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STUDY OF PURIFIED MICROCYSTIN-LR TOXICITY ON BIOCHEMICAL MARKERS IN BLOOD AND LIVER OF *CYPRINUS CARPIO* DURING ACUTE EXPOSURE

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Abstract

The results of present study showed, Study of purified hepatopeptide toxicity on biochemical markers in *Cyprinus carpio* during acute exposure, the purification of microcystin-LR from *Anabaena circinalis* was partially purified by using silica gel, then highly purified and collected by preparative high performance liquid chromatography, the concentration of purified microcystin-LR reached to 74.832 µg/ml. *Cyprinus carpio* was intraperitoneally injection with different concentration of microcystin-LR (20, 40, 60) µg/200g per day of body weight for 96 hours. The oxidative stress in *C. carpio* was determined via measuring biochemical markers such as antioxidants defense systems such as superoxide dismutase SOD, catalase CAT, glutathione peroxidase GPx, glutathione-S-transferase GST, and glutathione GSH and Malondialdehyde MDA, reactive oxygen species ROS, the concentration of Cytochrome P450 CYP450 and enzymes of liver function such as alkaline phosphatase ALP, glutamate oxaloacetate transaminase GOT and glutamate pyruvate transaminase activities GPT, The results of presents study shown concentration of MDA as well as ROS and activities of antioxidants such as SOD, CAT, GPx, GST,GSH and the concentration of CYP450 and enzymes of liver function such as ALP, GOT and GPT were significantly increased ($P<0.05$) with increasing dose of microcystin-LR as compared with control groups.

***Keyword*:** Purification, Toxicity of hepatopeptide, Microcystin-LR, Biochemical Markers

Introduction

Microcystin-LR are biologically active cyclic hepatopeptides, hepatotoxin which are mainly produced by most of cyanobacterial species in freshwater ecosystems (Xue *et al*., 2015). They possess considerable stability for their ring structures and spacer double bonds. microcystins are cyclic heptapeptides with two variable amino acids, of which more than 100 kinds of different structural variants have been identified. The most toxic microcystins is microcystin-LR, which is considered to be the most commonly occurring, distributed and abundant (Liu *et al*.,2015; Ma *et al*., 2016). Recent studies have suggested that Adda region plays an important role in the toxicity of microcystin-LR (Svircev 2010; Zhou *et al*., 2015; Khadairi *et al*., 2017). Cellular uptake of microcystins cannot directly diffuse through plasma membrane because of high molecular weight, complex structure, specificity of cell and hydrophilic so that required adenosine triphosphate (ATP)-dependent transporter (Campos and Vasconcelos, 2010). The transporters that was responsible for microcystins to enter the liver cell including bile acid transporter organic anion transporting polypeptides, Nat-taurocholateco transporting polypeptides, organic anion transporters and organic cation transporters (Klaassen and Lu 2008).

Toxicity mechanism of microcystin occur through inhibition of serine / threonine protein phosphatase 1 and 2A by binding with enzymes catalytic subunits and lead to disrupting of homeostasis cell was playing important role in regulating phosphorylation and dephosphorylation process in cell that it is catalyzed by phosphatase and kinase this lead to cause hyperphosphorylation cytoskeleton protein and finally cause promote tumor (Xing *et al*., 2006). ATP synthase (Mikhailov *et al.;* 2003) and aldehyde dehydrogenase identified or has be as further cellular targets of microcystins (Chen *et al.*; 2006, Al-Juboori *et al*., 2017).

The exposure to microcystins can be effected on intracellular regulatory processes and signal transduction, It can cause higher phosphorylation of structural proteins, cell necrosis, mitochondrial permeability transition, mitochondrial membrane potential alteration, formation of reactive oxygen species, induction of oxidative stress, oxidative damage of DNA, modulation of apoptosis, changes in cell proliferation and cytokinesis (Mikhailov *et al.*; 2003) while microcystins transformed into less toxic products by microcystin-glutathione conjugated lead to prevent covalently binding the microcystins with protein phosphatase and eliminated from body via urines and feces (Falconer, 2006). Many people died every year after drinking water from lakes or eating seafood contaminated by microcystin-LR, therefore, the World Health Organization (WHO) has stipulated that the provisional safety guideline for microcystin-LR in drinking water was 1.0 µg/L (Ma *et al*., 2016).

