblast cell line NIH-$3T_3$. The up-take study indicates that the toxin requires bile acid transporters. Many transporters are responsible for the uptake of endogenous

and exogenous chemicals into liver, including the organic anion transporting polypeptides (Oatps), Nat-taurocholatecotransporting polypeptide (Ntcp), organic anion transporters (Oats), and organic cation transporters. Among these transporters, the Oatps is responsible for the majority of uptake of microcystin (Trauner & Boyer 2003, Klaassen & Lu 2008).

The observed antidepressive activity of microcystin-FR in combination with Cyclosporin A in Swiss albino mice can also be correlated with an increased level in neurotransmitters and biogenic amines. The increase in the biogenic amine by microcystin-FR was also reflected in reducing the agitation or anxiety (introduced into the animals during non-soluble aversive situation) in Elevated plus maze test by increasing the time duration on open arm. The lack of significant difference between test animals and negative control in spontaneous locomotor activity test reveals no interference in locomotive activity of animals by microcystin-FR in Swiss albino mice. The lack of analgesic activity of 0.8 µg/kg Mcy-FR were clearly demonstrated with acetic acid induced writhing test showing lack of significant difference with negative controls. A comparison of antidepression like activity of 0.8 µg/kg Mcy-FR and standard antidepressants (Bupropion & Desvenlafaxine) showed that Mcy-FR combined with CsA combination is as effective as standard drugs.

The current study shows the presence of toxic cyanobacterial strains in the water blooms in the semi-desert region of Rajasthan, India. The extent of toxicity varied significantly among the invasive cyanobacterial species *Anabaena doliolum* and *Nostoc spongiaeforme.* While synergistic effects of the peptides dihydroMicrocystin-LR and [D-Asp3] microcystin-WR isolated from *A. doliolum* caused colon tumor in Swiss albino mice, the peptide microcystin-LR, [D-Asp3] microcystin-HtyR isolated from *N. spongiaeforme* causes intestinal tumor. Thus, the presences of toxic cyanobacteria in these water bodies pose a grave risk to the health of both

human beings and the cattle. The toxicity of these strains is influenced by the synthesis of various variants of microcystins and their coexistences. The synthesis of these microcystin variants are influenced mainly by the temperature prevailing in semi-desert region of Rajasthan, India. Other nutrimental factors such as nitrate and phosphate concentration have marginal effect. Light don't seem to have much role in biosynthesis of these toxic peptides. The hepatotoxicity of microcystin-FR has been effectively reduced by CsA. The protective action of CsA prompted us to investigate the microcystins for pharmaceutical application. The preliminary investigation demonstrates the antidepression and anxiolytic like effects of microcystin-FR in Swiss albino mice using a battery of behavioural models for depression. The antidepression and anxiolytic like property of Microcystin-FR can be correlated with the inhibitory action of PP_2A on the expression of transporter receptors, thereby increasing the amount of biogenic amine in synaptic cleft.

Chapter V

Summary

The current study investigates the *in-vivo* toxicological effects of microcystin variants present in two cyanobacteria strains (*Anabaena doliolum* and *Nostoc spongiaeforme*) isolated from the water bodies of Shekhawati region, Rajasthan. The study also reveals the antidepression and anxiolytic like effects of microcystin-FR in Swiss albino mice using a battery of behavioural model for depression. The major results finding are as follows.

1. The strains isolated from the site-1 and site-2 of Pilani were identified as filamentous, heterocyst forming *Anabaena doliolum* and *Nostoc spongiaeforme* respectively.
2. An acute exposure of crude methanolic extracts (100 µg protein/kg via i.p) of *A. doliolum* and *N. spongiaeforme* showed characteristic symptoms of microcystin toxicity i.e. restlessness, fast breathing, slow movement and loss in co-ordination leading to death within 5.4 and 4 hours respectively.
3. Chronic exposures (28 days: 46 µg/kg body weight) of crude extracts generate colon and intestinal tumour with *A. doliolum* and *N. spongiaeforme* respectively. The hepatotoxic natures of extracts were indicated by increases in the serum ALT, AST, ALP, LDH and MDA level.
4. TLC purified methylene chloride extracts from *A. doliolum and N. spongiaeforme* showed four spots each. The chronic toxicity studies with sub lethal dose of 20 µg/kg body weight via i.p, showed colon tumors with Ad spot-3 from *A. doliolum* and intestinal tumors with Ns spot-3 from *N. spongiaeforme*. They are found to be hepatotoxic and their toxicity decreases the bodyweight of Swiss albino mice.
5. A comparison of R_f, R_t values and UV spectral analysis of Ad spot3 and Ns spot 3 confirmed the presence of microcystins.

