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Inonotus obliquus aqueous extract prevents histopathological alterations in liver induced by environmental toxicant Microcystin

Pir Mohammad Ishfaq [a,c], Shivani Mishra [a], Anjali Mishra [a], Zaved Ahmad [a], Shovanlal Gayen [b], Subodh Kumar Jain [c], Swati Tripathi [d,*], Siddhartha Kumar Mishra [a,e,**]

[a] Cancer Biology Laboratory, Department of Zoology, School of Biological Sciences, Dr. Harisingh Gour Central University, Sagar, 470003, (M.P.), India
[b] Department of Pharmaceutical Sciences, Dr. Harisingh Gour Central University, Sagar, 470003, (M.P.), India
[c] Molecular Biology Laboratory, Department of Zoology, School of Biological Sciences, Dr. Harisingh Gour Central University, Sagar, 470003, (M.P.), India
[d] Amity Institute of Microbial Technology, Amity University, Noida, 201313, (U.P.), India
[e] Department of Biochemistry, University of Lucknow, Lucknow, 226007, (U.P.), India

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ABSTRACT

Environmental toxicants like microcystins are known to adversely impact liver physiology and lead to the increased risk for abnormal liver function and even liver carcinoma. Chaga mushroom (*Inonotus obliquus*) is reported for various properties mainly antibacterial, antiallergic, anti-inflammatory, antioxidant, and anticancer properties. This study was aimed to assess the effect microcystin (MC-LR) on histopathology of liver in mice and a preventive measure by using aqueous extract of *Inonotus obliquus* (IOAE). Adult Balb/c mice were administered with MC-LR at 20 μg/kg body weight, per day, intraperitoneal (i.p.) for 4 weeks. IOAE was treated to one group of MC-LR mice at 200 mg/kg body weight, per oral, for 4 weeks. Histological staining for liver structural details and biochemical assays for functions were assessed. The results of the study showed that MC-LR drastically reduced the body weight of mice which were restored close to the range of control by IOAE treatment. MC-LR exposed mice showed 1.9, 1.7 and 2.2-fold increase in the levels of SGOT, SGPT and LDH which were restored by IOAE treatment as compared to control (one-fold). MC-LR exposed mice showed reduced level of GSH (19.83 $\pm$ 3.3 μM) which were regained by IOAE treatment (50.83 $\pm$ 3.0 μM). Similar observations were noted for catalase activity. Histological examinations show that MC-LR exposed degenerative changes in the liver sections which were restored by IOAE supplementation. The immunofluorescence analysis of caspase-3 counterstained with DAPI showed that MC-LR led to the increased expression of caspase-3 which were comparatively reduced by IOAE treatment. The cell viability decreased on increasing the concentration of MC-LR with 5% cell viability at concentration of 10 μg MC-LR/mL as that of control 100% Cell viability. The IC_{50} was calculated to be 3.6 μg/ml, indicating that MC-LR is chronic toxic to AML12 mouse hepatocytes. The molecular docking interaction of NF-κB-NIK with ergosterol peroxidase showed binding interaction between the two and showed the plausible molecular basis for the effects of IOAE in MC-LR induced liver injury. Collectively, this study revealed the deleterious effects of MC-LR on liver through generation of oxidative stress and activation of caspase-3, which were prevented by treatment with IOAE.

1. Introduction

Microcystins are the type of environmental toxicants produced by several genera of cyanobacteria like *Microcystis*, *Planktothrix*, *Anabaena*, *Anabaenopsis*, and *Nostoc*. Microcystins have shown hepatotoxicity in mammals and have received increasing worldwide concern since last few decades because of their toxic nature [Wiegand and Pflugmacher 2005]. Microcystins have the capability to accumulate in the tissues of animals and can be transferred to other animal in food chain. Microcystins has been reported to exert toxicity in liver cells and death occurs mainly due to severe liver damage which starts with cytoskeletal disorganization and can induce lipid peroxidation, cellular disruption, cell blebbing, loss of membrane integrity, DNA damage, necrosis, apoptosis and by hemorrhagic shock [Batista et al., 2003; Martins et al., 2017; McDermott et al.,

* Corresponding author.
** Corresponding author. Department of Biochemistry, University of Lucknow, Lucknow, 226007, (U.P.), India.
E-mail addresses: swatitri@gmail.com (S. Tripathi), siddharthakm@yahoo.com (S.K. Mishra).

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1998]. More than 100 microcystin isoforms have been detected, among which microcystin-leucine arginine (MC-LR) is the most abundant and the most toxic variant of microcystin. The main target of MC-LR toxicity is the liver, causing necrosis, cytoskeletal damage, and increase in weight, pooling of blood, blabbing of hepatocytes and morphological alteration of microtubules [Fu et al., 2005]. The liver plays an important function in the detoxification of microcystins yet it causes liver damage mainly due to hemorrhagic shock [Brooks and Codd 1987; McDermott et al., 1998]. Thus, the intake of MC-LR in drinking water has been reported to result the occurrence of liver cancer in humans [Funari and Testai 2008a; Ueno et al., 1996].

Humans and animals are exposed to both low and high doses of MC-LR and *in vitro* studies have confirmed that dose as low as 0.01 μg/ml can induce DNA damage in hepatoma cell line HepG2 [Chen and Xie 2005]. In acute exposure, MC-LR damages DNA directly or indirectly through mutagenesis and ROS generation. Previous reports suggest that acute exposure of high doses of MC-LR, *in vitro* or *in vivo*, leads to the collapse and aggregation of both actin microfilaments and cytokeratin intermediate filaments around the nucleus [Falconer et al., 1992; Robinson et al., 1989]. While high dose of MC-LR ranging between 500 and 5000 μg/L becomes a chronic condition leads to degeneration and necrosis of hepatocyte, progressive fibrosis and infiltration of mononuclear leukocyte and liver tumor promotion in laboratory animals [Campos and Vasconcelos 2010; Nishiwaki-Matsushima et al., 1992]. High doses of MC-LR causes rupture of mitochondrial membrane and damage to the hepatic architecture with enormous intrahepatic hemorrhaging [Chen et al., 2005; McDermott et al., 1998]. MC-LR treated mice showed increased liver weight due to massive intrahepatic hemorrhage and pooling of blood in the liver [Funari and Testai 2008b]. Elevation of serum activity of enzymes indicates that hepatocellular damage has been previously reported in both acute and chronic toxicity studies [Dawson 1998]. Thus, MC-LR infestation needs to be checked from water bodies and then there remains a need of preventive measure from MC-LR-induced hepatotoxicity.