Material and Methods

Culturing of *Anabaena circinalis*

Uni-algae of *A. circinalis* were culturing according to (Tredici, 2004).

Extraction and Purification of microcystin-LR

The *A. circinalis* cell are freeze-thaw, three time before extraction to disrupt the cell wall lead to easy release of microcystin from cell and lyophilized cell of (5g) from *A. circinalis* had been extracted three time by solvent mixture of water:methanol:1-butanol 75:20:5 for one hour then sonication by path sonicator for two hour and the extracts were centrifuged at 15000 rpm for 30 min at 20 Ċ and the combined of supernatant. The combined supernatants would be air-dried at 35Ċ to remove methanol and 1-butanol and to concentrate to 3ml and microcystins in each extract detected by using Ultraviolet-Spectrophotometer at 238nm. (Namikoshi *et al*.,1993).

The purification of toxins has been performed according to Namikoshi *et al*. (1993) above extract was loaded on glass column (2 × 15cm) which contained Silica gel (75-250 mesh), then the column washed by 120 ml of Deionized water followed by 20% methanol and finally, the toxins was eluted by 80% methanol with flow rate 3ml/min, then The toxins fraction has been dissolved in absolute methanol and collection of microcystin-LR by preparative

high performance liquid chromatography (Lawton and Edwards, 2001).

Experimental Designs

Common carp in weight was ranged between 200±20 g, then put 10 individual of fish in each aquarium that its filled by 70 liter of dechlorinated tap water and leave it for three days to acclimate in appreciation laboratory condition at temperature (25±2), pH (7.5±0.1) and appreciate light with continuous aeration condition by using electrical air pumps apparatus and fish was divided into four group, each group contained ten individual of fish put in aquarium then intraperitoneal injection of fish species to different concentration of microcystin-LR that collected from preparative HPLC for (20, 40, 60) µg/kg per day for four days as acute exposure and then taken the blood and liver tissue to measure biochemical markers (Prieto *et al*., 2006).

Antioxidants Defense

Superoxide dismutase activity SOD has been determined by autooxidation of Pyrogallol according to Marklund and Marklund, (1974). While Catalase activities was measured according to procedure of Clariborn, (1985) and Aebi, (1974) but The glutathione peroxidase activity was investigated according to procedure of Rotruck *et al.* (1973), glutathione-S-transferase activity was determined by the method of Habig *et al*. (1974) Glutathione activity was determined according to the method of Moron *et al*., (1979) The acid soluble sulfhydryl groups form a yellow colored complex with dithionitrobenzene (DTNB).

Lipid peroxidation and reactive oxygen species ROS

Lipid peroxidation had been estimated by the Thiobarbituric acid assay for Malondialdehyde (MDA) concentration according to Aust, (1985) and Burtis, (1999) Whereas reactive oxygen species ROS was determined according to ELISA kit of Elabscience, china.

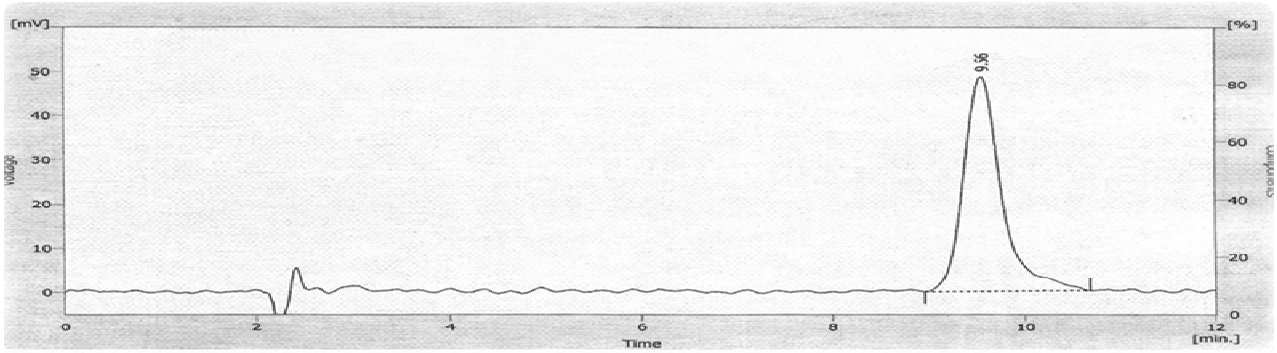
Cytochrome P540 concentration and Enzymes of liver function

