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Immunotoxicity of cynobacterial toxin Microcystin-LR is mitigated by Quercitin and himalaya tonic Liv52

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ABSTRACT

Microcystin-LR (MC-LR) has received worldwide concern for its hepatotoxicity with maximum acceptable daily intake of 0.0015 mg/L (1.5 μg/L) [Federal-Provinicial-Territorial-Committee-on-drinking-water-2002]. Comprehensive immunotoxicity data is still deficient with MC-LR. To curb the menace of MC-LR, Quercitin (QE), himalaya made hepatotonic Liv52 were studied. To investigate the immunotoxic properties of MC-LR, QE and Liv52, primary splenocyte cells prepared, cultured, and immunoproliferation assay with mitogens lipopolysaccharide (LPS) or concanavalin A, (Con A) was done for, immunophenotyping, cell cycle and apoptotic studies. In current study, we have divided the splenocytes into 4 groups, i.e., Group I: Normal saline, Group II: MC-LR (0.1 μM), Group III: MC-LR (0.1 μM) + QE (20 μM), and Group IV: MC-LR (0.1 μM) + Liv52 (25 μg/ml) and treated with maximum $< CC_{50}$ concentration. MC-LR enhanced proliferation of Con A and LPS stirred splenocytes at 24 h, whereas QE and Liv52 both act as antimitogenic. With combined mixture of MC-LR + QE, a significant increase in proliferation compared to mitogen or MC-LR was observed. MC-LR down-regulated expression of $CD19^+$, CD3e+, $CD4^+$, $CD8^+$, (1.05%), (18.9%), (8.9%), and (7.8%) respectively in comparison to Group I. Down-regulation of 10% and 28% is observed in $CD19^+$ and $CD4^+$ populations with MC-LR and QE. The Liv52 addition concealed MC-LR adverse properties in most effective way. MC-LR induced G1-phase significant declined cell cycle arrest at S phase (9.26%) and G2/M phase (26.31%) was observed. QE and Liv52 mask the activity of MC-LR. Further apoptotic study revealed that MC-LR treatment decreases late apoptotic cells compared to control with no significant change in live and early apoptotic cells. Although QE increased live cells and Liv52 significantly increased late apoptotic cells, these results suggest that a $<CC_{50}$ concentration (0.1 μM) of MC-LR may have immunosuppressive activity. Liv52 is found to be a more potent drug to counteract this cyanotoxin.

1. Introduction

MC-LR a toxic cynobacteria compound is normally released into stored freshwater by *Microcystis aeruginosa* commonly originated as a result of eutrophication (Carmichael et al., 2001). Excessive production, combined with the ability to withstand higher temperatures (up to 300 °C), leads MC-LR to build-up of high concentrations in the environment, posing potential sources of exposure for humans, animals, fish, and birds, primarily through the consumption of contaminated seafood, vegetables, and drinking water (Chen et al., 2016) (Organization, 2020). In aquatic ecosystems, MC-LR is known to be a potential environmental stress inducer and a potential health hazard. It is a secondary metabolite that threatens human health by contaminating drinking water and food chains (Stone and Bress, 2007) (Prabir and Katrina, 2021). MCs (microcystins) are mainly of two types as per literature first one is hepatotoxins which are cyclic heptapeptides and second one is neurotoxins mostly alkaloid (Carmichael and Boyer, 2016). Hepatotoxins are either seven amino acids ringed peptides (five common amino acids and two variable L-amino acids leucine/arginine at position 1, 2 in ring), called microcystins or five amino acids are known as nodularins secreted from *Microcystis* sp. and *Nodularia* sp. respectively. Microcystins structure is cyclo (-D-Ala-X-D-Me Asp-Z-Adda-D-Glu-Mdha-), X and Z represents L-amino acids, D-Me Asp is D-erythro-B-methylaspartic acid, and Mdha is N-methyldehydroalanine (Carmichael and Boyer, 2016) (van der Merwe, 2014) (Chorus and Welker, 2021). The toxicity in the microcystin cyclic structure is imparted by Adda, (2S, 3S, 8S,

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9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4, 6-dienoic acid. Approximately more than 12 genera has been reported among those main genera are *Anabaena, Microcystis, Nodularia, Nostoc and Oscillatoria* (Prabir and Katrina, 2021). Other genera that produces hepatotoxin that were not characterized yet are *Aphanizomenon, Cylindorspermopsis, Coelospherium and Gloeotrichia*. (Prabir and Katrina, 2021) (Carmichael and Boyer, 2016) (van der Merwe, 2014). MC-LR inhibits the activities of phosphatases PP1 and PP2A which lead to excessive protein phosphorylation, which leads to cytoskeleton misregulation, cell proliferation, and apoptosis (Komatsu et al., 2007). MC-LR has been linked to a variety of toxicities, including hepatotoxicity, nephrotoxicity, reproductive toxicity, growth, and developmental toxicity, immunotoxicity, and neurotoxicity (Li et al., 2021). Humans can exhibit symptoms after exposure to cyanotoxins, including fever, headaches, muscular and joint aches, blisters, stomach cramps, diarrhoea, vomiting, mouth ulcers, and allergic reactions. These effects might emerge from minutes to days after exposure, Seizures, hepatic failure, respiratory arrest, and rarely death in severe cases. It is considered neurotoxins, hepatotoxins, and dermatoxins that are harmful to the nervous system, liver, and skin (Al-Khazraji et al., 2022a). New studies of effects in other systems, including hematologic, renal, cardiac, reproductive, and gastrointestinal effects, have been published (Yoshida et al., 1998a). Long-term exposure to low levels of microcystins has even shown to enhance the cell proliferation leading to hepatic cancer (Moore, 2023). The immune system has been reported to be constituted by a variety of immune cells and several soluble mediators that help the body to defend itself. Lymphoproliferation assays, Immunophenotyping, Cell cycle phase distribution, Apoptosis assay, etc. provide an in-depth view to understand the effect of toxins on the immune system (Yadav et al., 2016). However, the study of immunomodulatory properties of MC-LR is still in prefatory stage. Prior studies disclosed destruction instigated by MC-LR can be restricted by antidotes, free radical hunters, antioxidant enzymes, and inhibitors and regulators of calcium channels (Hermansky et al., 1991). Catechins earlier used oxidative stress reducer and averter of changes caused due to MC-LR (Xu et al., 2007). Left-handed flavonol and silymarin found more effective against liver toxicity provoked by MC-LR (Jayaraj et al., 2007).