Many synthetic drugs being used in the treatment of liver diseases have serious side effects, owing to which there is an increasing focus to follow systemic research methodology and to evaluate scientific basis for traditional herbal medicines that are claimed to possess hepatoprotective activity [Kanter et al., 2005; Sharma and Gupta 2015]. A wide range of chemical agents have been analyzed for possible hepatoprotective effect. Most of these are synthetic and many are known to produce undesirable side effects. Increasing attention is being given worldwide on therapeutic effects of naturally occurring substances like vitamins, minerals, amino acids, small bioactive peptides [Domitrovic and Potocnjak 2016]. Therefore, there has been a considerable interest in the role of complementary and alternative medicines for the treatment of liver diseases [Madrigal-Santillán et al., 2014]. Natural products have received considerable attention in recent years because of their reduced side effects compared with synthetic drugs [Farghali et al., 2015; Srivastava and Shivanandappa 2010]. A large number of plants and formulations have been claimed to have hepatoprotective activity [Zaidman et al., 2005].

Mushrooms have been extensively researched for their health promoting benefits apart from being low calories, high in minerals, essential amino acids, vitamins and fibers [Valverde et al., 2015]. *Inonotus obliquus*, commonly known as chaga, is a macro-fungus mushroom belonging to the hymenochaetaceae family of Basidomycetes. Chaga is a remarkable medicinal mushroom which has been used in traditional medicine to treat a variety of ailments such as stomach disease, liver-heart problems, blood purification gastrointestinal disorers and pain relief [Kim 2005; Mishra et al., 2012a]. Chaga mushroom has been reported for beneficial properties for human health, such as anti-bacterial, anti-allergic, anti-inflammatory and antioxidant activities [Mishra et al., 2012a]. We have reported that an aqueous extract of *Inonotus obliquus* (IOAE) suppressed intestinal inflammation and colorectal carcinoma via downregulation of Wnt/β-catenin signaling and NF-κB-iNOS-Cox-2 pathways [Mishra et al., 2013; Mishra et al., 2012a]. An aqueous extract of *Inonotus obliquus* mushroom was reported for its potent hepatoprotective effects against tert-butyl hydroperoxide (t-BHP)-induced oxidative liver injury in the primary cultured rat hepatocyte [Hong et al., 2015]. These attributes prompted us to investigate the hepatoprotective activity of *Inonotus obliquus* in MC-LR induced conditions. Therefore, this study was designed to determine the hepatoprotective and antioxidant activities of the extract obtained from *Inonotus obliquus* against MC-LR-induced hepatotoxicity in mice. Hepatoprotective activity was determined by assaying the histopathological signatures and biochemical indices relating to liver physiology and function.

2. Materials and methods

2.1. Preparation of Inonotus obliquus Aqueous Extract (IOAE)

The aqueous extracts from *Inonotus obliquus* (IOAE) was prepared as described earlier [Mishra et al., 2012b]. The fruiting bodies (10 gm) of Chaga mushroom were dried in air under the shade. Then the dried fruiting bodies were mechanically ground to a fine powder. The powder was then extracted twice with 100 ml of warm water (40 °C) for 3 h. The filtrates of both extractions were collected and filtered at room temperature. The filtrate was dried in a rotary evaporator under vacuum at 40 °C and stored at refrigerated condition in airtight containers in the dark. IOAE was freshly prepared to a deliverable concentration by dissolving IOAE powder in distilled water at a final concentration of 200 mg/kg of body weight when delivered to mice.

2.2. MC-LR administration and IOAE treatment to mice

Animal experiments were carried out by approval and in accordance with the Institutional Animal Ethical Committee of the Institute (Registration N379/GO/ReBi/S/01/CPCSEA). Balb/c male mice, 6–8 week old, weighing from 18 to 22 gm were housed in the animal house with a 12 h: 12 h light-dark cycle. Mice were maintained at standard laboratory conditions, fed with standard laboratory diet, and given free access to water. Mice were randomly divided into three groups with five mice in each group. Group I (control) mice received phosphate-buffered saline (PBS) as vehicle control to a maximum 20 μl per day, i.p., for 4 weeks. MC-LR (*Sigma*-Aldrich) was diluted in PBS and administered to Group II and III mice, at 20 μg/kg b.w. per day, i.p. for 4 weeks. Group III mice were orally administered with IOAE at 200 mg/kg b.w. per day, for 4 weeks. Mice in groups I and II were given with equivalent volume of distilled water (as vehicle control for IOAE). After total 8 weeks of experiment, mice were euthanized, and liver was expatriated.

2.3. Measurement of body weight of mice

Body weight of mice in each group was recorded at an interval of 3 days starting from the day of treatment with the help of a laboratory weighing balance. The body weight values (gm) for each individual in the group were averaged as mean with standard deviation (SD).

2.4. Histological examination of liver

Histological analysis was performed by using standard procedure with suitable modification as per experimental requirements [Kaur et al., 2013]. Liver tissues were processed through several changes of alcohol and then serially dehydrated for embedding. Tissues blocks were prepared in wax and sections of 6 μm thickness were cut by using rotary microtome. Sections were stained with haematoxylin and eosin (H&E) and visualized under light microscope (Magnus MLX).

2.5. Analysis of serum glutamate-oxaloacetate-transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT)

For biochemical studies, blood was collected from mice in each group

after euthanasia, and serum was separated. The activity of SGOT and SGPT was analyzed from the serum of mice in each treatment group using Erba Manheim Assay kit (Mannheim, Germany) according to the manufacturer's instructions. The values were calculated in IU/L and then compared between treatment groups as fold change. The enzyme activity in control was set to fold one.

2.6. Measurement of lactate dehydrogenase (LDH) activity

LDH activity was spectrophotometrically measured by the standard methodology with suitable modifications [Kornberg 1955]. Briefly, the reaction mixture (1 ml) was prepared which contained 20 mM Tris-Cl (pH 7.4), 3 mM NADH, 1 mM sodium-pyruvate, and the sample from mice of different treatment groups. Decrease in the absorbance was recorded at 340 nm after every 30 s for 3 min. The enzyme activity was calculated as IU/L and then compared between treatment groups as fold change, considering the enzyme activity in control as one-fold.