6. MALDI TOF MS and FTIR analysis infer the presence of microcystin variants such as dihydromicrocystin–LR (m/z=996.8219), [D-Asp3]-microcystin-WR (m/z=1051.954), microcystin-LR (m/z=995.472) & 995.638) in Ad spot 3 of *A. doliolum* and [D-Asp3]microcystin-HtyR (m/z=1043.756), microcystin-FR (m/z=1029.521), microcystin-RR (1037.970), microcystin-LR (m/z= 995.638) in Ns spot 3 of *N. spongiaeforme.*
7. HPLC purified compounds when administered individually did not show colon or intestinal tumour formation. The prominent colon tumors were observed when two compounds of Ad spot 3 (dihydromicrocystin-LR and [D-Asp^3]microcystin-WR) were co-administered with a dose of 0.35 µg/kg each in Swiss albino mice. Similar type of results were observed in the induction of prominent intestinal tumors when two compounds of Ns spot 3 ([D-Asp^3]microcystin-HtyR and microcystin-LR) were co-administered with a dose of 0.5 µg/kg each.
8. No significant change in total microcystin concentration was observed with respect to change in nutrimental factors such as phosphate, nitrate, temperature and light. However synthesis of different variants of microcystin was found to depend on the concentration of phosphate, nitrate, and temperature.
9. Decrease in phosphate concentration (0.5mM to 0mM) increases the synthesis of less toxic microcystin peptides. A similar effect is observed with increasing the concentration of nitrate (0mM to 0.5mM).
10. Lower temperature (15°C) significantly increased the synthesis of more toxic variants in both *A. doliolum* and *N. spongiaeforme.* A temperature range of 22-26°C was found to be suitable for the synthesis of different variants of microcystin in *A. doliolum* and *N. spongiaeforme.*

11. Microcystin-FR showed lesser accumulation in the liver as it showed only 57% increase in liver weight as compared to the control. The other variants showed almost 100% increase in liver weight suggesting the lower accumulation of microcystin-FR.
12. Docking studies indicate that the bending orientation, especially fatty acid side chain of microcystin variants with PP_2A receptor determine the severity of toxicity.
13. Lower toxicity of microcystin-FR can be attributed to the presence of bulky phenylalanine which not only forms a nonbonding interaction with Cys-269 but also makes the benzene ring of ADDA moiety to rotate in anticlockwise direction to make nonbonding interaction with Valine 189, Proline 190 and His 191 of PP_2A receptor.
14. Toxicity of demethylated derivatives was found to be lower than the methylated variants due to lesser interaction and different orientation with PP_2A receptor.
15. 40% reduction in Force swim test (FST) and Tail suspension test immobility showed definite role of microcystin-FR (0.8 µg/kg body weight) as antidepressive agent. The observed antidepressive activity of microcystin-FR in Swiss albino mice could be attributed to increased level of neurotransmitters and biogenic amines.
16. The increase in the biogenic amine by microcystin-FR was also reflected in reduced agitation or anxiety in Elevated plus maze test by increasing the time duration on open arm to 89%.
17. Cyclosporin-A provides 100% protection over the hepatotoxicity of microcystin-FR.
18. Spontaneous locomotive activity test reveals that the antidepression like effects of microcystin-FR does not interfered with the normal locomotory activity of animals.
19. The lack of analgesic activity of Mcy-FR was demonstrated by acetic acid induced writhing test which showed lack of significant difference with negative controls.

Future Scope of the Work

- The tumour promoting genes that are responsible for the induction of colon and intestinal tumours can be studied upon microcystins exposure, in order to use these compounds to develop an effective *in-vivo* animal model for screening of anticancer or antitumor drugs.
- The genes regulating the synthesis of different variants can be studied in order to understand the genetic basis of synthesis of these cyclic peptides in different strains of cyanobacteria.
- Estimation of biogenic amine at neuronal synaptic level will help us to understand the pharmacological role of microcystin variants over the availability of specific role of specific monoamines.

