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Research Article

ANTIDEPRESSIVE-LIKE EFFECT OF MICROCYSTIN-FR IN SWISS ALBINO MICE TESTED BY A BATTERY OF BEHAVIOURAL DEPRESSION MODELS

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ABSTRACT

Microcystin-FR a cyclic heptapeptide was isolated from *Nostoc spongiaeforme* and purified by thin layer chromatography, MALDI TOFF Ms and HPLC methods. The Swiss albino 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). A 40% decrease in immobility in both TST and FST was seen 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 seem to interfere with the normal locomotory activity of mice. The histopathology, serum aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) assays confirm the hepatotoxic nature of microcystin-FR. A dose of 8mg/kg body weight of Cyclosporine-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.

Keywords: Microcystin-FR, Cyanotoxins, *Nostoc spongiaeforme*, Tail suspension test, Force swim test, Depression models

INTRODUCTION

The words of eminent physiologist Claude Bernard "Poisons can be employed as a means for destruction of life or as agents for the treatment of the sick" seems to be appropriate for the usage of Microcystins-FR (Hepatotoxin) for screening antidepressive activity. Microcystins belong to the family of cyclic hepta-peptides having a molecular weight of 824 to 1,044 daltons and are most widely distributed among the toxic cyanobacteria[1-3]. 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, andMdha is N-methyl-dehydroalanine[2]. The Adda moiety is responsible for toxicity by binding to the protein phosphatases[4, 5]. 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[6, 7]. Variants of microcystins have been isolated from *Microcystis, Anabaena*, *Nostoc, Planktothrix* with greater variation in different amino acids with methylated and nonmethylated forms[8]. Among different variants, the microcystin LR shows maximum toxicity followed by nonmethylated and methylated forms[6]. The microcystin LR is reported to cause restlessness, fast breathing, slow movement and loss of co-ordination leading to death[8]. Our previous studies on chronic and acute toxicity of DihydroMicrocystin–LR, [D-Asp3] Microcystin-WR and [D-Asp3] Microcystin-HtyR have shown severe blood pooling and disorganization of hepatocytes[9]. Studies on 293-hSERT cell lines showed that microcystin-LR inhibits protein phosphatase 2A (PP_2A) activity[10-12]. Since the role of PP_2A is well established in regulating the biogenic amine, which is indirectly linked to depression[4, 5], it prompted us to investigate the role of microcystins in reducing the depression effect created by battery of established depression test models. In the present study, we report that one of the two variants of microcystins i.e. microcystin-FR present in *Nostoc spongiaeforme* acts as antidepressant in Swiss albino mice model.

MATERIALS AND METHODS

Thin layer chromatography separation

Microcystin variants were purified from *Nostoc spongiaeforme* isolated from Shekawati region of Rajasthan. The culture was grown in Allen Arnol medium without fixed nitrate at 25±1^{0}C under continuous illumination of 400lux. The 18 days old culture was harvested to extract microcystins variants. 8.0gms of dried cells were treated with methanol (50 ml) thrice for 60 mins with continuous mixing in magnetic stirrer at 4^{o}C. The supernatant of extracts were collected and concentrated by rotator apparatus (Buchi R-210). The concentrated extracts were partially purified and visualized by thin layer chromatography (TLC)

MALDI TOF analysis

The partially purified microcystins (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.

HPLC purification

TLC purified microcystins were dissolved in Phosphate buffer (pH 6.2) and further purified in high performance liquid chromatography (HPLC), using reverse phase (RP) C-18 column (Alltima 250mm x 4.6mm, 5µm) as stationary phase. 0.01 M ammonium acetate: acetonitrile (64:36, v/v) mixture was used as mobile phase with the flow rate of 1ml/min. All purified microcystin variants were collected with the help of UV detector at the wavelength of 238 nm. The microcystin-LR 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^{0}C for further studies.