2.7. Measurement of catalase activity

Catalase activity was measured by the decomposition of hydrogen peroxide (H_2O_2) by standard methodology [Bergmeyer and Gawehn 1974]. H_2O_2 has absorption maximum at 240 nm and absorption declines with the decomposition of H_2O_2. The difference in extinction per unit time is a measure of the catalase activity. The reaction mixture (1 ml) contained H_2O_2-phosphate buffer and the reaction was started by the addition of suitably diluted sample. The decrease in absorbance at 240 nm was recorded after every 30 s for 3 min. The enzyme activity was calculated as IU/L.

2.8. Measurement of glutathione (GSH) level

Glutathione (GSH) assay was performed by assaying the oxidation of substrate 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and generated a yellow-colored complex that can be read at 412 nm. The absorbance is directly proportional to the amount of GSH. GSH concentration was measured by the standard method with suitable modification. Briefly, the assay mixture (200 μl) contained Tris-HCl buffer pH 8.0 with 100 μl of DTNB/Ellman's reagent (2 mM in 50 mM of sodium acetate). The liver tissue sample was extracted in 100 mM phosphate buffer pH 7.4 and was then added to the reaction mixture in 10x dilution. The assay mixture containing yellow colored product was measured at 412 nm in an ELISA microplate reader. The enzyme activity was calculated as IU/L.

2.9. Immunofluorescence for Caspase-3

Liver tissues processed for immunofluorescence examination, were fixed in 10 % buffered formalin and then embedded in molten paraffin. Then the waxed blocks were deparaffinized and rehydrated in ethanol series. The blocks were then rinsed in distilled water and washed in 10 mM Tris buffer saline pH 7.4. Then slides were immersed in 100 mM citrate buffer (pH 6.0) and antigen retrieval was done using microwave oven. Sections were then blocked for 30 min with diluted goat normal serum (Santa Cruz Biotechnologies, USA) and incubated overnight at 4 °C with anti-Caspase-3 antibody (Santa Cruz Biotechnology, USA) at 1:300 dilutions in PBS. Sections were then incubated with goat anti mouse IgG-FITC secondary antibody (GeNei, India) at 4 °C for 4 h. The slides were then counterstained with DAPI at 0.5 mg/ml concentration (Himedia, India). The sections were visualized under Confocal Laser Scanning Microscope (NIS- Elements AR 4.2, Nikon A1, Japan) at magnifications 10×, 20X and 40X.

2.10. Culture of alpha mouse liver 12 (AML 12) mouse hepatocytes

Alpha mouse liver 12 (AML 12) cells have been established from the hepatocytes of a male mouse transgenic for human (TGF α) transforming growth factor α (ATCC CRL-2254). The AML 12 cells were stored in liquid nitrogen for further use. Further the cells were then thawed for 2 min in a dry bath at 37 °C, transferred to tissue culture flask diluted with (Dulbecco's Minimum Essential Medium) DMEM that was aided with 10% fetal bovine serum (FBS), 1% streptomycin and penicillin, and incubated at 37 °C in a humidified incubator (New Brunswick, Eppendorf) with 5% CO_2 to allow cell growth and form a monolayer of the cells.

2.11. Assessment of AML12 hepatocytes cell viability

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide] assay was performed for evaluating the cytotoxicity and cell proliferation activity in AML 12 mouse hepatocytes exposed to MC-LR. AML 12 mouse hepatocytes were maintained in DMEM in standard culture conditions. AML 12 cells were seeded at 10,000 cells/well in 96-well plates for 24 h. Then cells were treated with variable concentrations of MC-LR or vehicle control for 48 h. Cells were incubated with 20 μl of MTT solution (5 mg/mL) and cell growth was assessed in each well under an ELISA microplate reader at absorption maxima 550 nm. Cell viability was assessed by comparing the total percent cell viability as compared to control (100 %).

2.12. Molecular docking to probe interaction of Inonotus obliquus compound ergosterol peroxide

The docking studies were performed by Autodock Vina (UCSF Chimera) and analyzed by Autodock tools 1.5.6rc3**.** The three-dimensional structures of anticancer compounds ergosterol peroxide were downloaded in.sdf format from Pubchem database (https://pubchem.ncbi.nlm.nih.gov/) with Pubchem ID: 5318517. Structure was downloaded in.sdf format and converted to.pdb format with Open Babel tool for docking analysis. Hydrogen bonds were added, and the energy was minimized using CHARMm force field. The 3-D crystal structure of the p53, NF-κB and beta-catenin with PDB ID: 1pes, 1oy3 and 2gl7 (http://www.rcsb.org/pdb/) with resolution 1.5 Å, 2.05 Å and 2.6 Å were downloaded. The bonds, bond orders, hydrogen, charges and flexible torsion were assigned if they were missing in UCSF Chimera. Evaluation of the results was done by sorting the different complexes with respect to the predicted binding energy, root mean square deviation values (RMSD values) and presence of hydrogen bonds and the lowest energy conformation with hydrogen bonds was considered as the most trustable solution.

2.13. Statistical analysis

The results were expressed as mean ± standard deviation (SD) from three independent repeats. Statistical analysis of the results was performed using Microsoft Office Excel and SPSS v.17 (SPSS Inc., USA). The result within groups was compared by Students t-test for determining the level of significance between control and the experimental groups. The P value < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Preventive effect of IOAE on MC-LR-induced body weight loss

MC-LR exposure (20 μg/kg b.w./day, i.p. for 4 weeks) caused a significant decline in the net body weight of animals as compared to the control. Decrease in net body weight gain might be related to inhibition of DNA synthesis by MC-LR treatment which in turn results in inadequate protein turnover and could also alter the concentrations of enzymes, structural proteins, receptors and different secretions in the body [Mrdjen et al., 2018; Naik et al., 2011; Zegura 2016]. Such inhibition of growth by MC-LR exposure could be due to fewer food intake, decrease in protein levels and alteration in metabolic activities as observed in this study. The results of our study have showed that IOAE prevented the

decrease in body weight of mice due to MC-LR treatment (Fig. 1a). The average body weight of mice in control group was 25.98 ± 0.50 gm which was drastically reduced by exposure to MC-LR. MC-LR-exposed groups showed average body weight 17.49 ± 0.90 gm with statistically significant (P = 0.001) as compared to control. While administration of IOAE to MC-LR treated group caused notable improvement in the body weight. IOAE-treated group showed average body weight 23.72 ± 0.62 gm which was statistically significant (P = 0.0005) as compared to MC-LR group. Previous Studies have shown significant decrease in the body weights of mice exposed to MC-LR [Parola and Robino 2001; Shen et al., 2003]. While chaga mushroom was earlier reported to have improved body weight of mice models of obesity and suppression of inflammation in the liver and spleen of high-fat diet-induced obesity in mice [Na et al., 2019]. It also maintained the body weight in mouse models of Lewis lung carcinoma as well as it suppressed the growth and spontaneous metastasis of tumor in mice [Arata et al., 2016]. Thus, chaga mushroom extract acting to restore the body weight loss in mice exposed to MC-LR is an important preliminary preventive signature in inflammatory liver diseases.