Contemporary scientific studies focus on herbal formulations and different bioactive compounds that can be potential future drugs for different problems (Al-Khazraji et al., 2022a) (Maoyuan et al., 2021). This includes Flavonoids namely anthoxanthins, anthocyanidins, flavonols/flavans/flavan-3-ols, flavanones, and isoflavonoids (Shrinet et al., 2021). Quercetin (QE) a flavonol commonly found in almost all the fruits and vegetables that are consumed on daily-hood is capable antioxidant and can promote the expression of anti-apoptotic proteins to control toxin-induced apoptosis (Zhang et al., 2014). To compare the effect of QE, Liv.52 a well-known commercially available formulation was included in this study. The formulation contains a high concentration of pharmacologically active phenolic compounds. It is a hepatotonic with a hepatoprotective role against xenobiotic-induced hepatic damage, and detoxification activity and is common usage in the treatment of liver disorders in both human and animal models (Al-Khazraji et al., 2022b). The US EPA has emphasized the need to investigate immunotoxicological responses of toxins present in water bodies. In the current study, we paid attention on the in-vitro consequences of MC-LR on T-cell and B-cell responsiveness and on the mechanism involved in the immunotoxicity by inspecting MC-LR provoked changes in the lymphocytes such as its proliferation, cell cycle distribution, and inclding cell deaths.

2. Material methods

2.1. Chemicals and reagents

MC-LR was purchased from Algal Chem Inc. with purity ≥95% (Taipei City, Taiwan). Lipopolysaccharide (LPS), propidium iodide (PI), Concanavalin A (Con A), FITC (Fluorescein isothiocyanate), AnnexinV, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), and all the reagents used were procured from Sigma Chemical Co (St. Louis, MO). RPMI-1640 media was purchased from Gibco, Thermo Fisher Scientific (USA) antibodies such as Anti-CD19-Alexafluor700, Anti-CD3e-APC-Cy7, Anti-CD4-FITC, and Anti-CD8-APC, etc. for immunophenotyping were procured from BD Biosciences (San Diego, California). Tritiated thymidine was acquired from Amersham Life-science (Uppsala, Sweden).

2.2. Animal ethics statement

The authentic and approved procedures for animal care and use were followed as mentioned in the ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines (du Sert et al., 2020); the Institutional Animal Care guide lines and Ethical Committee of Institute of Science, Banaras Hindu University, Varanasi approved procedures were followed during the experiments. All animals were kept at 12 h light and dark cycle in the animal house the temperature of is always maintained at 25 ± 2 °C also *ad libitum* water and appropriate foods were provided consistently (Yadav et al., 2016). All the experimentations were done according to the protocol followed in the institutional practices categorically approved by the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animal), Government of India (2007) (CPCSEA Registration No.- F.Sc./88/IAEC/2016–17/1628 dated Feb 27, 2017).

2.3. Splenocyte culture

Single mouse was sacrificed each time innocuously according to the guidelines and protocol (Yadav et al., 2016) provided by the Institutional Animal Ethical Committee, Institute of Science, BHU, India. Spleens were removed and washed in cold phosphate-buffered saline (4 °C), and the cell suspension was prepared in RPMI 1640 medium by mincing spleen tissues. The RBCs were lysed and removed by using RBC lysis buffer (0.15 M NH_4Cl, 1 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.4). Later on the cells were rinsed twice with incomplete medium before centrifugation (300×*g*). The cells pellets were resuspended in complete RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% antibiotic solution maintained at 37 °C temperature with 5% CO_2. 2×10^5 cells/well in 100 μl was incubated overnight for growth and adaptation.

2.4. Cytotoxicity assays

MTT assay was done to assess the cytotoxicity of MC-LR, Quercetin, and Liv52 on primary splenocytes (Mosmann, 1983). The cells (2×10^5 cells/well in 100 μl) were exposed to various concentrations of the MC-LR (0.1 μM), QE (20 μM), and Liv52 (25 μg/ml)). MTT (0.5 mg/ml concentration) was added to the cell culture plate after 24 h of incubation and incubation was extended for another 2 h. The culture supernatant was discarded and the adhered cell monolayer cells with matrix was dissolved in 100 μl DMSO (dimethyl sulfoxide) and read plate was read at 550 and 660 nm wavelength in an Elisa plate reader (BioTek Instruments Inc, Vermont, USA).

2.5. Groups

The treatment groups were based on the results of the cytotoxicity assay and divided into four groups of primary splenocytes with the highest non-cytotoxic that is $<CC_{50}$ concentration. Group I: Normal saline, Group II: MC-LR (0.1 μM), Group III: MC-LR (0.1 μM) + QE (20 μM), and Group IV: MC-LR (0.1 μM) + Liv52 (25 μg/ml).

2.6. Lymphoproliferation assays

Con-A and LPS stimulators were added to invigorate blastogenesis and proliferation in lymphocyte T and B cells populations in single cells splenocyte culture respectively. Final concentration of 5 μg/ml of ConA and 10 μg/ml of LPS was added to the cultured monolayer splenocytes along with test compounds and reincubated for 72 h at 37 °C in CO_2 incubator. The absorption of tritiated thymidine compounds in spleenocyte cells was used to assess the relative fold proliferation of cells. 18 h before the endpoint, tritiated thymidine (2 μCi/ml) was given to the cultures. The cells were subsequently harvested using glass fiber filters a Nunc cell harvester. The filters containing cells were transferred to scintillation cocktail-W (Sisco Research Laboratories Pvt. Ltd) for β-counts and to record data on the β-counter (Hewlett-Packard, Palo Alto, CA).

2.7. Immunophenotyping

The highest non-cytotoxic $< CC_{50}$ concentration for cells was used to treat the different groups. After 24 h incubation the splenocytes were harvested and used for the immunophenotyping studies. Cell surface specific marker antibodies were used to label splenocytes to identify and separate populations of lymphocyte B and T cells in all treated groups. Anti-CD19, FITC-conjugated anti-CD4, APC-conjugated CD8, and APC-Cy7-conjugated anti-CD3e antibodies were used for the cell surface staining for 20 min keeping on ice with staining buffer (2% FBS, 0.1% sodium azide in PBS). The properly stained splenocyte single cells were twice washed using wash buffer (0.1% sodium azide in PBS), centrifuged to make pellet and later re-suspended in 500 μl PI solution (1 μg/ml). The resuspended samples of splenocyte cells were kept on ice for 1 h and then examined with a flow cytometer. The analysis data were acquired by gating the $CD19^+$ and $CD3^+$ splenocyte cells populations in a dot plot showing living cells. $CD3^+$ subpopulations were categoricaly separated from $CD4^+$ and $CD8^+$ subpopulations.

2.8. Cell cycle phase distribution

Splenocytes that had been treated with different groups of compounds were washed twice and fixed in 70% ethanol for 2 h keeping at −20 °C. Fixed cells (1×10^5 cells) were again washed twice in first in PBS and then stained for 30 min with PI & Triton X-100 PBS solution (2 mg RNase A free of DNase and propidium iodide (PI) 200 μl of l mg/ml added in 10 ml of 0.1% (v/v) Triton X-100/PBS) before being examined in a flow cytometer (BD FACS Calibur, BD, NJ, USA) and cell fluorescence was measured.