Cytochrome P450 concentration had been determined by ELISA Kit BioScience alkaline and The activity of phosphatase ALT, Aspartate transferase AST and Alanine transferase ALT had been measured for supernatant of a blood and liver tissue of rats and fish by reflotron plus roche and

Results

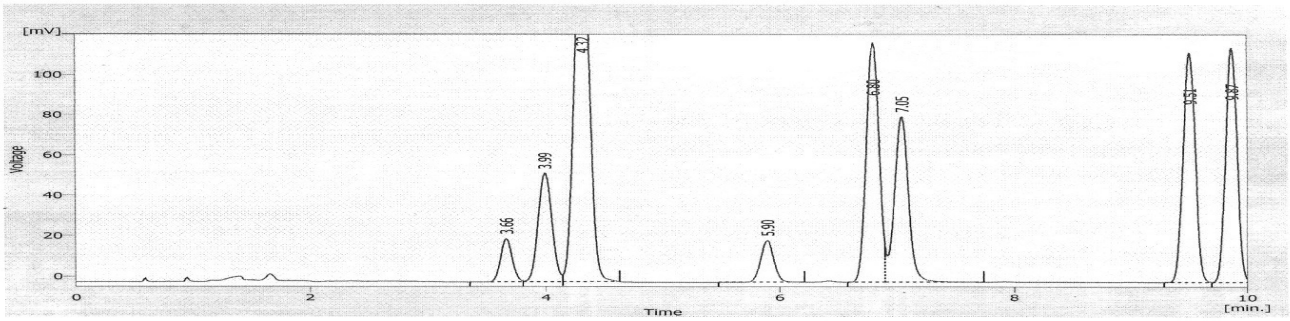
Extraction and purification of microcystin-LR

The extraction of *A. circinalis* has been extracted by water: methanol: butanol and partially purified by silica gel column, then analyzed by preparative HPLC to detect the present of microcystin-LR and toxin concentration was determined by comparing peak area and retention time of analytical standard of microcystin-LR with peak area and retention time of extraction of *A. circinalis*, the retention time of analytical standard of microcystin-LR were 9.55 min (Fig. 1) and it's concentration was 10µg/ml and *A. circinalis* retention time was 9.51min (fig. 2) and it's concentration was 74.832 µg/ml, then highly purified and collected of microcystin-LR by preparative HPLC.



	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]
1	9.557	1327.449	48.527	100.0	100.0	0.39
	Total	1327.449	48.527	100.0	100.0	

Fig. 1 : Peak of microcystin-LR standard that used in study, analyzed by preparative HPLC



	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	3.660	146.419	21.218	2.6	3.0	0.11
2	3.990	440.865	53.571	7.9	7.6	0.13
3	4.320	1603.258	179.109	28.8	25.4	0.15
4	5.897	162.746	20.357	2.9	2.9	0.13
5	6.800	894.032	118.688	16.1	16.8	0.12
6	7.047	690.062	81.712	12.4	11.6	0.13
7	9.510	827.859	113.786	14.9	16.1	0.12
8	9.870	800.429	116.298	14.4	16.5	0.11
	Total	5565.672	704.739	100.0	100.0	

Fig. 2 : Chromatography of preparative HPLC at absorbance 238nm for *Anabaena circinalis*