3.2. IOAE improved the liver function alterations induced by MCLR

The liver function tests (LFT) is referred to as a biochemical assay for the enzymes of hepatic panel that provide information about the physiological functions of a liver [Johnston 1999]. The biochemical assays for liver transaminases aspartate transaminase (AST or SGOT) and alanine transaminase (ALT or SGPT) were performed as useful biomarkers of liver injury in mice model which designate some degree of intact liver function [Blann 2014]. SGOT is released into blood when there is a liver injury and the blood SGOT levels increase which is biochemical marker. SGOT or AST is a pyridoxal phosphate (PLP)-dependent transaminase enzyme that catalyzes the reversible transfer of an α-amino group between aspartate and glutamate mainly in the liver and to some extent in heart, skeletal muscle, kidneys, red blood cells (RBCs) and brain [Blann 2014].

The activity of SGOT and SGPT were assayed as IU/L and compared among different treatment groups as fold-change as compared to control considering as one-fold enzyme activity. The results show that MC-LR exposure to mice caused an about two-fold (1.9-fold) increase in the levels of SGOT as compared to control with statistical significance (P = 0.005) (Fig. 1b). Further the treatment of MC-LR exposed mice with IOAE caused a notable reduction in SGOT activity. IOAE reduced the levels of SGOT to 1.1-fold which was statistically significant (P = 0.003) as compared to MC-LR group, while statistically not significant as compared to that of control (P > 0.05). Likewise, MC-LR exposed mice showed an increased level of SGPT (1.7-fold) as compared to control with statistical significance (P = 0.001) (Fig. 1c). Further the treatment of MC-LR exposed mice with IOAE reduced the level of SGPT to 1.3-fold which was statistically significant (P = 0.015) as compared to MC-LR group, while marginally significant as compared to that of control (P = 0.046). Thus, IOAE restored the liver biochemical function to a level close to control. Some of the liver diseases show only an initial mild alteration in LFT yet severe hepatic diseases can be of crucial importance especially cirrhosis and hepatocellular carcinoma (HCC). Thus, the LFT is performed as a crucial determinate associated with functionality of hepatic cells as well as cellular integrity.

Serum AST level and serum ALT level, and their ratio (AST/ALT ratio) are generally measured as clinical biomarkers for liver pathophysiology [Nyblom et al., 2006]. This test ratio provides a classification of liver disease which helps in understanding the disease status and thus disease management protocols are accordingly developed. In this study, we found that MC-LR induced ALT and AST levels (IU/L) were in the ratio of 3.32 which is suggestive of inflammatory livery injury or alcoholic liver injury whichever the case may be [Nyblom et al., 2006]. This ratio was

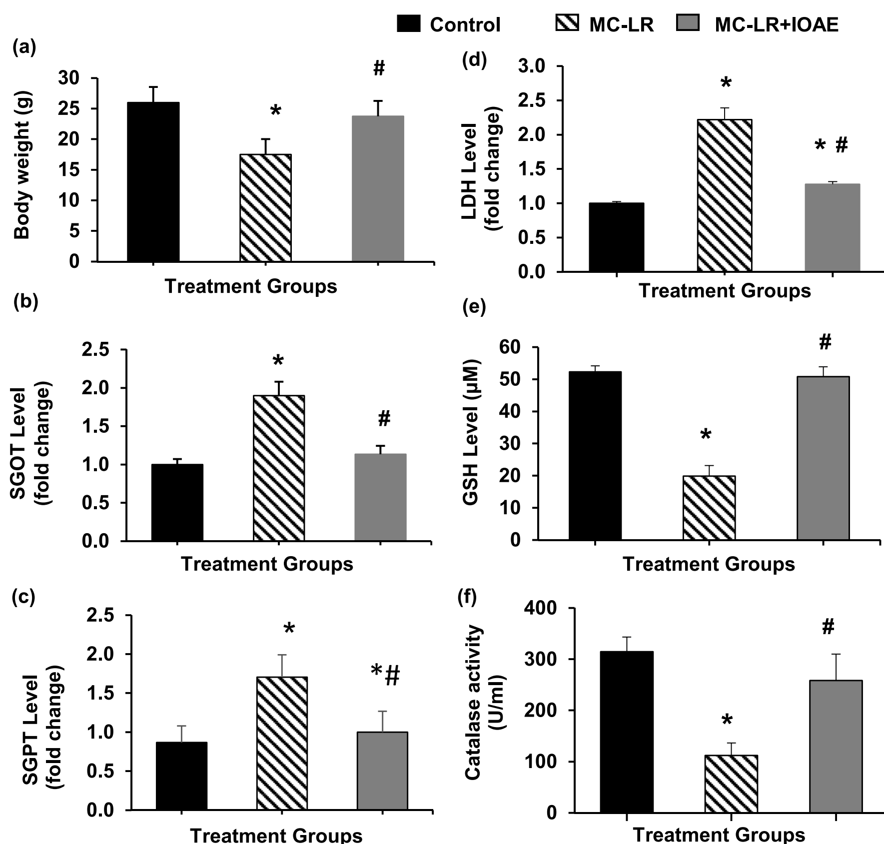

Fig. 1. (a) Effect of MC-LR and chaga treatment on the body weight of mice. Body weight of five mice (n = 5) in each group was recorded (in gm) at day 28 and presented as measn±SD. **(b, c) Effect of IOAE on MC-LR-treated liver SGOT and SGPT.** SGOT and SGPT level was assayed from three mice (n = 3) in each group and values calculated in IU/L were represented as fold change. Considering control group as one-fold. **(d) Effect of IOAE on MC-LR-treated liver LDH activity.** LDH level was assayed from three mice (n = 3) in each group and values calculated in IU/L were represented as fold change. Control group was considered as one-fold and other groups were compared to the control. **(e, f) Effect of IOAE on GSH concentration and catalase activity in MC-LR-treated mice.** GSH concentration (μM) and catalase (U/ml) enzyme activity was assayed from three mice (n = 3) in each group and values were calculated as mean ± SD. Balb/c mice were randomly divided into three groups with five mice in each group. Group I (control) mice received phosphate-buffered saline as vehicle control to a maximum 20 μl per day, i.p., for 4 weeks. Group II and III mice were treated with MC-LR at 20 μg/kg b.w. per day, i.p. for 4 weeks. Group III mice were orally administered with IOAE at 200 mg/kg b.w. per day, for 4 weeks. Mice in groups I and II were given with equivalent volume of distilled water (as vehicle control for IOAE). *P < 0.05 vs control; #P < 0.05 vs MC-LR.