2.9. Apoptosis

For apoptotic assay experiments, splenocytes from the control and treated groups were directly tagged with annexin V-FITC and P). The splenocyte cells were twice washed using ice kept cold PBS then centrifuged and pellet were resuspended in 1X Binding Buffer (0.1 M HEPES/NaOH (pH 7.4), 1.4 M NaCl, and 25 mM $CaCl_2$) at 1×10^5 cells/ml final concentration. Transfer 100 μl of the resuspended cells with concentration of 1×10^5 cells to a 2 ml culture tube. 5 μl each from FITC Annexin V (1 μg/ml) and PI (100 μg/ml) were added to the samples. After a gentle vortexing, the reagents were added into the resuspended cells to incubate at room temperature (RT) at 25 °C in the dark for 15 min. Finally 400 μl of 1X Binding Buffer was added to each sample containing tube and flow cytometry (BD FACS Calibur, BD, NJ, USA) was performed within 1 h.

2.10. Data analysis

Graph Pad Software Inc. Prism version 9 was applied for statistical analyses. The data is presented as a mean standard deviation. Analysis of variance was used to examine the data (one-way ANOVA Tukey test). Significance statistical value is $p < 0.05$.

3. 3. Results

3.1. Cytotoxicity assay

Cytotoxicity assay for MC-LR, QE, and Liv-52 showed a $<CC_{50}$ concentration -dependent response. The non-cytotoxic $< CC_{50}$ concentration was determined separately for MC-LR, QE, and Liv-52. The $< CC_{50}$ obtained was 0.1 μM, 20 μM, and 25 μg/ml respectively. The $< CC_{50}$ concentration of MC-LR (1 μM–10 μM), QE (50 μM–1000 μM) and Liv-52 (50 μg/ml – 100 μg/ml) were found toxic to the cells (Fig. 1). Therefore, in whole experiments the non-toxic $< CC_{50}$ concentration of MC-LR, QE & Liv-52 were used to study the functional response of both T and B lymphocyte cells.

3.2. Lymphoproliferation assays

MC-LR (0.1 μM), the non-cytotoxic dose that is $<CC_{50}$ concentration was used for the treatment of splenocytes showed insignificant stimulation and increase in proliferative responses on ConA (8.9%) and LPS (1.4%). Similar way in the QE (20 μM), and MC-LR (0.1 μM), showed increased proliferation response in the presence of both mitogens. We observed MC-LR along with QE up-regulates or enhanced the functional responses of T and B lymphocyte cells as compared to MC-LR alone. The MC-LR treated splenocytes in combination with Liv-52 (25 μg/ml) decreased or down-regulated the proliferative response on ConA or LPS stimulation and suppressing the lymphocyte response (Fig. 2).

3.3. Immunophenotyping

We observed that presence of MC-LR affected the B and T lymphocytes relative distribution in the cultured splenocytes (Fig. 3A and B). MC-LR diminished the relative percentage of $CD3^+$ cells (18.9%) in group II significantly, but a slight reduction in $CD19^+$ cells (1.05%) was observed. In group III $CD3^+$ cells were insignificantly affected by MC-LR while significant decrease (10.1%) of $CD19^+$ splenocyte cells population was observed compared to group II. However, Liv52 masked the effects of MC-LR in group IV, resulting in enhanced the B (2.6%) and T (28.8%) cells population compared to group II (Fig. 3C and D). The effects in each group were further resolved the T cells into $CD4^+$ and $CD8^+$ subpopulations. In group II, MC-LR causes the reduction of $CD4^+$ (8%) and $CD8^+$ (7.8%) cell population. But in group III, QE and MC-LR down-regulated the $CD4^+$ cells (26%) percentage high significantly but $CD8^+$ cells (0.4%) remained unaffected. Where as, in the group IV, MC-LR with Liv52 enhanced the both $CD4^+$ (19.7%) and $CD8^+$ (28.5%) percentage (Fig. 3E and F).

3.4. Cell cycle phase distribution

The cell cycle analysis of MC-LR treated splenocyte cells in respect to untreated control, reflected alterations in different cell cycle phases (Fig. 4A–D). MC-LR induced G1 phase cell cycle arrest but relative percentage of cell cycle phase significantly decreased in the S phase (9.26% ↓) and G2/M phase (26.31% ↓). The effects of MC-LR were masked by QE and Liv-52. The MC-LR supplemented with QE showed relatively decreased percentage of the G1 phase (6.7%↓) and subsequently significantly increased percentage of arrest in both the S phase (10.91%↑) and G2/M phase (48.54%↑). Similarly, MC-LR and Liv-52 treated cells illustrated a significantly decreased relative percentage of the G1 phase (12.3%↓) with significant increase in the S phase (31%↑) and G2/M phase (76%↑) (Fig. 4E–H).

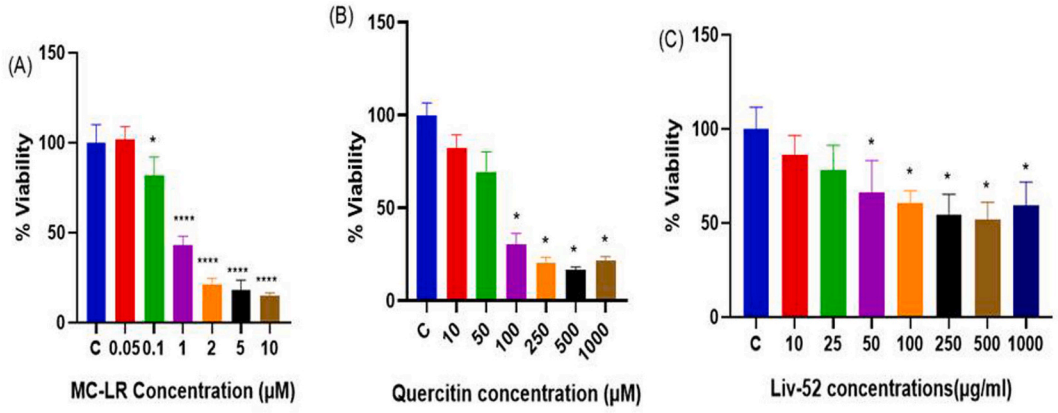

Fig. 1. Cytotoxicity assay of MC-LR (A), QE (B) and Liv52 (C) on splenocyte cultured cells. The MTT assay experiment was performed after incubation of splenocyte cells with cynobacterial MC-LR for 24 h. Data given represents mean ± SEM of three experiments (in each experiment single mouse was used) in respect to untreated cells (percent control) (*$p < 0.05$, ****$p < 0.0005$ significant with respect to the control).