Animals

All animal studies were carried out in accordance with approved protocol and guidelines of Institutional Animal Ethics Committee

(IAEC/RES/13/09). Swiss albino male mice of age group 2 to 3 weeks with the weight of 25-35gms were procured from Chaudhary Charan Singh Agricultural University, Hissar, India. They were housed in standard animal house condition with commercial standard mash feed and water *ad libitum*. Experimental sessions began after 3 weeks of quarantine period. The animals were used only once for each experiments and strictly acclimatized to the experimental room for one hour before testing.

Drugs

Propylene glycol (PG) was purchased from Merck (India). Amphetamine was purchased from Sigma alrich (India). Cyclosporine-A (CsA) was obtained as a gift from Sun Pharma Limited (India); Desvenlafaxine was from IPCA laboratories (India) and Bupropion was from Ranbaxy research laboratories (India).

Methodology

The animals were randomized into control and experimental groups with each group containing six animals (n=6). All microcystin samples were dissolved in Phosphate buffered saline (PBS) (pH 7.4) and their sub-lethal concentration as established earlier[9] were used in subsequent experiments. Cyclosporine-A was dissolved in propylene glycol and administered 30 minutes before injecting microcystin –FR in test animals. The dosage of cyclosporine-A (8mg/kg body weight) and the positive controls such as Bupropion (10mg/kg body weight) and Desvenlafaxine (4mg/kg body weight) were chosen from the previous reports[14, 15]. All samples and controls were administered intraperitonally (i.p) only once in a day for 28 days.

Hepatotoxicity

The serum of the animals were collected on 28th day after 8 hours of the sample administration and hepatotoxicity of the test drug were analyzed by serum Aspartate aminotransferase (AST), and Lactate dehydrogenase (LDH) assays by commercially available(Spinreact kits).

Histopathology

The liver of all animals were collected on 28th day and perfused with normal saline. They were fixed with buffered formalin. The fixed tissue were dehydrated with the ascending concentration of ethyl alcohol and cleared with xylene. Section of 2-3mm thick was taken from the lobes with maximum diameters and processed for embedding in paraffin. Section of 4-5µm were cut and strained with Ehrlich haematoxylin/eosin.

Antidepression Activity

Tail suspension test

Behavioural despair was induced by tail suspension according to the procedure of Pandey *et al*[16] with slight modification. The study was conducted only after 30 minutes of drug administration. The animals were suspended individually using adhesive tapes from a horizontal rod 50cm above the flat surface of the tabletop. The point of attachment on the tail was 2cm from the tip. 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 the number of second spent immobile.

Force swim test

The animals were forced to swim individually in a glass jar (25x12x25cm) containing water column of 15cm at a temperature of 24±3^{o}C for a period of six minutes as described by Porsolt *et al*[17]. 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. 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.

Elevated plus maze test

The test was performed on the experimental protocol standardized by Ramamoorthy *et al*[18] with slight modification. The anxiety of Swiss albino mice was measured by allowing the animals to move freely over an elevated maze height 50cm from the ground. The apparatus consists of two open and two closed arms having walls (50cmx10cmx50cm), arranged alternatively with a common central platform (10cmx10cm). The open arm was provided with 2mm high ledge to prevent falling of mice. The apparatus was indirectly illuminated with a ceiling-fronting lamp (60W), which was placed 100cm above the apparatus. The animals were placed at the centre of apparatus; head turned towards an open arm, then 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.

Spontaneous locomotive activity

The spontaneous locomotive activity was assessed as per method proposed by Boissier & Simon[19] with slight modification. The mice were placed individually in a square arena (30cmx30cm), with wall painted black. 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.

Statistics Analysis

All the data were expressed as mean ± standard error of mean (s.e.m). One-way ANOVA was performed for all animal studies at the significance level of $p<0.05$. The significance level was evaluated by using Bonferroni's Multiple Comparison/ Tukey's Multiple Comparison/ Dunnett's Multiple Comparison tests. Each value is a representation of the mean of test conducted on six animals.