reduced to 2.53 when MC-LR exposed mice were treated with IOAE. These biochemical tests assessing liver function are useful in the evaluation and management of hepatic dysfunction and liver pathophysiology [Blann 2014; Johnston 1999]. *Inonotus obliquus* was earlier shown to protect against hepatic injury against t-BHP-induced oxidative liver injury in the primary cultured rat hepatocyte. Chaga mushroom extract significantly suppressed the levels of ALT and AST induced by t-BHP exposure [Hong et al., 2015]. In this purview, the preventive effects of IOAE by lowering the levels of SGOT and SGPT in MC-LR-treated mice are considerable for natural supplementation for prevention of liver inflammation and injury.

3.3. IOAE prevents LDH release in liver induced by MC-LR

Lactate dehydrogenase (LDH) is a prominent enzyme in glycolysis which catalyzes the conversion of pyruvate to lactic acid and vice versa. LDH activity increases in cancer or diseased cells because glucose is used as the major energy source through glycolysis and lactic acid is the by-product of glycolysis [Eventoff et al., 1977]. Therefore, during diseased conditions, cells need continuous energy supply for growth and glycolysis proceeds at faster rate than needed by citric acid cycle. Anaerobic metabolism is turned on and lactate is produced from pyruvate [Rogatzki et al., 2015]. While LDH is abundantly expressed in different tissues, its level in blood remains at a normal lower level. However, when tissue damage process occurs, as in case of liver injury, LDH releases from hepatic cells into the bloodstream. Not only liver disease, also the conditions of cardiac dysfunction, anemia, skeletal muscle trauma, osteological diseases, carcinoma and viral infections increase the levels of LDH in blood [Eventoff et al., 1977].

Results demonstrate that MC-LR exposure to mice caused a dramatic increase in the LDH levels by 2.2-fold as compared to that of control with statistical significance ($P = 0.007$) (Fig. 1d). Increased LDH level by the treatment of MC-LR to mice indicated the hepatic injury caused by the environmental toxicant. Furthermore, treatment of MC-LR exposed mice with IOAE cased a notable reduction in the levels of LDH. It restored the LDH level to 1.3-fold with statistical significance ($P = 0.038$) as compared to that of MC-LR group, and marginally close to that of control ($P = 0.042$).

Release of the liver enzyme LDH into the blood stream has been used as marker of MC-LR induced liver damage [Giannini et al., 2005]. Previous studies have also noted an increase in LDH level in hepatocytes exposed to MC-LR [Woolbright et al., 2017]. Increased LDH levels are therefore considered indicators of hepatotoxicity and has been reported in acute as well as chronic toxicity studies [Kotoh et al., 2011]. Our results are in consistent with previous reports where MC-LR has shown significant increase in LDH levels in liver indicating tissue damage [Jayaraj et al., 2007; Woolbright et al., 2017]. Restoration of LDH activity by *Inonotus obliquus* was also shown in the model of hepatic injury induced by t-BHP exposure to the primary cultured rat hepatocyte [Hong et al., 2015]. Thus, these observations add to our other findings that IOAE lowered the levels of LDH in MC-LR-treated mice which appears to be a considerable natural supplement against inflammatory liver injury.

3.4. IOAE suppressed the oxidative stress induced by MC-LR

Microcystins are known to act by inhibiting phosphatases as well as by inducing oxidative stress. Although its mechanism is not elucidated, microcystins exposure can lead to an excessive formation of reactive oxygen species (ROS) which culminate in to cellular oxidative damage [Amado and Monserrat 2010]. Microcystins are also reported to incorporate the triggering of glutathione depletion and then consequently increase the ROS concentration in cells. In particular, microcystins are reported to interact with the cellular hyperphosphorylation state by inhibiting phosphatases. Microcystins act as potent inhibitors of protein phosphatases 1 and 2 A and cause disruption of the cytoskeleton system leading to cell death [Amado and Monserrat 2010; Martins et al., 2017]. Notably the hyperphosphorylated cellular environment induced by microcystins may modulate the antioxidant enzymes system and can contribute to the generation of ROS. MC-LR was reported to affect the antioxidant system in liver and gills of fish. MC-LR increases the activity of glutathione S-transferase (GST) by 74% and glutathione peroxidase (GPx) by 217%, as well as it decreased the superoxide dismutase (SOD) activity by 47% in liver [Martins et al., 2017]. Thus, microcystin induced liver toxicity may involve generation of oxidative stress as a mechanistic aspect, which was further investigated in this study. While reduction of the oxidative stress generation may serve to prevent the effects of MC-LR in liver.

In this study, the effect of MC-LR exposure to GSH level and catalase activity was assayed and compared among the treatment groups. MC-LR exposure to mice caused a notable decline in GSH level (19.83 ± 3.3 μM) as compared to control (52.33 ± 1.8 μM) (Fig. 1e). Further the treatment of MC-LR exposed mice with IOAE restored the levels of GSH to $50.83 + 3.0$ μM. The decline in GSH by MC-LR was statistically significant ($P = 0.003$) as compared to control, and the increase in GSH by IOAE was statistically significant ($P = 0.006$) as compared to MC-LR group. Likewise, the effect of MC-LR exposure to the activity of catalase enzyme was assayed and compared among the treatment groups. MC-LR exposure to mice caused a notable decline in the catalase activity (112 ± 24 U/ml) as compared to control (314 ± 29 U/ml) (Fig. 1f). Further the treatment of MC-LR exposed mice with IOAE restored the levels of catalase enzyme activity to the level of 258 ± 51 U/ml. The reduction in the catalase activity by MC-LR was statistically significant ($P = 0.001$) as compared to control, while the improvement of catalase activity by IOAE was statistically significant ($P = 0.010$) as compared to MC-LR group. These observations show that chaga extract prevented the oxidative stress and ROS generation by MC-LR as indicated by maintaining the levels of GSH and catalase.