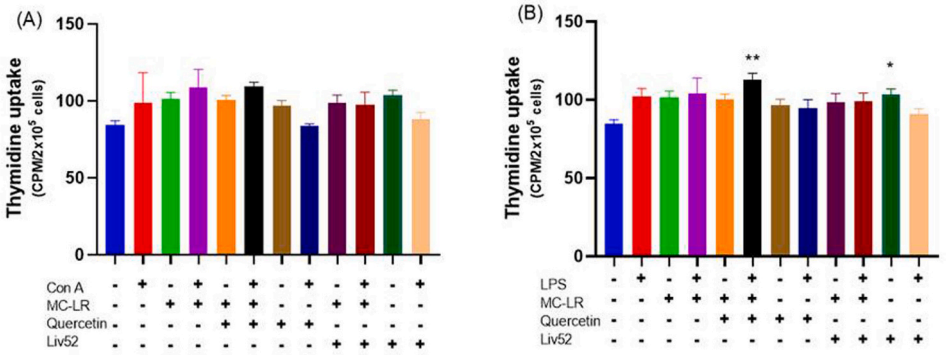

Fig. 2. Proliferative assay of splenocyte cells in the presence of MC-LR (G-II) in the presence of (A) Con A and (B) LPS and in combitions with QE (G-III) and Liv52 G-(IV). Splenocytes single cell suspension cultured with or without Con A (5 mg/ml) or LPS (10 mg/ml) in combinations of MC-LR incubated for 72 h were used for proliferation and [3H] thymidine uptake assay. Data represent mean ± SEM of 5 sets (*$p < 0.05$ & **$p < 0.005$ significant with respect to control).

3.5. Apoptosis assay

The early and late apoptotic assay of splenocyte cells was done by conventional flow cytometry using two types of labeling dyes Annexin V-FITC and PI. The unstained (Annexin V-FITC $^-$/PI) determines the presence of viable cells while due to a lack of plasma membrane integrity, the early apoptotic cells exhibited different characteristic of Annexin V-FITC$^+$/PI$^-$ staining patterns, The cells in late apoptotic phase exhibited Annexin V-FITC$^+$/PI$^+$ patterns of staining (Fig. 5A). In this study late apoptotic cells were found lower in numbers in MC-LR-treated cells (Group-I) (17.9%) than in control (Group-II) (21.33%), however, no major change was observed in necrosis, early apoptosis, and live cells. Splenocytes exposed with MC-LR and QE (Group-III) showed a significantly higher percentage of live cells (57.7%) whereas both early (25.46%) and late (15.22%) apoptotic cells were reduced in culture. When Group-IV was compared with control, late apoptotic cells increase was (25.9%). However, overall percentage of necrotic cells was unchanged in all treated groups (Fig. 5B).

3.6. 4. Discussion

in vitro screening of compounds for risk assessment turns out to be a preferable option rather than *in vivo* animal studies for assessing immunotoxicity. However, in the search for alternative to animal models, novel *in vitro* approaches have emerged, which, apart from saving time and effort; offer an advantage over *in vivo* studies. The European Centre for the Validation of Alternative Methods (ECVAM) also promotes methods that reduce the use of laboratory animals to a bare minimum (Gennari et al., 2005).

Although various researches on the toxicity of MC-LR have been published, less information on immunological activity and counter application of QE as well as Liv52 is known. The current study was carried out to investigate the immunomodulatory role of MC-LR and combinations with its counteracting natural bioactive compounds on splenocytes via lymphoproliferative assay, immunophenotyping, cell-cycle study, and apoptotic study. Cultured splenocytes were exposed to different $< CC_{50}$ concentration of MC-LR, QE, and Liv52 for 24 h to determine their immunotoxic potential. MTT cell viability assays showed the highest non-cytotoxic $< CC_{50}$ concentrations of hepatotoxic MC-LR along with hepatoprotective QE, and Liv52. The reported non-toxic dose is 1ug/L in the drinking water set by the WHO for MC-LR (Chorus, 1999). These non-toxic $< CC_{50}$ concentrations of MC-LR, QE and Liv52 i.e. 1 μM, 20 μM, and 25 μg/ml respectively were used for further immunotoxic experiments because there cytotoxic concentration ($<CC_{50}$) for survival for 50% cells population was lower although significant decrease in the proliferation of the cells have been noted. The consequences of a MC-LR was evaluated on the mitogen-stimulated lymphoproliferation response that indicats lymphocyte functional activity. This test is used for the testing of Immunotoxicity (Kaminski et al., 2008) (Descotes, 2006) of MC-LR toxicant. We observed that MC-LR acts as a mitogen for both types of lymphocytes T and B cells to enhance the proliferation of splenocyte cells upon stimulation with well known recommended stimulator Con A and LPS synergistically. However, presence of QE or the reference hepatoprotective drug Liv52 with MC-LR