RESULT

Partially purified TLC spots showed presence of two variants of microcystin i.e. microcystin-FR and microcystin-RR with m/z valve of 1029.521 and 1037.970 respectively (Figure 1). The RP HPLC purification of these variants showed the R_t values of 7.8 mins and 12.1 mins respectively (Figure. 2).

Antidepressant Activity

A 40% decrease in immobility of Swiss albino mice in both tail suspension test and force swim test was observed with the animals treated with 0.8µg/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. The animals which are dosed with a combination of 0.8µg/kg McyFR and 8mg/kg CsA showed 55% decrease in FST immobility as compared to controls, whereas the same combination caused 84% TST immobility (Figure 3)

Elevated Plus Maze Test

The time spent in open arm by the animals treated with 0.8µg/kg McyFR + 8mg/kg CsA was 89% of the time spent by positive control

animals. The animals treated with CsA alone (8mg/kg McyFR) showed similar effect as that of negative control group suggesting that CsA has no prominent role in Anxiolytic activity.

Spontaneous locomotive activity

In order to understand the microcystin induced locomotive disorder, the spontaneous locomotive activity test was conducted. As shown in Table 1, the locomotive score was almost same for the mice treated with CsA alone or in the combination with 0.8µg/kg McyFR compared to the negative controls.

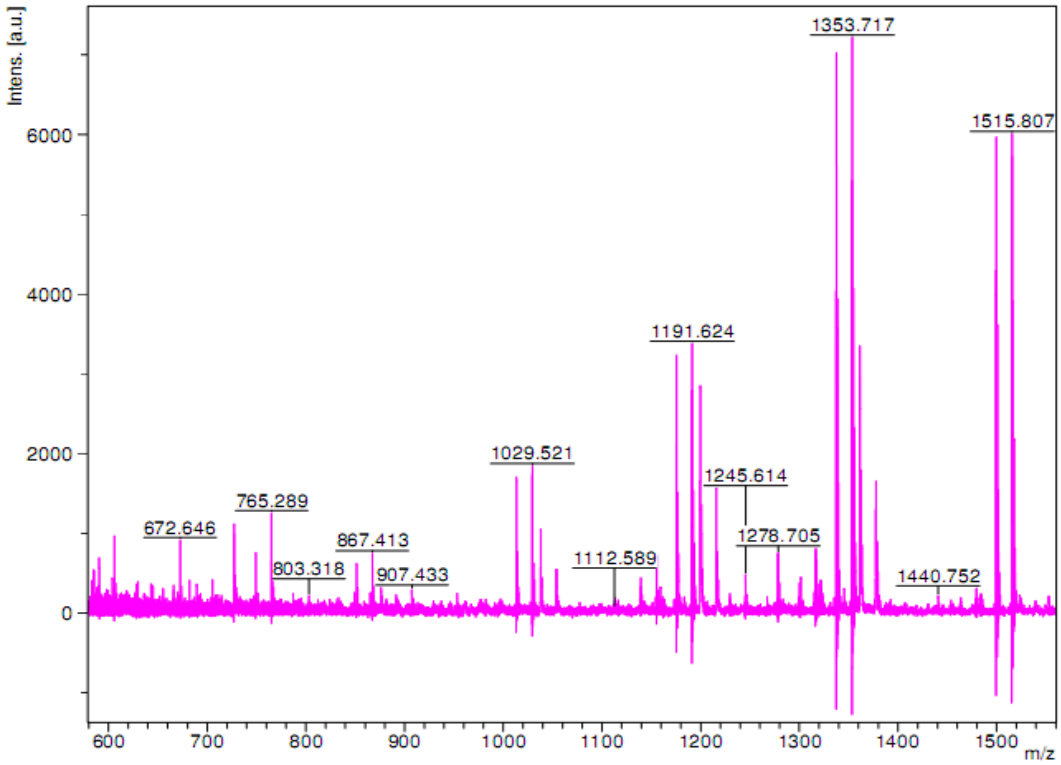

Fig. 1: MALDI TOF MS spectra of partially purified TLC spot of *N. spongiaeforme*: The partially purified TLC samples were mixed with equal amount of α-cyano-4-hydroxycinnamic acid and dissolved in buffer containing 1: 2 of acetonitrile and 0.1% TFA