The effect of microcystin-LR toxin on *C .carpio*

The result of present study had been showed the biochemical markers in blood and liver of *C. carpio* during acute exposure period to microcystin-LR, The activity of SOD in blood and liver control was (2.35, 2.71)U/mg respectively, Whereas SOD activity in treatment of blood were (3.96, 4.17, 6.05) U/mg and in liver treatment were (3.98, 6.23, 8.95) U/mg in doses (20, 40, 60) µg /200g b.w respectively (Fig. 3). the significant difference was found in activity of CAT in blood and liver between control and treatments, the highest activity of CAT in blood (69.63)U/mg was recorded in dose 60 µg /200g b.w. and lowest activity of CAT (4)U/mg recorded in dose 20 µg /200g b.w when compared with control (9.12) U/mg While, the highest activity of CAT in liver(92.93)U/mg was recorded in dose 60 µg /200g b.w and lowest activity of CAT (50.63) U/mg was recorded in dose 20 µg /200g b.w. when compared with control group (28.47) U/mg (Fig. 5).

The result was showed GPx significantly change, the highest and lowest activities in blood (63.86, 38.37) µmol/ml respectively While, in liver (98.71, 51.6) µmol/ml in (60, 20) µg /200g b.w. respectively Fig. 6). The GST activity appeared significant variation between control and treatments, GST activity in control blood and liver were (27.82, 31.08)U/mg respectively While GST activity in treated blood were (37.2, 49.62, 61.83)U/mg and in treated liver were (44.73, 67.66, 78.19)U/mg in three doses respectively (Fig. 7). the GSH activity showed significant variation between control and treatments, GSH was recorded the highest and lowest activities in treated blood (85.8 - 72.3) µmol/ml respectively While, in treated liver (103.6, 88.92) µmol/ml when compared with control (27.82 and 41.42) µmol/ml respectively (Fig. 8).

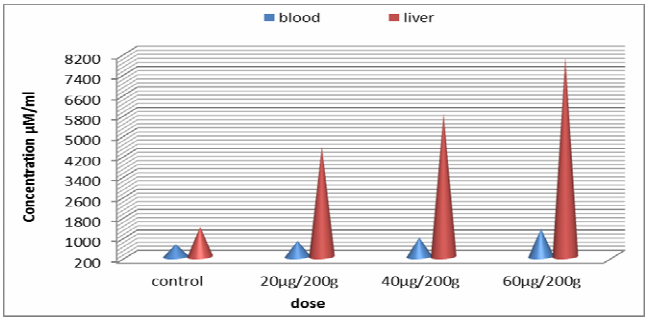


Fig. 3 : Concentration of total antioxidants in blood and liver of *C. carpio* after acute exposure period by purified microcystin-LR

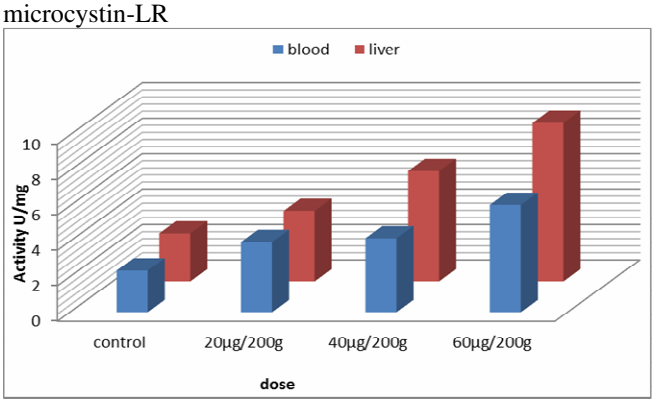


Fig. 4 : Activity of superoxide dismutase in blood and liver of *C. carpio* after acute exposure period by purified microcystin-LR

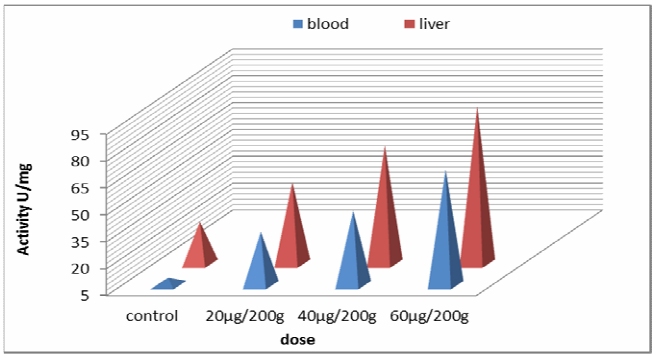


Fig. 5 : Activity of Catalase in blood and liver of *C. carpio* after acute exposure period by purified microcystin-LR