Glutathione (GSH) is a tripeptide (L-γ-glutamyl-L-cysteinylglycine) with multiple functions in living organisms. As a carrier of an active thiol group in the form of a cysteine residue, it acts as an antioxidant either directly by interacting with reactive oxygen/nitrogen species (ROS and RNS) and electrophiles or by operating as a cofactor for various enzymes [Chen et al., 2013]. Glutathione is considered as a well-known antioxidant and constitutes the first line of protection against increased level of ROS. The conjugation of MC-LR with GSH is one of the main routes of its excretion [Martins et al., 2017]. Catalase is an antioxidant enzyme responsible for the detoxification of H_2O_2. Treatment of mice with MC-LR caused a significant decline in the levels of GSH and catalase. Co-treatment of MC-LR treated mice with IOAE prevented and improved the activity of catalase and levels of GSH in liver towards normal.

3.5. IOAE prevented the MC-LR induced hepatotoxic histological alterations

The toxicity of microcystins is known to cause hepatocellular damage which can be characterized by histopathological staining. MC-LR was found to affect the liver histology as characterized by rounding of hepatocytes, including condensed cytoplasm and contact loss between hepatocytes. It also included an apparent lysis of hepatocyte membranes as necrotic cells, and an appearance of pyknotic or apoptotic nuclei and apoptotic signs [Fischer and Dietrich 2000]. In this study, MC-LR treatment with mice showed significant histopathological alterations in liver (Fig. 2A). Results show that MC-LR caused the disrupted organization of cytoskeleton in the hepatocytes, which might interrupt metabolism of liver under effects of oxidative stress. Morphological alterations in hepatic injury caused by MC-LR were characterized by centrilobular fibrosis and inflammation. Earlier reports suggest that MC-LR induced hepatotoxicity is closely linked with intracellular formation of ROS and apoptosis in hepatocytes [Jiang et al., 2013]. Previously it was reported that MC-LR induced significant damage to hepatocytes, therefore leading to hemorrhage and necrosis in mice [Chen et al., 2005]. This study showed that the liver of control group showed normal hepatic lobules,

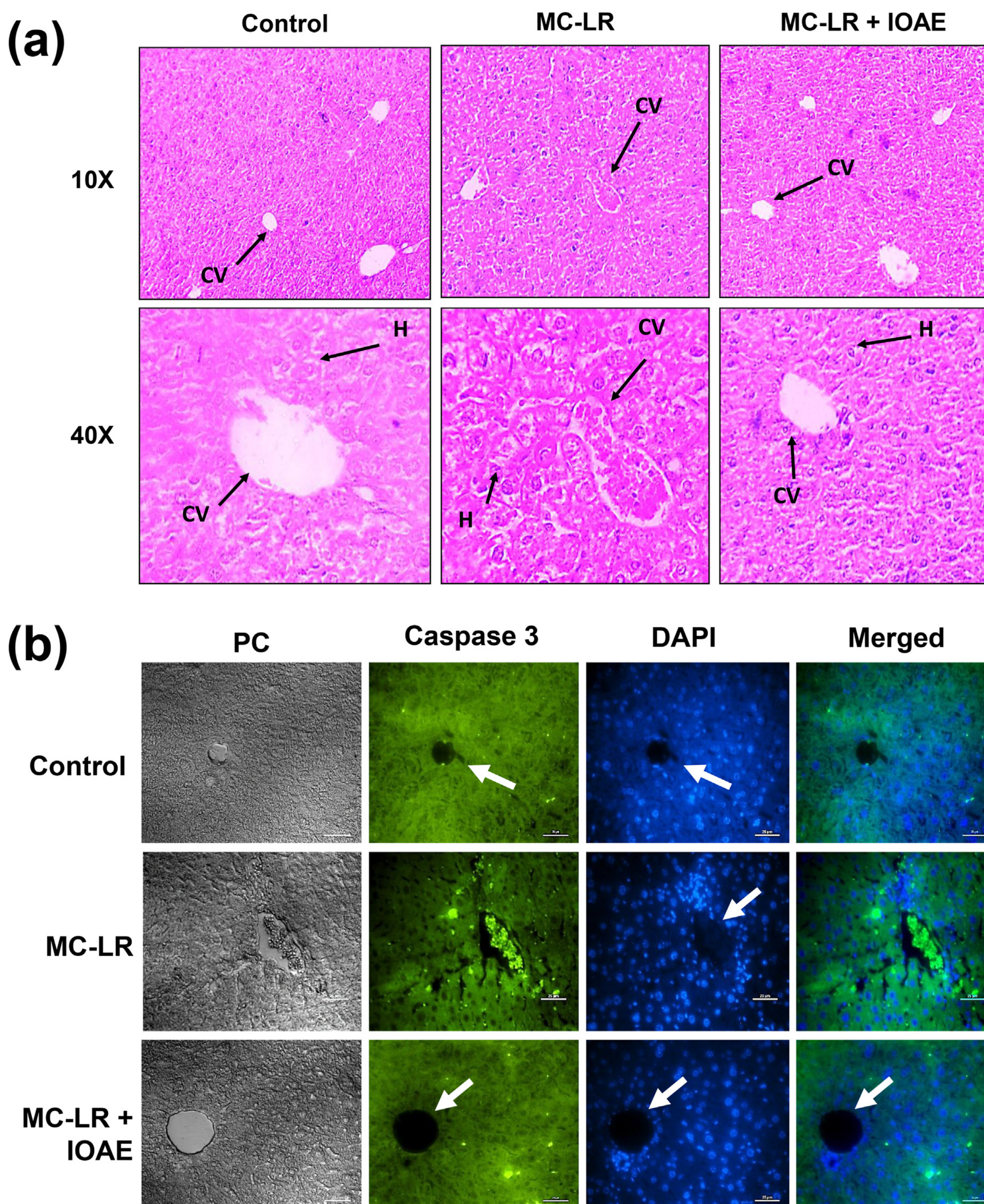

Fig. 2. (a) Protective effects of IOAE on MCLR-induced histological changes in liver. Histological examination of IOAE-treated liver exposed to MCLR was analyzed by staining with H&E and imaged under light microscopy at 10X and 40X magnification. CV, central vein; H, hepatocyte. T. S. of the liver of the control mice showing normal appearance of liver tissue with normal hepatic cord pattern, nuclei are located centrally in hepatocytes. T. S. of the liver of the mice treated with MC-LR showing dilation of central vein, vacuoles and hyalinization of hepatocyte). T. S. of the liver of mice treated with MC-LR and IOAE showing normal hepatocytes with well-arranged hepatic cords, large round nuclei in most cells. **(b) Immunofluorescence assessment of MC-LR-induced caspase-3 and effects of IOAE**. Liver sections were treated with caspase-3 antibody followed by FITC-labelled goat anti-mouse secondary antibody. Tissue sections were counterstained with DAPI. Imaging was performed for phase-contrast and immunofluorescence images under Confocal Laser Scanning Microscope at 40X magnifications. Red arrows denote increased expression and the white arrow shows central veins. PC, phase-contrast; DAPI, 4′,6-diamidino-2-phenylindole. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