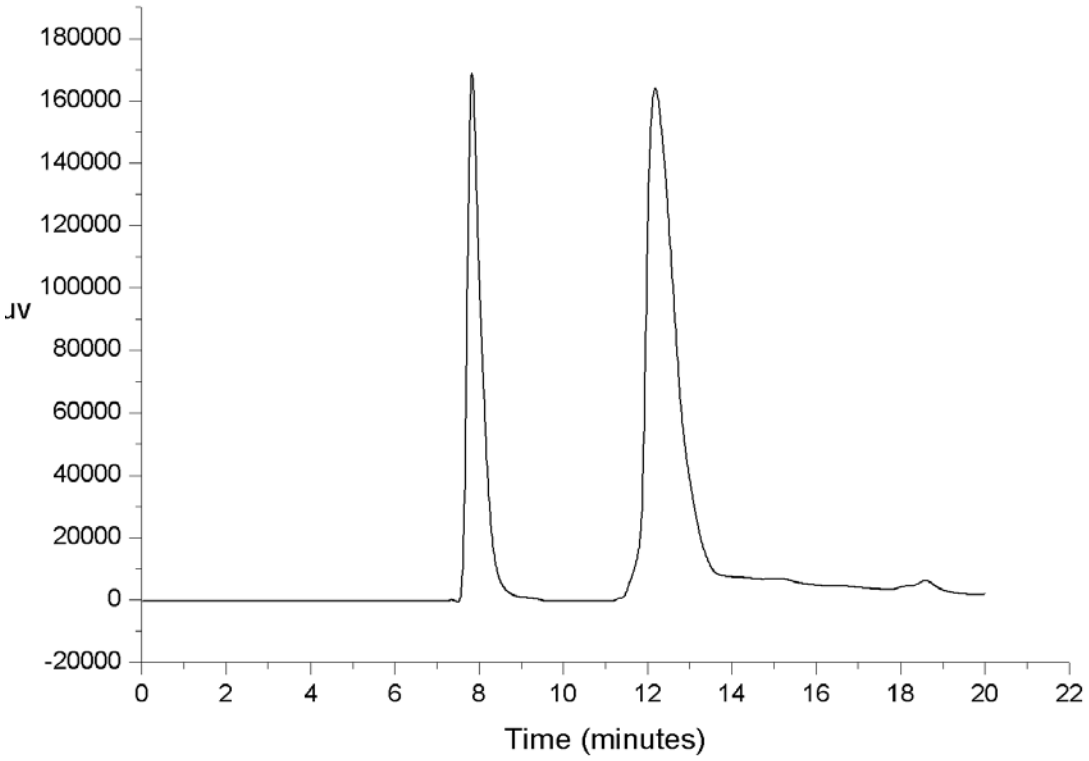

Fig. 2: RP-HPLC elution profile of Microcystin-FR from partially TLC purified spot of *N. spongiaeforme*.