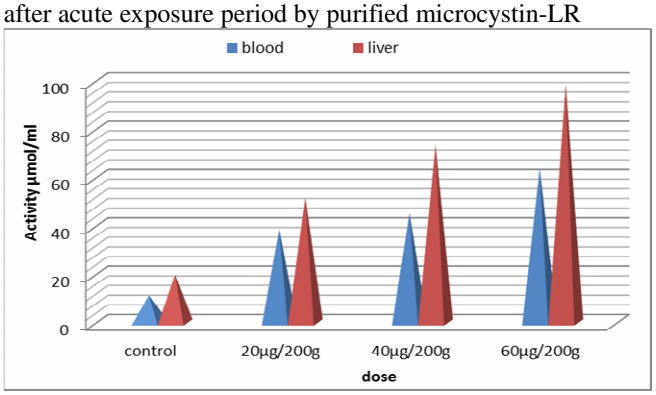


Fig. 6 : Activity of glutathione peroxidase in blood and liver of *C. carpio* after acute exposure period by purified microcystin-LR

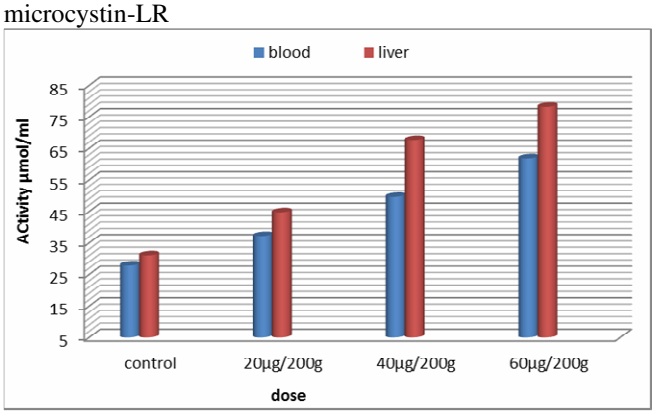


Fig. 7: Activity of glutathione-S-transferase in blood and liver of *C. carpio* after acute exposure period by purified microcystin-LR

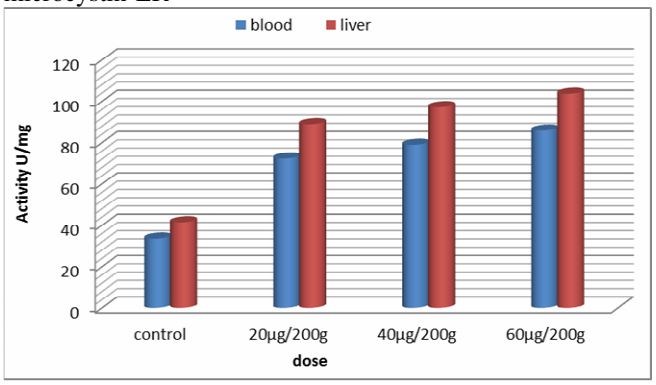


Fig. 8: Activity of glutathione in blood and liver of *C. carpio* after acute exposure period by purified microcystin-LR

The concentration of MDA in blood and liver tissue control were (0.32-0.74) µmol/ml respectively While, in treated blood were (1.61, 1.85, 2.4) µmol/ml and in treated