hepatic cord pattern, and hepatocytes. While mice exposed to MC-LR alone showed disturbed liver pathology showing widespread apoptotic cells, hemorrhage, cytoplasm shrinkage and condensed chromatin, degeneration of hepatic cells more pronounced and severe liver lesion showed congestion and necrotic areas (Fig. 2a). These results suggested that MC-LR induced significant damage to the liver probably by generation of ROS. Furthermore, the treatment of MC-LR-exposed mice with IOAE showed improved histopathological structures. IOAE treated mice

showed reduced areas of necrosis and hemorrhaging, and more normal tissue structures as compared with MC-LR group. We also observed that the hemorrhage in liver cells was reduced by IOAE treatment which indicates that IOAE is able to prevent histopathological damages of hepatic cells caused by microcystin.

H&E-stained microscopic examinations of MC-LR exposed liver sections revealed the cord-like parenchymal architecture was lost mainly in the perilobular regions. Similar observations were observed by other study with extended hepatocellular injury by MC-LR as characterized by condensed microvesicular steatosis of hepatocytes, diffuse focal necrosis, pyknotic nuclei, and degeneration in the pancreatic acinus with necrotic cells [Atencio et al., 2008]. Microcystins induced different enzymatic alterations and histopathological changes in liver, as shown by treating fish with microcystin variants MC-LR and MC-RR at a dose of 500 μg/kg. Microcystins caused significant changes in the activities of acid and alkaline phosphatases in liver and kidneys [Atencio et al., 2008]. Microcystin treatment has been reported to cause pathological lesions in hepatic tissues, such as megalocytosis, necrotic process, and microvesicular steatosis [Hooser et al., 1991]. Collectively these observations suggest that limiting the oxidative stress may improve the biochemical and histological alterations in liver as induced by environmental toxicants like microcystins.

3.6. IOAE suppressed the expression of caspase-3 in MC-LR exposed mice liver

Apoptosis may contribute to the MC-LR-induced hepatic injury in mice apart from oncotic necrosis in hepatocytes [Batista et al., 2003; Woolbright et al., 2017]. Apoptosis is amongst the most common mechanisms for the deleterious effects of MC-LR in tissues. MC-LR induced cell death in several animal models with characterization of elevated levels of ALT and AST, and hemorrhagic necrosis [Chen and Xie 2016; Kleppe et al., 2015]. Expression of caspase 3 in liver tissue is often enhanced during the conditions of inflammation and injury. MC-LR when treated to the primary rat hepatocytes showed that apoptosis induction as well as MC-LR (120 μg/kg) administration to C57BL/6 J mice caused apoptotic cell death in hepatocytes [Woolbright et al., 2017]. MC-LR increased the expression of capase-3 in the liver. Its treatment to human hepatocytes showed positive staining with propidium iodide which is an indicator of apoptosis as indicated by plasma membrane instability [Woolbright et al., 2017], and cytoskeletal disorganizations [Batista et al., 2003]. Thus, targeting apoptosis or necrosis in liver cells may be a critical mechanism for preventing microcystin-induced liver injury.

Therefore, we measured the expression of caspase-3 by immunofluorescent staining of liver sections in different treatment groups. Liver tissue sections were treated with caspase-3 antibody followed by FITC-labelled secondary antibody. The tissue sections were counterstained with DAPI, and imaging was performed for phase-contrast and immunofluorescence images under Confocal Laser Scanning Microscope at different magnifications (Fig. 2b). Results show that MC-LR exposed mice showed an increased expression of caspase-3 in liver tissues as observed by caspase-3-FITC fluorescence patches as compared to control. While treatment of MC-LR exposed mice with IOAE showed a reduced immunofluorescence of caspase-3 in liver sections, as compared to that of MC-LR group. Likewise, the MC-LR exposed liver sections showed morphed nuclear structures when counterstained with DAPI. These also showed swollen and larger sized nuclei, and nuclear content fragmentation in MC-LR exposed liver section. These nuclear signs were indicative of apoptosis and necrosis induction under influence of MC-LR, while these nuclear changes were lowered to larger extent in MC-LR exposed mice when treated with IOAE.

These results clearly indicate that treatment of IOAE significantly lowered the caspase-3 expression which corroborates to the apoptosis preventing effects of IOAE in MC-LR induced liver toxicity in mice. MC-LR has shown the sensitivity to caspase activation especially exogenous activation of caspase-3, indicating the cell death process, and undergoing necrosis [Batista et al., 2003; Fladmark et al., 1999]. Thus, MC-LR induced apoptosis and necrosis are apparent fundamental mechanisms in clinically relevant animal models. This is even more striking that caspase-3 inhibition may prevent apoptosis induced by MC-LR toxicity, which was observed by administration of IOAE in mice model. These observations further correlate with the anti-inflammatory and antioxidant effects of IOAE in inflammatory liver injury and hepatotoxicity by environmental toxicant microcystin.

3.7. Effect of microcystin-LR on growth of hepatic cells

Normal hepatic cells (AML12: alpha mouse liver 12 cells) were cultured and the effect of MC-LR was assessed on cell growth using MTT assay. AML 12 cells were exposed to increasing levels of MC-LR for 48 h. The cytotoxic effect of MC-LR on AML 12 mouse hepatocytes is shown in Fig. 3a. Cell viability percentage values of MC-LR treated cells were compared to the control (100% cell survival) to determine any significant differences. The percentage for cell viability were recorded at different concentrations of MC-LR (μg/mL). Cell viability percentage was high at low concentrations of MC-LR. The cell viability decreased on increasing the concentration of MC-LR with 5% cell viability at concentration of 10 μg MC-LR/mL as that of control 100% Cell viability. The IC_{50} was calculated to be 3.6 μg/ml, indicating that MC-LR is chronic toxic to AML 12 mouse hepatocytes.