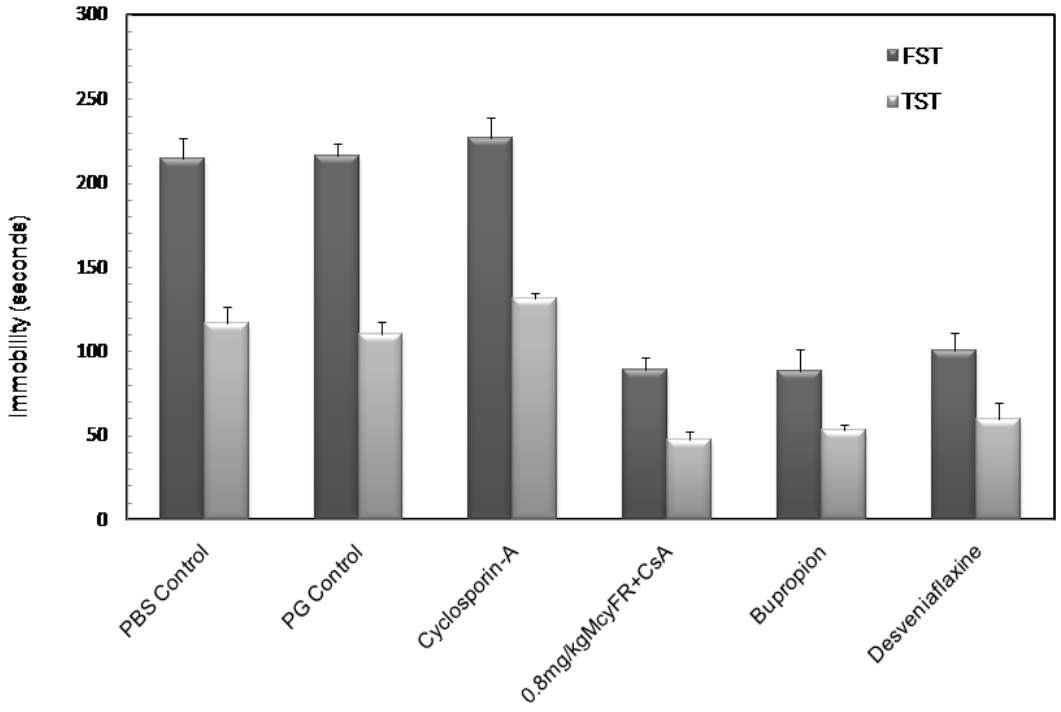

Fig. 3: Antidepressive activity of Micrcocystin-FR along with Cyclosporine-A in Tail suspension test (TST) and Force swim test (FST). One away ANOVA and Bonferroni's Multiple Comparison Test was employed to determine the significance level.

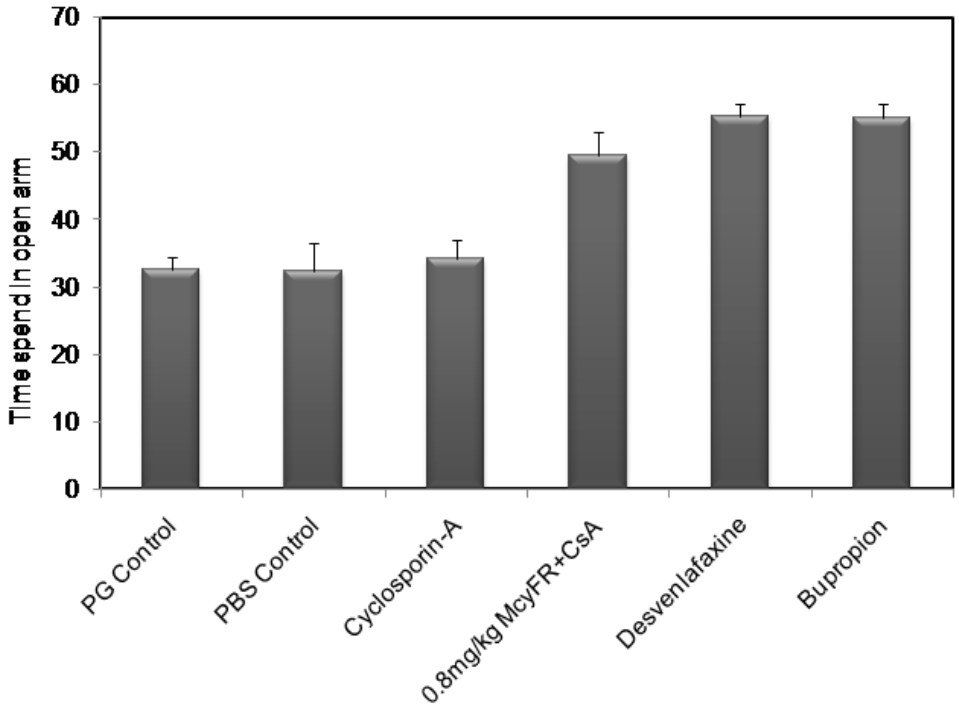

Fig. 4: Anxiolytic effect of Micrcocystin-FR along with Cyclosporine-A in Elevated Plus Maze test. One away ANOVA and Bonferroni's Multiple Comparison Test was employed to determine the significance level

Table 1: 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.