liver tissue were (1.75, 3.98, 4.73) µmol/ml in doses (20, 40, 60) µg /200g b.w respectively (fig. 9) ROS concentration was significantly difference between control and treatments, the highest and lowest concentration of ROS was recorded in treated blood (95.9, 58.31)P/ml While, in treated liver (172.7 - 68.12)P/ml respectively when compared with control (8.14 and 12.11) P/ml respectively (fig. 10). The Cyt.P450 concentration was presented significant variation between control and treatment, it's concentration in blood and liver control were (189.1, 422.3)P/ml respectively While, in treated blood were (241.2, 482.3, 677.1)P/ml and in treated liver were (499.4, 606.7, 778)P/ml in doses (20, 40, 60) µg /200g b.w respectively (fig. 11). The activity of enzyme liver function (Alkaline phosphatase, GOT, GPT) were recorded highest and lowest value in treated blood (165.7- 104.3) , (182.3-116.8) and (146.7 – 86.5) U/l respectively and in treated liver tissue were (157.4- 126.3) , (191.4-148) and (187.3-137) U/l respectively in doses (60, 20) µg /200g b.w. (Fig. 12).

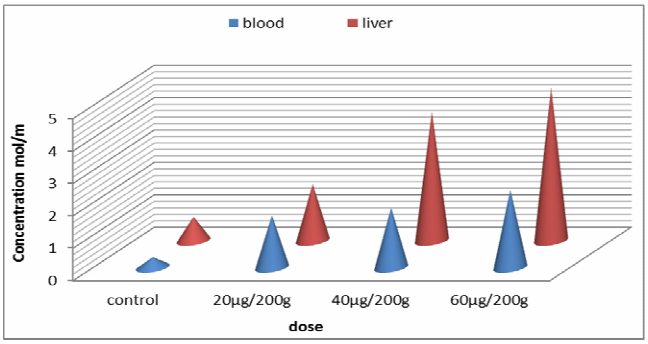


Fig. 9 : Concentration of Malondialdehyde in blood and liver of *C. carpio* after acute exposure period by purified microcystin-LR

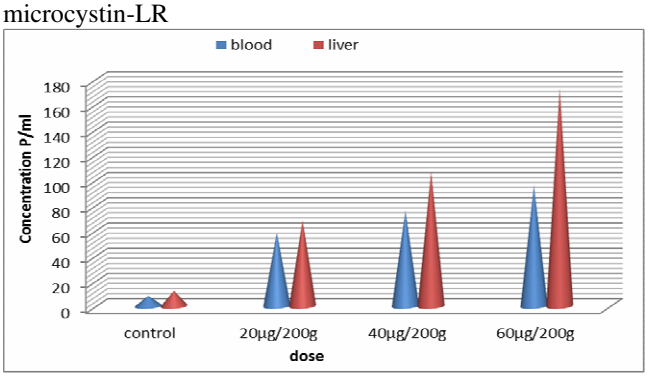


Fig. 10 : Concentration of reactive oxygen species in blood and liver of *C. carpio* after acute exposure period by purified microcystin-LR

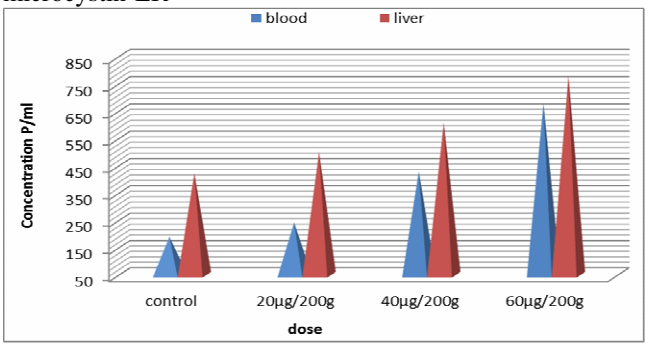


Fig. 11 : Concentration of Cytochrome P450 in blood and liver of *C. carpio* after acute exposure period by purified microcystin-LR

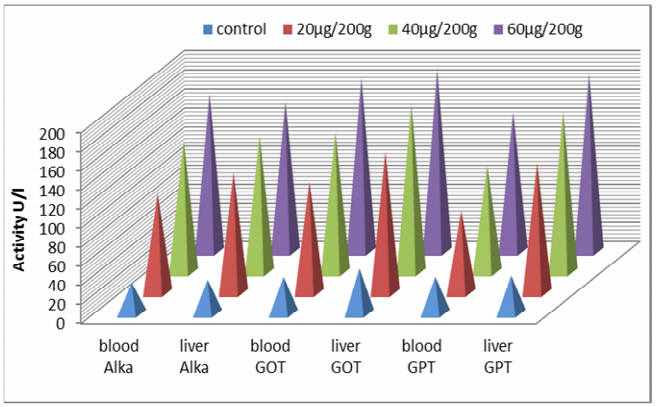


Fig. 12 : Activities of Alkaline phosphatase, glutamate oxaloacetate transaminase and glutamate pyruvate transaminase in blood and liver of *C. carpio* after acute exposure period by purified microcystin-LR