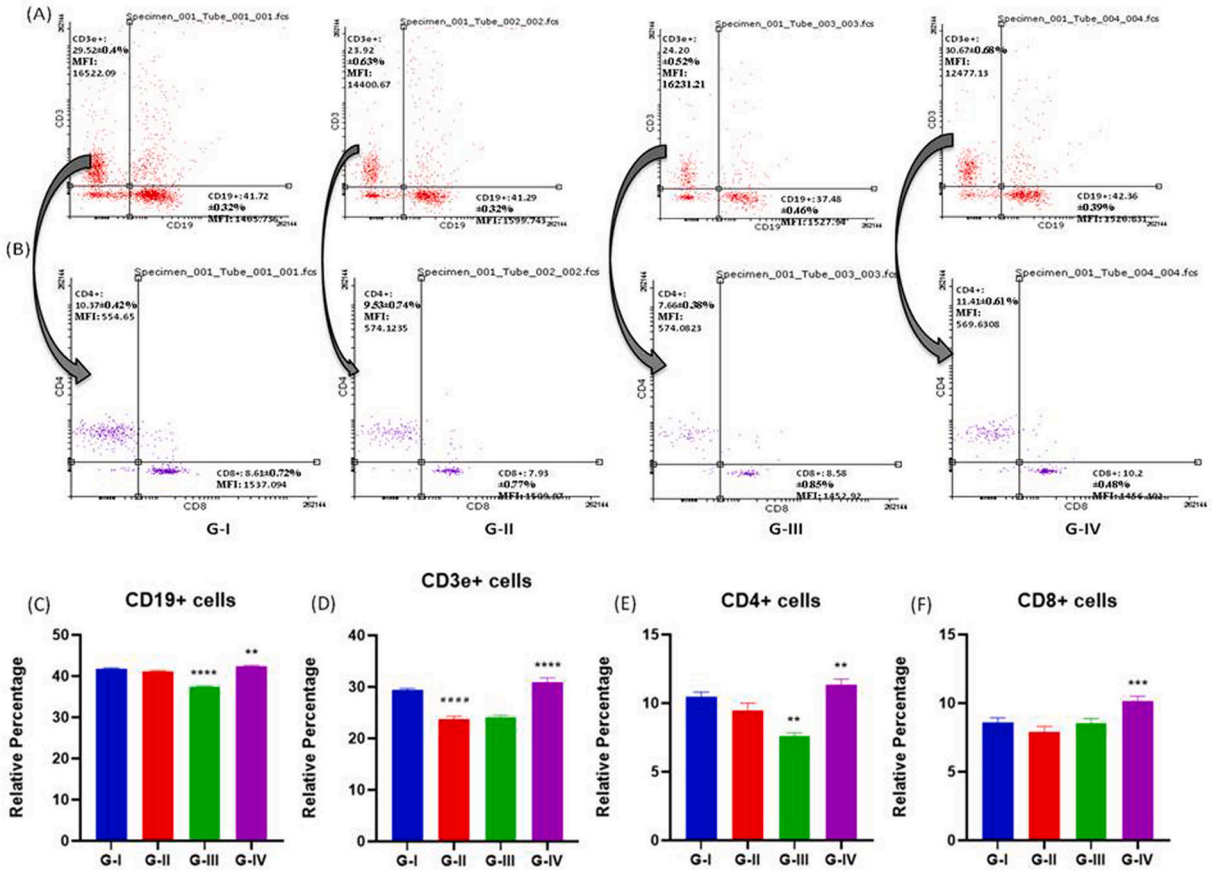

Fig. 3. (A) Relative percentage of B-cells ($CD19^+$) and T-cells (CD3e+) upon exposure to only MC-LR (G-II), MC-LR + QE (G-III) and MC-LR + Liv52 (G-IV) on the sub-populations of $CD4^+$ and $CD8^+$ lymphocyte cell populations. (B) Histogram representation of Flow cytometric data from a typical experiment is shown. The values represent mean ± SE of four experiments while the corresponding graph represents the relative percentage (C–F) ($^{\#\#\#\#}p < 0.00005$ significant with respect to control). ($^{**}p < 0.005$, $^{***}p < 0.0005$ & $^{****}p < 0.00005$ significant with respect to MC-LR).

produced no changes in the expression when compared with MC-LR alone, but masking the effect of Con A. As lymphocyte activation and proliferation are required for adaptive immune responses, adequate proliferation of splenocyte cells in response to a mitogenic stimulators is considered as an indicator of immunity expression (Gao et al., 2005).

The immune cells dysfunction is primarily assesed by phenotyping assay of lymphocytes cells (Schwulst et al., 2008). The different types of immune cells conatin specific cell surface markers, to represent a reliable indicator of their specific activation phenotype. Some cell surface markers has been found to express upon the activation of immune cells as reported by earlier scientists (Mesquita Júnior et al., 2010). Alarcón et al. (2003) reported CD3e as an unique T-cell and TCR complex marker (Mesquita Júnior et al., 2010). The interaction of CD3e to the MHC complex on APC (antigen-presenting cells), enables the receptor complex to send out signals [] to be assessed during the FACS analysis. CD4/CD8 co-receptors specific molecules are known to initiate TCR signal transduction pathway to modulate TCR signaling (Pitcher and Van Oers, 2003). The down regulation in the CD4/CD8 co-receptors expression has been noticed by the MC-LR inhibitor leading to decrease in the T-cell co-stimulation, activation, and proliferation culminating into the decrease in the relative expression of co-receptor molecules CD3e, CD4, and CD8 in spleenocyte cells as the interruption in theTCR complex formation might be due to the inhibition of factors responsible for the proliferation of CD3e, CD4 and CD8 lymphocyte cells. The statement is very well supported by the earlier reports that is related about the expression profile of certain cytokines such as IL-2 and IL-6 followed by decreased expression of T and B-lymphocytes proliferation (Shi et al., 2004), (Welten et al., 2020) In current study, the decline in the expression of T-cell receptors and CD3e co-receptors is in concomitant decrease in the expression of overall receptors number of $CD4^+$ and $CD8^+$ lymphocyte cells depicting the MC-LR mediated immunotoxicity in the splenocytes. QE couldn't masked the impact of MC-LR on splenocyte cells that is on the numbers of CD3e cells but a significant down-regulation in $CD4^+$ cells and negligible change in $CD8^+$ cells was observed that might be due to expression of certain specific cytokines that regulates or we can say affects the expression and proliferation of these immunological factors that is CD3e, $CD4^+$ and $CD8^+$ (Welten et al., 2020).

A cell grows and divides to create a copy of itself during the highly regulated and conserved cell cycle. When cells experience DNA damage, a wide variety of specific check-point kinases is expressed and activated, which causes the cell cycle halt to give chance to lesions are to be repaired. During the G1 phase of the cell cycle decisions for DNA replication and the finalization of cell division are to be determined (Smith-Garvin et al., 2009). Therefore, we investigated that how in current study MC-LR and its combinations with QE and Liv52 affect the cell cycle progression. After 24 h, a significant G1 arrest was observed when MC-LR was exposed to the splenocytes but the presence of combinations QE and Liv52 mask the consequence of MC-LR. Masking effects of MC-LR to cause G1 arrest by QE and Liv52 might be due to the binding of QE with MC-LR. In contrast in another study no significant changes was observed after the treatment of MC-LR on HepG2 cells after treatment up to 24 h (Díez-Quijada Jiménez et al., 2020). But S-phase cell cycle arrest was reported on Chinese hamster ovary cells, in response to MC-LR treatment (Zhang et al., 2016). Overall it is hypothesized that the treatment of MC-LR caused the cell growth