S. No	Treatment	Locomotor Score
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 (0.8µg/kg + CsA 8mg/kg)	300.3 ± 7.329

Hepatotoxicity

The animals treated with 0.8µg/kg microcystin FR 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 (Figure 5) suggesting a definite role of CsA in protecting McyFR induced hepatotoxicity.

Animals treated with 0.8µg/kg body weight McyFR alone showed swollen livers and changes in normal architecture of hepatocytes (Figure 6A). Addition of 8mg/kg body weight CsA to McyFR treated animals showed no significant change in liver morphology when compared to the controls (Figure 6B). The liver body weight index 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 (Figure 7).

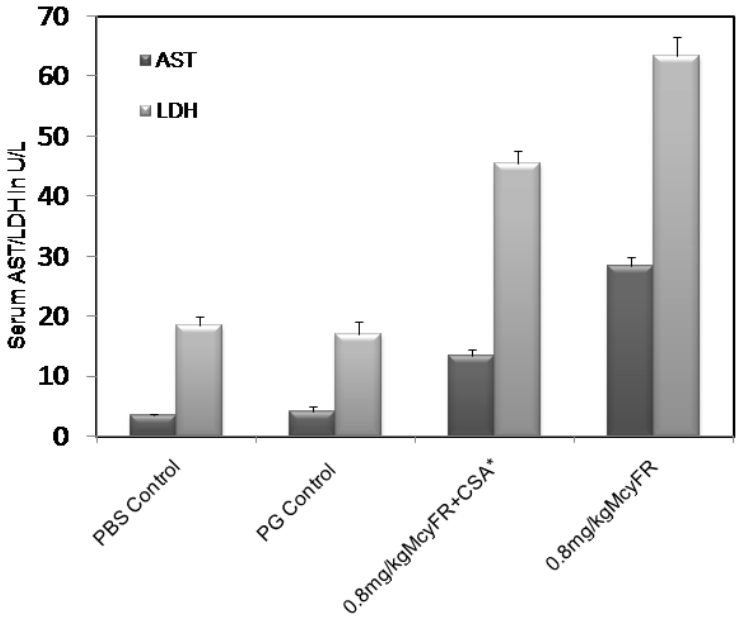

Fig. 5: Chemoprotective efficiency of CsA against the hepatotoxic effect of Microcystin-FR on liver enzymes AST & LDH levels. One way ANOVA and Tukey's Multiple Comparison Test Test was carried to find the significance level. *p<0.001 as compared between 0.8mg/kg Mcy-FR+CSA and 0.8mg/kg Mcy-FR

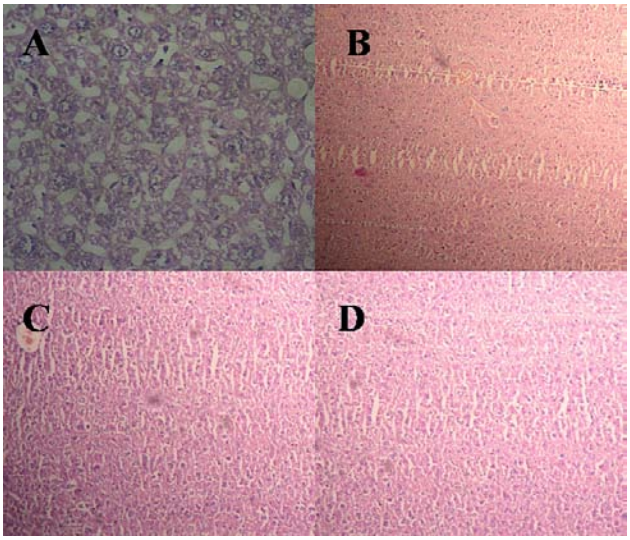

Fig. 6: Histomicrographs of perfused liver of Swiss albino mice injected with (A) 0.8µg/kgMcyFR alone (B) 0.8µg/kgMcyFR+8mg/kg CsA (C) PBS Control (D) PG Control (PG)