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Appendix I

List of Publications

1. **Ashwin KR**, Ramani K and Verma SK (2011) Induction of colon tumor and intestinal tumor in *Swiss Albino mice* by the dihydromicrocystin-LR, [D-Asp3]microcystin-WR and [D-Asp3]microcystin-HtyR produced by the cyanobacterial strains isolated from water bodies in semi-desert region of India. South Asian Journal of Experimental Biology 1: 188-197.
2. **Ashwin KR**, Ramani K and Verma SK (2008) Tumorigenic property of filamentous cyanobacteria isolated from stagnant of Pilani. Indian Journal of Pharmacology 40: S167.
3. **Ashwin KR** and Verma SK (2011) Factors regulating the synthesis of different variants of microcystin in cyanobacteria from semi desert region of India. Natural Product Research (Communicated).
4. **Ashwin KR**, Sushil Y and Verma SK (2011) Antidepressive-like effect of microcystin-FR in *swiss albino mice* tested by a battery of behavioural depression models. International Journal of Pharmacy and Pharmaceutical Sciences (Accepted).

Books / Chapters Contributed

1. **Ashwin KR** and Verma SK (2009) Secondary metabolite production of cyanobacteria. Khattar JIS, Singh DP and Kaur G (Eds), Algal Biology and Biotechnology, I. K. International Publishing House Pvt. Ltd. India. pp. 205-215.
2. **Ashwin KR** and Verma SK. (2011) Cyanotoxins: Pharmacological and ecological effects. Dhingra HK, Jha PN and Bajpal P (Eds), Current Topics in Biotechnology and Microbiology, Lambert Academic Publishing Gmbh & Co. KG. Germany. pp. 311-329.

Awards/ Fellowships

1. **Received best poster award** in International Symposium on Applied Phycology and Environmental Biotechnology, BITS, Pilani. (October 29-31, 2007) on "Partially purified antibacterial compound from cyanobacteria isolated from local polluted water body of Pilani".
2. Recipient of **Senior Research Fellowship** (SRF) awarded by the Council of Scientific & Industrial Research (CSIR), India during the period of doctoral research (April, 2007- till date).

Conference Presentations

1. **Ashwin KR**, Senthil N and Verma SK (2010) Tumorigenic property of *Anabaena doliolum and Nostoc spongiaeforme* from Shekawati region of Rajasthan. In: International Symposium on Phycological Research (February 25-27), Centre of Advances Study in Botany, Banaras Hindu University, Varanasi, India. pp. 135.
2. **Ashwin KR** and Verma SK (2009) Tumorogenic property of *Anabaena doliolum* isolated from Pilani. In: National Symposium on Phycology in India: Basics to Applied (February 12-13), Department of Botany, Punjabi University, Patiala, India. pp. 92.
3. **Ashwin KR** and Verma SK (2008) Tumorigenic property of cyanobacteria isolated from polluted water bodies of Pilani. International Conference on the Interface of Chemistry-Biology in Biomedical Research (February 22-24), Department of Chemistry, BITS-Pilani, India. pp. 295.
4. **Ashwin KR** and Verma SK (2007) Partially purified antibacterial compound from cyanobacteria isolated from local polluted water body of Pilani. International Symposium on Applied Phycology and Environmental Biotechnology, Department of Biological Sciences, BITS-Pilani (October 29-31), BITS-Pilani, India. pp. 66.

Appendix II

Biography of Prof. S. K. Verma

Prof. Sanjay Kumar Verma has obtained master's degree in Genetics from Banaras Hindu University (BHU), Varanasi with specialization in Cyanobacteria and Applied Phycology before completing Ph.D in the area of Environmental Biotechnology from the same university. He worked as Post-Doctoral Research Fellow at University of Hyderabad in the area of Microbial and Molecular Genetics. Prof. Verma has handled several research projects related to bioremediation and biodegradation of toxic industrial waste, development of recombinant bacterial biosensors for environmental applications, funded by Bhabha Atomic Research Centre (BARC), Council of Scientific and Industrial Research (CSIR), University Grants Commission (UGC) and Department of Science & Technology (DST). Currently he is working as a Professor, Department of Biological Sciences, Birla Institute of Technology and Science, Pilani. His major research interest lies in biochemistry of cyanobacteria, Environmental Biotechnology and Molecular Biology.

Biography of Mr. R. Ashwin Kumar

Mr. Ashwin Kumar completed M.E. in Biotechnology from Birla Institute of Technology and Science, Pilani. He continued his Ph. D programme in the same institute from 2005 to 2012. He was a recipient of Senior Research Fellowship from Council of Scientific and Industrial Research (CSIR). Ashwin Kumar has an active interest in Pharmaceutical Biotechnology and Molecular Toxicology. He has also been involved in teaching various courses in Department of Biological Sciences, BITS-Pilani. He has published research articles in renowned international and national journals and presented papers in various national and international conferences.