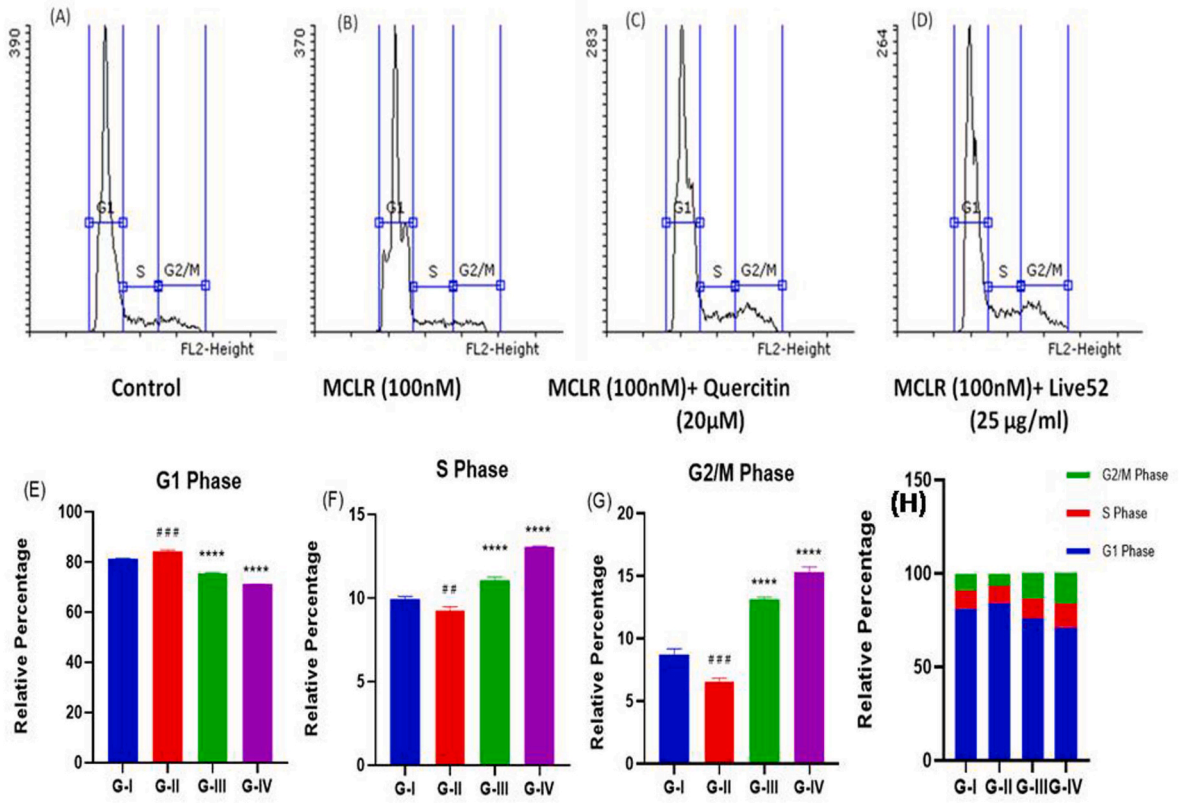

Fig. 4. Cell cycle phase distribution of only MC-LR treated (G-II) and with combination of QE (G-III) and Liv5 (G-IV) 2 was assesed upon 72h of incubation. Flow cytometric analysis was done of PI labeled cells (A, B, C and D). Histogram representation of Cell cycle different phases is represented (E, F, G and H) with mean ± SEM of three experiments (in each experiment single mouse was used) in respect of the percent of untreated cells. (Significant $^{\#\#\#}p < 0.005$, with respect to control and $^{****}p < 0.0005$ with respect to MC-LR).

inhibition that may be due to cell cycle arrest at different phases but mainly due to G1 phase arrest that stops the progression of cell cycle to the S-phase which is DNA replication phase.

In multi-cellular organisms, the process of controlled cell death is called apoptosis. It is necessary for several processes, such as healthy cell turnover, proper development, and drug-induced cell death. Uncontrolled apoptosis, triggered by some medications and chemicals, radiation, and other factors different disease conditions can result in cancer, autoimmune disorders, neurodegenerative diseases, and ischemic damage (Fuchs and Steller, 2011). It has been widely established that MC-LR exposure can cause abnormally excessive levels of oxidative stress, resulting in induced apoptosis in mammalian cells (Amado and Monserrat, 2010). According to Jiang et al. after 12 h of treatment, MC-LR could significantly induce apoptosis in carp liver cells (Jiang et al., 2013). The centrilobular regions of mice given sub-lethal toxin < CC_{50} concentration showed a detectable intensity of staining and significant concentrations of apoptotic cells (Yoshida et al., 1998b). We also obtained consistent results that showed an increased number of early and late apoptosis cells when MC-LR treatment was given. However, when QE added along with MC-LR the combination, increased the number of live cells significantly after treatment of 24 h.

4. Conclusions

Overall outcomes demonstrated that the MC-LR has a slight proliferative response on splenocytes, whereas QE shows synergy with MC-LR and significant immunomodulation can be detected by the proliferation of splenocytes. MC-LR exposure affects the functional response of Lymphocyte T and B-cells directly leading to the activation of overall the adaptive and innate immune systems, corroborated by the arrest of cell cycle at the G1 phase and the synergy of QE or Liv52 governed the S & G2/M phase arrest. Moreover, in MC-LR exposed splenocytes, a population of T-cells with the low expression level of CD4 and CD8 co-receptors was present, although the relative distribution of T-cells was significantly higher in the synergy with Liv52. But MC-LR also induces apoptosis, but QE masks the effect and leads to significantly increased numbers of live cells. Furthermore, the mechanistic study deciphered the MC-LR induced immunosuppression was due to the modulation of expression patterns and expression levels of various activation receptors, co-receptors, co-stimulatory molecules, including various regulatory cytokines, responsible to play a vital role to activate and regulate particularly adaptive immunity. For further study it would be interesting to characterize population getting affected and also to learn more about MC-LR-induced immunomodulation on animal system. Though much in-depth research at molecular level is necessary to apprehend the immunomodulatory properties role of MC-LR, the current findings suggest an immunosuppressive response of MC-LR that may increase the susceptibility of the exposed population to disease.

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Author's contribution

Kriti Shrinet: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization. Arvind Kumar: Conceptualization, Methodology, Investigation, Resources, editing, Project administration, Supervision.