Discussion

The results of present study were revealed that microcystin-LR induce oxidative stress in *C. carpio* fish that are intraperitoneally injected during acute period, the oxidative stress occurred as result of an imbalance between oxidants and antioxidants (Kamper *et al*., 2009) or may occur due to the reactive oxygen species overproduction and to the cellular antioxidants levels decreased, therefore the free radicals and reactive oxygen species that are generated in tissue and subcellular compartment are efficiently scavenged by the antioxidant defense systems to protect the cells against DNA damage, protein oxidation and lipid peroxidation (Prieto *et al*., 2006). The results of this study shown, The liver was more affected than blood in *C. carpio* because the microcystin are potent hepatotoxins in mammals and fish (Towner *et al*., 2002; Jos *et al*., 2005).

The result of presents study was shown, the SOD activities in blood and liver of *C. carpio* significantly elevated during acute period because the SOD plays essential role in scavenging of superoxide free radical, which helps to maintain a balance between oxidants and antioxidants (Prieto *et al*., 2006). The CAT activities significantly increased in blood and liver of *C. carpio* during acute period, due to high production of reactive oxygen species such as hydrogen peroxide (Liu *et al*., 2016) this results agreement with study reported the CAT activities which known to be able to remove and convert reactive oxygen species ROS such as hydrogen peroxide to oxygen and water (Sinciska *et al*., 2006; Mohammed *et al*., 2011) and This results coincide with study of Li *et al*. (2003) showed stimulated SOD and CAT activities induced by microcystin-LR, the CAT and SOD activities increased 2.7 times compared with control during 96 hours, the activity of SOD increased in blood and liver induced by microcystin-LR (Puerto *et al*., 2009).

The GPx was catalyzed the reduction of H_2O_2 to oxygen and water by using glutathione as a substrate (Mates *et al*., 1999).the result of presents study pointed the GPx activity also significantly increase in blood and liver of *C. carpio* during acute due to the response of antioxidants systems to microcystin-LR and to reflect the adaptation to oxidative condition (Li *et al*., 2003). This results correspond with study reported increase the GPx activity in blood and liver of common carp after exposure to microcystin (Li *et al*., 2003; Mohammed *et al*., 2011). Pinho *et al.* (2005) the elevated in

SOD, CAT, and GPx activities indicate the formation of ROS in target organism due to microcystin-LR exposure.

The GST enzyme plays important roles in detoxification of microcystins via conjugation to GSH so that GST activity was used to indicate microcystins detoxification in organisms (Pflugmacher *et al*., 2006). The activity of GST after exposed to microcystins was either stimulated or inhibited in animals (Best *et al*., 2002). In the results of present study, the significant increase observed in GST activity in serum of blood and liver of *C. carpio* in acute may be due to stimulated biotransformation reactions and higher rates of microcystin-LR conjugation, and high transcription of cytosolic GST and microcystin-LRmay be on the phasell detoxification systems lead to variations in gene transcription of GST (Li *et al*., 2010).

GSH is the essential compound in the regulation of intracellular redox status and its considered as an important cofactor in many metabolic reactions (Van Bladeren, 2000). The results of presents study, observed significant elevation of GSH in both blood and liver in *C. carpio* during acute period may be due to a compensatory response induced by imbalance in the redox state of the cell as the result of excessive H_2O_2 production (Campolo *et al*., 2006). This result correspond with Moreno *et al*., (2005) showed that the activity of enzyme of glutathione was significantly increased in microcystin-LR in treated liver and blood, GSH activities in *C. carpio* was significantly increased because the GSH level increases as a results of overproduction for protection the cells from oxidative stress, therefore the capacity to increase GSH synthesis in response to increased demands on GSH utilization (Maher, 2005; Khadairi *et al*., 2017).