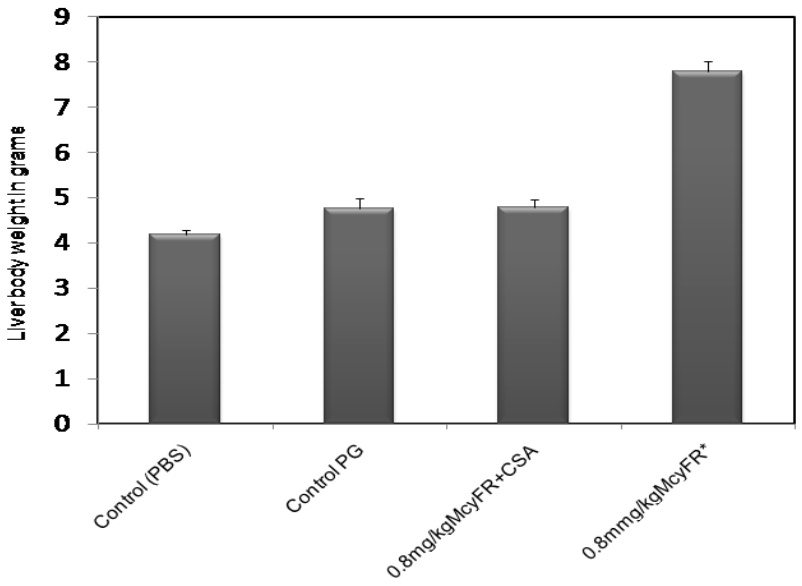

Fig. 7: Liver body weight index in Swiss albino mice treated with: 0.8µg/kgMcyFR*(P<0.01) alone and in combination with 8mg/kg CsA body weight (P>0.05), PBS Control and PG Control. One way ANOVA and Dunnett's Multiple Comparison Test was carried to find the Mean, SEM and significant level

DISCUSSION

Microcystins induced toxicity is known to be mediated through the inhibition of PP_2A activity in hepatocytes[20]. Our previous studies on chronic and acute doses of microcystins have shown breakdown of sinusoids, severe blood pooling and disorganization of hepatocytes[9]. MALDI TOF Ms analysis of TLC purified samples revealed the presence of two variants of microcystins in *N. spongiaeforme*: Microcystin-FR (1029.521 m/z) and Microcystin-RR (1037.970 m/z). Microcystin-FR was used in the present study due to its mild hepatotoxic effect as compared to the severe hepatotoxic effect caused by microcystin-LR or microcystin-RR[6].

The psychological status of normal animals when subjected to a non–soluble aversive situation does alter between agitation and immobility[16, 18]. 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[16, 21- 24]. 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 lead to the accumulation of serotonin (5-HT), dopamine (DA) and nonepinephrine (NE) in neuronal cleft[10-13]. 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[10]. Studies on effective chemoprotectant over hepatotoxicity of microcystins have shown 100% protection in mice by Rifampin and Cyclosporine A[14]. Out of these Cyclosporin A was chosen in the present study for its effectiveness in reducing microcystin uptake by hepatocytes.

The observed antidepressive activity of microcystin-FR in combination with Cyclosporine 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. A comparison of antidepression like activity of 0.8µg/kg Mcy-FR and standard antidepressants (Bupropion & Desvenlafaxine) showed that Mcy-FR combined with CsA combination is as effective as standard drugs.

CONCLUSION

The preliminary investigation demonstrates the antidepression and anxiolytic like effects of microcystin–FR in Swiss albino mice using a battery of behavioural model 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.

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