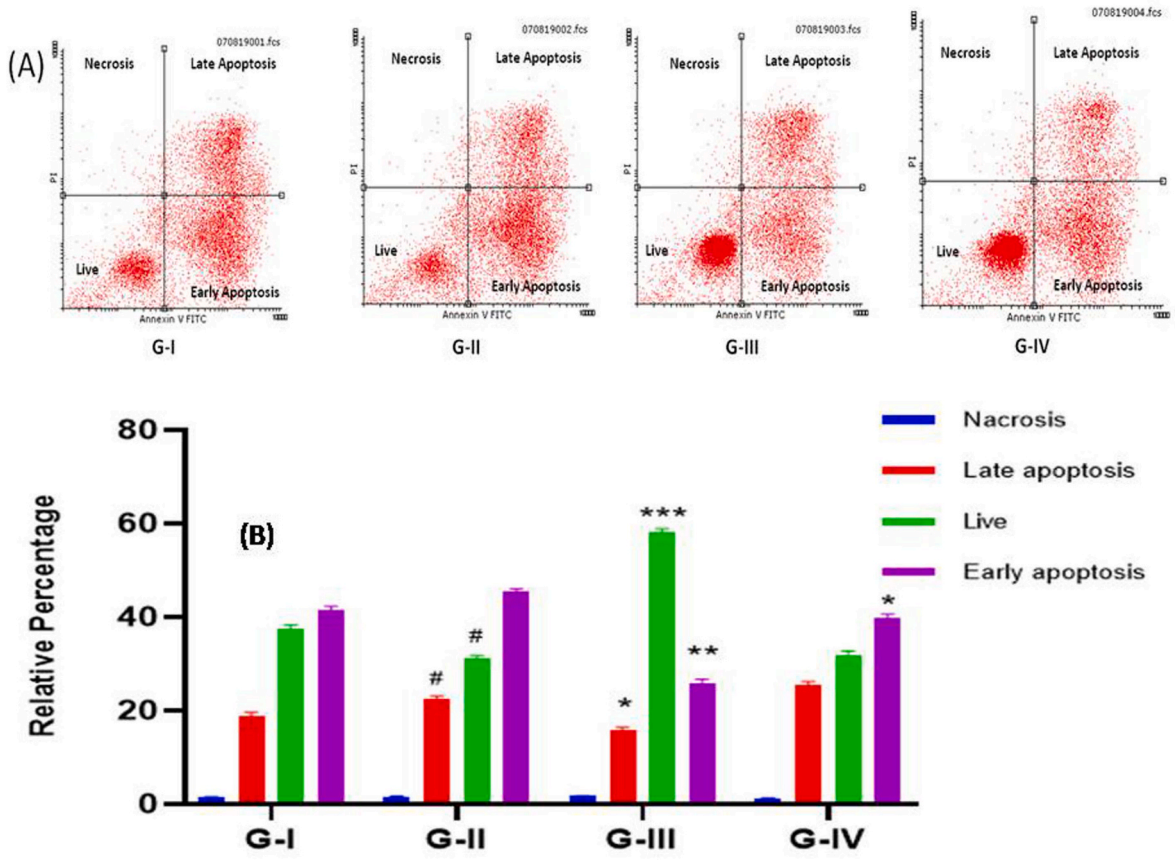

Fig. 5. (A) Assesment of apoptosis & Necrosis using Flow cytometry and Annexin V-FITC/PI staining of splenocytes cells treated and stained for 48 h(B) Histogram representation of necrotic, late apoptotic, early apoptotic and live cells percentage in MC-LR (G-II) and in combination with quercetin (G-III) and Liv52 (G-IV) treated group in relative to control. ($^{\#}p < 0.002$, significant with respect to (G–I) control $^{*}p < 0.05$, $^{**}p < 0.005$ & $^{***}p < 0.0005$ with respect to (G-II) MC-LR.

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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.

Data availability

No data was used for the research described in the article.

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References

Al-Khazraji, K.A., Khalid, A., Hashim, M.K., Hashim, M.K., Abdulla, S.K., Abdulla, M.K., Khudhair, I.H., Abbas, W.K., 2022a. Role of Liv. 52 in non-infectious chronic liver disease. Global J. Health Sci. 14, 1–76. https://ideas.repec.org/a/ibn/gjhsjl/v14y2022i7p76.html.

Al-Khazraji, K.A., Khalid, A., Hashim, M.K., Hashim, M.K., Abdulla, S.K., Abdulla, M.K., Khudhair, I.H., Abbas, W.K., 2022b. Role of Liv. 52 in non-infectious chronic liver disease. Global J. Health Sci. 14, 1–76. RePEc:ibn:gjhsjl:v:14:y:2022:i:7:p:76.

Alarcón, B., Gil, D., Delgado, P., Schamel, W.W.A., 2003. Initiation of TCR signaling: regulation within CD3 dimers. Immunol. Rev. 191, 38–46. https://doi.org/10.1034/j.1600-065x.2003.00017.x.

Amado, L.L., Monserrat, J.M., 2010. Oxidative stress generation by microcystins in aquatic animals: why and how. Environ. Int. 36, 226–235. https://doi.org/10.1016/j.envint.2009.10.010.

Carmichael, W.W., Boyer, G.L., 2016. Review health impacts from cyanobacteria harmful algae blooms: implications for the north American great lakes. Harmful Algae 54, 194–212. https://doi.org/10.1016/j.hal.2016.02.002.

Carmichael, W.W., Azevedo, S.M., An, J.S., Molica, R.J., Jochimsen, E.M., Lau, S., Rinehart, K.L., Shaw, G.R., Eaglesham, G.K., 2001. Human fatalities from cyanobacteria: chemical and biological evidence for cyanotoxins. Environ. Health Perspect. 109, 663–668. https://doi.org/10.1289/ehp.01109663.

Chen, L., Chen, J., Zhang, X., Xie, P., 2016. A review of reproductive toxicity of microcystins. J. Hazard Mater. 301, 381–399. https://doi.org/10.1016/j.jhazmat.2015.08.041.

Chorus, I., 1999. J. Bartram Toxic Cyanobacteria in Water: a Guide to Their Public Health Consequences, Monitoring, and Management. E & FN Spon, London. https://doi.org/10.1201/9781003081449.

Chorus, I., Welker, M., 2021. Toxic Cyanobacteria in Water: A Guide to Their Public Health Consequences, Monitoring and Management, second ed. CRC Press. https://doi.org/10.1201/9781003081449.

Descotes, J., 2006. Methods of evaluating immunotoxicity. Expet Opin. Drug Metabol. Toxicol. 2, 249–259. https://doi.org/10.1517/17425255.2.2.249.

Díez-Quijada Jiménez, L., Hercog, K., Štampar, M., Filipič, M., Cameán Fernández, A.M., Jos Gallego, Á.M., Žegura, B., 2020. Genotoxic effects of cylindrospermopsin, microcystin-LR and their binary mixture in human hepatocellular carcinoma (HepG2) cell line. Toxins 12, 778. https://www.mdpi.com/2072-6651/12/12/778#.

du Sert, N.P., Ahluwalia, A., Alam, S., Avey, M.T., Baker, M., Browne, W.J., Clark, A., Cuthill, I.C., Dirnagl, U., Emerson, M., Garner, P., Holgate, S.T., Howells, D.W., Hurst, V., Karp, N.A., Lazic, S.E., Lidster, K., MacCallum, C.J., Macleod, M., Pearl, E. J., Petersen, O.H., Rawle, F., Reynolds, P., Rooney, K., Sena, E.S., Silberberg, S.D., Steckler, T., Würbel, H., 2020. Reporting animal research: explanation and elaboration for the arrive guidelines 2.0. PLoS Biol. 18 https://doi.org/10.1371/JOURNAL.PBIO.3000411.

Fuchs, Y., Steller, H., 2011. Programmed cell death in animal development and disease. Cell 147, 742–758. https://doi.org/10.1016/j.cell.2011.10.033.

Gao, J., Lauer, F.T., Dunaway, S., Burchiel, S.W., 2005. Cytochrome P450 1B1 is required for 7, 12-dimethylbenz (a)-anthracene (DMBA) induced spleen cell immunotoxicity. Toxicol. Sci. 86, 68–74.

Gennari, A., Ban, M., Braun, A., Casati, S., Corsini, E., Dastych, J., Descotes, J., Hartung, T., Hooghe-Peters, R., House, R., 2005. The use of in vitro systems for evaluating immunotoxicity: the report and recommendations of an ECVAM workshop. J. Immunot. 2, 61–83. https://doi.org/10.1080/15476910590965832.

Hermansky, S.J., Stohs, S.J., Eldeen, Z.M., Roche, V.F., Mereish, K.A., 1991. Evaluation of potential chemoprotectants against microcystin-LR hepatotoxicity in mice. J. Appl. Toxicol. 11, 65–73. https://doi.org/10.1002/jat.2550110112.

Jayaraj, R., Deb, U., Bhaskar, A.S.B., Prasad, G., Rao, P.V.L., 2007. Hepatoprotective efficacy of certain flavonoids against microcystin induced toxicity in mice. Environ. Toxicol. An Int. J. 22, 472–479. https://doi.org/10.1002/tox.20283.