3.8. *Inonotus obliquus* compound ergosterol peroxide interacts with NF-κB

In this computational study model, we examined that how *Inonotus obliquus* may interact with the genes and proteins in cell growth and inflammation regulatory pathways. To perform this study, a compound already purified and characterized from the extract of Chaga mushroom was selected from our previous report [Kang et al., 2015]. The test compound Ergosterol Peroxide was shown to be a potent anti-inflammatory and anticancer agent in the model of intestinal inflammation and colorectal cancer [Kang et al., 2015]. Ergosterol Peroxide was shown to downregulate the β-catenin and NF-κB signaling pathways in colorectal cancer cells and inhibited cell growth and inflammation. We examined that how *Inonotus obliquus* interacts with NF-κB through molecular docking tools. NF-κB regulates the expression of genes involved in many processes that play a key role in the development and progression of cancer such as proliferation, migration, and apoptosis. Therefore, molecular modeling experiment was planned. The PDB ID: 4DN5 [Liu et al., 2012] corresponding to the crystal structure of NF-κB-inducing kinase (NIK) was screened from the database using autodock software. The NIK is the critical component in the non-canonical NF-κB signaling pathway. Aberrant activation of NIK is associated with several disorders including inflammation and cancers. The crystal structure of NIK shows a complex with adenosine 5'-O-(thiotriphosphate) at a resolution of 2.5 Å, R-Value Free: 0.222, and R-Value Work: 0.182 [Liu et al., 2012]. This truncated NIK protein is a catalytically active structure with an N-terminal extension of 60 amino acid residues with a kinase domain. As the NIK is a constitutively active kinase, it leads to constitutive non-canonical NF-κB signaling with enhanced B-cell adhesion and apoptosis resistance [Liu et al., 2012]. The molecular docking and binding simulation study of NF-κB with *Inonotus obliquus* compound Ergosterol Peroxide interactions are shown in Fig. 3b.

The docking results show that Ergosterol Peroxide represents numerous binding sites in NF-κB NIK where the binding residues were multiple in number. In the study, we used molecular dynamics simulations to evaluate the stability and dynamics of the interactions in the proposed Ergosterol Peroxide-NIK complex. Ergosterol Peroxide from *Inonotus obliquus* was identified to be an anticancer drug agent that may act via interacting with NF-κB [Kang et al., 2015]. Ergosterol Peroxide-NIK binding was evaluated mainly by constructive binding mode of Ergosterol Peroxide on NF-κB binding sites with better harmony with human protein crystal structure. Protein 4DN5 interaction with

Fig. 3. (a) Toxicity of MC-LR on AML 12 mouse hepatocytes. The cells were treated with serial dilutions of MC-LR (0–10 μg/mL). Cell viability was measured by MTT assay as described in the methodology. Absorbance readings were taken from each well of the 96-well plate and cell survival was counted as percentage viable cells as compared to control. The control cell viability was set to 100%. **(b) Molecular docking study for interaction of Ergosterol Peroxide with NF-κB-inducing Kinase (NIK)**. The crystal structure of NF-κB-inducing Kinase (NIK) with PDB ID: 4DN5 from *Homo sapiens* [Liu et al., 2012] was used in docking study.

Ergosterol peroxide showed a sound binding affinity of −9.5. In the molecular docking phase Ergosterol Peroxide packed against the amino acid residues ARG 363, GLY364, SER 365, ARG366, ARG368, SER361 and PRR463. Thus, the docking interaction of NIK with ergosterol peroxidase could reveal the molecular basis for the effects of IOAE in liver injury model created in this study by using MC-LR.

4. Conclusions

Environmental toxicant microcystins have shown the capability to accumulate in the tissues and cause hepatotoxicity in mammals [Greer et al., 2018; McLellan and Manderville 2017]. Persistent exposure of MC-LR can lead to severe hepatic injury and liver carcinoma by causing liver cell death due to cytoskeletal disorganization, lipid peroxidation, cellular disruption, cell blebbing, loss of membrane integrity, DNA damage and hemorrhagic shock. This study examined the preventive effects of chaga mushroom extract on MC-LR-induced liver toxicity which showed notable reduction in body weight of mice and significant histopathological alterations. MC-LR caused significant elevation of the LFT markers. The mechanism behind deleterious effects of MC-LR may include induction of oxidative stress as observed by alteration in GSH and catalase levels in the liver. MC-LR increased the levels of LDH in liver suggesting altered oxidative metabolism and mitochondrial dysfunction [Ait Abderrahim et al., 2021; Li et al., 2015]. IOAE was found to be effective against MC-LR by restoring the physiological and biochemical alterations. IOAE inhibited the oxidative stress in mice and prevented the apoptotic response. The cell viability decreased on increasing the concentration of MC-LR with 5% cell viability at concentration of 10 μg MC-LR/mL as that of control 100% Cell viability. MC-LR induced apoptosis in hepatocytes which was retained by IOAE treatment. MC-LR showed chronic toxicity to AML 12 mouse hepatocytes with IC_{50} 3.6 μg/ml. While exploring the molecular mechanism for the effects we utilized molecular docking interaction of NF-κB-NIK with ergosterol peroxidase, a single compound from chaga mushroom extract [Kim et al., 2015]. Molecular docking studies showed the binding interaction between NF-κB-NIK with ergosterol peroxide which indicates the plausible molecular mechanism associated with the effects of IOAE. Collectively, the observations suggest that chaga supplementation exerted antioxidant and anti-inflammatory effects in reversing the alterations caused by MC-LR in the liver of mice. Yet a limitation of this study remains to explore other single molecules apart from ergosterol peroxide and to identify specific molecular mechanism. Therefore, the data presented add a significant contribution in the field of environmental toxicity especially for cyanobacterial blooms exposed areas or populations worldwide and its natural prevention by using chaga mushroom.

CRediT authorship contribution statement

Pir Mohammad Ishfaq: Performed primary experiments, Data analysis, Wrote the initial manuscript draft. **Shivani Mishra:** Performed primary experiments, Data analysis. **Anjali Mishra:** Performed supporting experiments. **Zaved Ahmad:** Performed supporting experiments. **Shovanlal Gayen:** Performed molecular docking experiments. **Subodh Kumar Jain:** Supervised overall research work, revised the manuscript. **Swati Tripathi:** Supervised overall research work, revised the manuscript **Siddhartha Kumar Mishra:** Conceptualized the research work, edited the manuscript and supervised overall research work.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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