Lipid peroxidation play key role in generation of pathogenesis disease in organism, that occur by oxidative stress through imbalance in low production of antioxidant defense and high production of reactive oxygen species that lead to increase in lipid peroxide level (Pasupathi *et al*., 2009). The results of present study showed that the concentration of malondialdihyde was significantly increased in blood and liver of *C. carpio* during acute because microcystin-LR causes oxidative stress and increased lipid peroxidation in primary of rats hepatocyte (Ding *et al*., 1998). Many studies demonstrated that lipid peroxidation and oxidative stress increases in tissue of different species of organism as a result of being exposed to microcystin-LR (Prieto *et al*., 2006), the result of this study agreed with study by Jos *et al*. (2005) in tilapia fish have demonstrated the increases in the MDA level in blood and liver that had been intraperitoneally exposed to microcystins. The reactive oxygen species ROS are chemical species an atom or molecules with one or more unpaired electron in valence shell which makes it unstable; short lived and highly reactive; therefore it can be reacted quickly with other compound to capture electron to become again stable which causes oxidation and peroxidation of protein, lipid and DNA lead to significant cellular damage and even tissue or organ failure (Tripathy, 2016), the major source of ROS in biological systems are due to cellular metabolism such as mitochondrial electron transports, endoplasmic reticulum oxidation, peroxisomes and environmental factor such as toxic substance, radiation, etc.(Tandon *et al*., 2005). In results of present study, the ROS concentration was significantly increased in blood and liver of *C. carpio* during acute due to microcystin-LR induced oxidative stress that occur through an imbalances between the rate of production of reactive oxygen species and the rate of removal of these ROS by antioxidants defense systems (Jos *et al*., 2005; Tripathy, 2016). Many of studies reported the ability of microcystin-LR to induce the formation of ROS that lead to oxidative stress in many organism such as mice , rats and fish (Moreno *et al*., 2005).

Cytochrome P450 belong to a super family of hememonooxygenase that are catalyzed oxidation of lipid, steroidal hormones and numerous xenobiotic chemicals such as drug, carcinogen and environmental pollutants, the Cyt P450 that are involved in metabolizing endogenous substance and biotransformation of microcystins through converting from lipophilic into less toxic hydrophilic (Singh *et al*., 2011). The results of presents study was showed the concentration of Cyt P450 was significantly elevated in blood and liver of *C.carpio* during acute period, due to Cyt P450 activity was changed can reflect the presence of microcystin-LR lead to alter the Cyt P450 expression or enzyme activity in various organism (Zhang *et al*.,2015). in another study was showed that Cyt P450 activity change that induce by microcystin-LR in zebrafish and mice (Li *et al*., 2013).

The alkaline phosphatase (ALP) plays a significant role in phosphate hydrolysis and in membrane transport as well as is a good bio-indicator of stress in biological systems, the importance of measuring alkaline phosphatase is to check the liver dysfunction and the cellular membrane health (Banaee *et al*., 2011). Alkaline phosphatase (ALP) is an enzyme found in different body tissues; in liver, is produced by the cells lining the small bile ducts (Agrahari *et al*., 2007). Increased activity of ALP in blood may be related to hepatic tissue damage and dysfunction due to microcystin-LR toxicity, the elevation in ALP activity on may be due to an increase in transphosphorylation activity or strongly correlated with some form of damage to the hepatobiliary systems (Sharma, 1990). While elevated GOT, GPT levels in serum indicate to injury of hepatic parenchymal tissue (Wendy and Brickwell, 2007).

The results of presents study showed that the alkaline phosphatase, GOT and GPT were significantly elevated in blood and liver of *C.carpio* during acute period, due to over-phosphorylation of vital cellular protein, cytoskeletal disorder, microfilament decomposition, plasmatorrhexis and evening causing hepatic hemorrhage and metabolic disorders in mammals (Liu *et al.,* 2016).

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