Jiang, J., Shan, Z., Xu, W., Wang, X., Zhou, J., Kong, D., Xu, J., 2013. Microcystin-LR induced reactive oxygen species mediate cytoskeletal disruption and apoptosis of hepatocytes in Cyprinus carpio L. PLoS One 8, e84768. https://doi.org/10.1371/journal.pone.0084768.

Kaminski, N.E., Kaplan, B.L.F., Holsapple, M.P., 2008. Toxic responses of the immune system. Casarett & Doull's Toxicology The Basic Science of Poisons 12, 485–555.

Komatsu, M., Furukawa, T., Ikeda, R., Takumi, S., Nong, Q., Aoyama, K., Akiyama, S., Keppler, D., Takeuchi, T., 2007. Involvement of mitogen-activated protein kinase signaling pathways in microcystin-LR–induced apoptosis after its selective uptake mediated by OATP1B1 and OATP1B3. Toxicol. Sci. 97, 407–416. https://doi.org/10.1093/toxsci/kfm054.

Li, B., Liu, Y., Zhang, H., Liu, Y., Liu, Y., Xie, P., 2021. Research progress in the functionalization of microcystin-LR based on interdisciplinary technologies. Coord. Chem. Rev. 443, 214041 https://doi.org/10.1016/j.ccr.2021.214041.

Maoyuan, J., Feiya, S., Zhen, Z., Xiao, M., Tianhui, G., Chaomei, F., Peng, L., 2021. *Andrographispaniculata* (Burm.f.) Nees and its major constituent andrographolide as potential antiviral agents. J. of Ethnopharmaco. Elsevier 272, 113954.

Mesquita Júnior, D., Araújo, J.A.P., Catelan, T.T.T., de Souza, A.W.S., Cruvinel, W. de M., Andrade, L.E.C., da Silva, N.P., 2010. Immune system-part II: basis of the immunological response mediated by T and B lymphocytes. Rev. Bras. Reumatol. 50, 552–580. https://doi.org/10.1590/S0482-50042010000500008.

Moore, C., 2023. Invited Perspective: Decentralized CyanoHAB Monitoring Is Needed to Understand Public Health Risks, vol. 131. https://doi.org/10.1289/EHP12948.

Organization, W.H., 2020. Cyanobacterial Toxins: microcystinsGuidelines for Drinking Water Quality and Guidelines for Safe Recreational Water Environments, vol. 6. WHO/HEP/ECH/WSH/2020. https://apps.who.int/iris/bitstream/handle/10665/338066/WHO-HEP-ECH-WSH-2020.6-eng.pdf.

Pitcher, L.A., Van Oers, N.S.C., 2003. T-cell receptor signal transmission: who gives an ITAM? Trends Immunol. 24, 554–560. https://doi.org/10.1016/j.it.2003.08.003.

Prabir, K.K., Katrina, C., 2021. Recent trends in the detection of freshwater cyanotoxins with a critical note on their occurreence in Asia. Trends in Environmental Analytical Chemistry 32, e00150. https://doi.org/10.1016/j.teac.2021.e00150.

Schwulst, S.J., Muenzer, J.T., Chang, K.C., Brahmbhatt, T.S., Coopersmith, C.M., Hotchkiss, R.S., 2008. Lymphocyte phenotyping to distinguish septic from nonseptic critical illness. J. Am. Coll. Surg. 206, 335–342. https://doi.org/10.1093/toxsci/kfi176.

Shi, Q., Cui, J., Zhang, J., Kong, F.X., Hua, Z.C., Shen, P.P., 2004. Expression modulation of multiple cytokines in vivo by cyanobacteria blooms extract from Taihu Lake, China. Toxicon 44, 871–879. https://doi.org/10.1016/j.toxicon.2004.08.010.

Shrinet, K., Singh, R.K., Chaurasia, A.K., Tripathi, A., Kumar, A., 2021. Bioactive compounds and their future therapeutic applications. In: Nat. Bioact. Compd. Elsevier, pp. 337–362. https://doi.org/10.1016/B978-0-12-820655-3.00017-3.

Smith-Garvin, J.E., Koretzky, G.A., Jordan, M.S., 2009. T cell activation. Annu. Rev. Immunol. 27, 591–619. https://doi.org/10.1146/annurev.immunol.021908.132706.

Stone, D., Bress, W., 2007. Addressing public health risks for cyanobacteria in recreational freshwaters: the Oregon and Vermont framework. Integr. Environ. Assess. Manag. An Int. J. 3, 137–143. PMID: 17283602.

van der Merwe, D., 2014. Freshwater cyanotoxins. In: Biomarkers Toxicol. Elsevier, pp. 539–548. https://doi.org/10.1016/B978-0-12-404630-6.00031-2.

Welten, R.D., Meneely, J.P., Elliott, C.T., 2020. A comparative review of the effect of microcystin-LR on the proteome. Expo Health 12, 111129. https://doi.org/10.1007/s12403-019-00303-1.

Xu, C., Shu, W.-Q., Qiu, Z.-Q., Chen, J.-A., Zhao, Q., Cao, J., 2007. Protective effects of green tea polyphenols against subacute hepatotoxicity induced by microcystin-LR in mice. Environ. Toxicol. Pharmacol. 24, 140–148. https://doi.org/10.1016/j.etap.2007.04.004.

Yadav, A., Kumar, A., Das, M., Tripathi, A., 2016. Sodium benzoate, a food preservative, affects the functional and activation status of splenocytes at non cytotoxic dose. Food Chem. Toxicol. 88, 40–47. https://doi.org/10.1016/j.fct.2015.12.016.

Yoshida, T., Makita, Y., Tsutsumi, T., Nagata, S., Tashiro, F., Yoshida, F., Sekuima, M., Tamura, S.-I., Harada, T., Maita, K., 1998a. Immunohistochemical localization of microcystin-LR in the liver of mice: a study on the pathogenesis of microcystin-LR-induced hepatotoxicity. Toxicol. Pathol. 26, 411–418. https://doi.org/10.1177/019262339802600316.

Yoshida, T., Makita, Y., Tsutsumi, T., Nagata, S., Tashiro, F., Yoshida, F., Sekuima, M., Tamura, S.-I., Harada, T., Maita, K., 1998b. Immunohistochemical localization of microcystin-LR in the liver of mice: a study on the pathogenesis of microcystin-LR-induced hepatotoxicity. Toxicol. Pathol. 26, 411–418. https://doi.org/10.1177/019262339802600316.

Zhang, H., Wu, Y., Fang, W., Wang, D., 2014. Regulatory effect of quercetin on hazardous microcystin-LR-induced apoptosis of Carassius auratus lymphocytes in vitro. Fish Shellfish Immunol. 37, 278–285. https://doi.org/10.1016/j.fsi.2014.02.015.

Zhang, S., Liu, C., Li, Y., Imam, M.U., Huang, H., Liu, H., Xin, Y., Zhang, H., 2016. Novel role of ER stress and autophagy in microcystin-LR induced apoptosis in Chinese hamster ovary cells. Front. Physiol. 7, 527. https://doi.org/10.3389/fphys.2016.00527.