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Research Article

THE BIOCHEMICAL ALTERATION AND DNA DAMAGE IN RATS (*RATTUS RATTUS*) AFTER CHRONIC INTRAPERITONEALLY INJECTION TO PURIFIED MICROCYSTIN-LR FROM *ANABAENA CIRCINALIS*

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ABSTRACT

Objective: This study determined the effect of purified microcystin-leucine arginine (MC-LR) on biochemical and DNA damage parameters in rats.

Methods: Utilization of preparative high-performance liquid chromatography in analysis, purification and collection of MC-LR, then intraperitoneally injection of purified MC-LR to rats. At the end of exposure, animals were sacrificed, and liver cell was isolated to measure the biochemical markers such as superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) as well as measured malondialdehyde (MDA), reactive oxygen species (ROS) and cytochrome P450 (Cyt P450), and DNA damage markers such as comet length, tail length, and tail moment were measured with the single cell gel electrophoresis also called comet assay.

Results: The present results showed significantly increased activities of SOD as well as concentration of MDA, ROS with increasing concentration of MC-LR but the activities of CAT and GSH, as well as Cyt P450, were significantly decreased with increasing MC-LR dose while makers of DNA damage such as comet length, tail length, and tail moment also significantly increased with increasing MC-LR dose.

Conclusion: This study demonstrated that chronic exposure to MC-LR toxin can induce alteration of biochemical and DNA damage markers.

Keywords: *Cyanobacteria*, Purification, Microcystin-leucine arginine, Biochemical markers, DNA damage markers.

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INTRODUCTION

Microcystins (MCs) are hepatotoxins with cyclic peptides that contain seven amino acid linked by peptide bonds in the configuration of cyclic. The variants of different MC has been formed mainly because the α-amino acids variations that are founding at positions two and four; for example, in MC-LR stands for leucine (L) and arginine (R) and a molecular weight of MC was ranged between 800 and 1000 daltons, its soluble in water and can't be able to cross through lipid membrane of animals, plants and bacteria and also its resistant to temperature, chemical hydrolysis, and oxidation [1]. MCs are known to effect on many organisms, from microorganism to mammals [2]. MCs can produce by different genera of *Cyanobacteria* such as *Anabaena, Nostoc, Planktothrix,* and *Microcystis* [2]. The World Health Organization has been determined acceptable of the maximum limit of MC-LR in drinking water was 1 μg L-1 and for foods was 0.04 μg kg-1 day-1 consider as tolerance daily intake of MCs [3]. *Cyanobacteria* produce various metabolites that are antibacterial, antifungal, antimalarial, anticancerous, and antitumor [4,5].

The MC-LR can effect at molecular level, the major targets of MCs-LR are inhibition of serine-threonine protein phosphatases (PP). Inhibition of PP can stimulate through increasing proteins phosphorylation, that leads to disrupt of the microfilament of cytoskeleton and cell shape loss that can cause the liver cell destruction, which may lead to intrahepatic hemorrhage or liver failure [6].

The ability of MCs to generate oxidative stress can lead to increase the generation of reactive oxygen species (ROS) and oxidative damage, such as lipid peroxidation and the DNA damage [6,7]. The ROS are scavenging by the defense of antioxidant system which are antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) peroxidase and GSH reductase, and non-enzymatic antioxidants such as reduced GSH [8]. When the antioxidant defense has not enough to remove the ROS or ROS are produced in a large quantity, they can stimulate oxidative damage resulting in lipid peroxidation, chromosome aberration and induce DNA damage through the 8-oxo-deoxyguanosine formation (8-oxo-dG) consider as a marker of oxidative DNA damage [9].

METHODS

Culturing of *Anabaena circinalis*

Uni-algae of *Anabaena* sp. were obtained from University of Baghdad and take of 10 ml of isolate of *A. circinalis* in log phase which added to a flask contained 90 ml of BG11 media and incubated at 27±2°C with a photo period of 8 hrs darks: 16 hrs lights for 14 days, this flask that contained 100 ml growth of *A. circinalis* would transport to flask contained 900 ml of media and incubated for 14 days and ultimately the growth of *A. circinalis* in the flask that contained 1000 ml, would be transported to pools 15 L and harvested after 3-5 days at stationary phase (MC-LR was formed in this phase) and concentrated by centrifugation at 3000 rpm for 15 minutes and lyophilized by oven at 35°C for 48 hrs, repeated culturing of each species of *Anabaena* sp. 4 time to obtain large amount of biomass [10].

Extraction and purification of MC-LR

Animal ethical approval: All procedures performed in studies involving animal were in accordance with the Ethical Standards of the Institutional and/or National Research Committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The *A. circinalis* cell are freeze-thaw, 3 times before extraction to disrupt the cell wall leads to easy release of MC from cell and lyophilized cell of 5 g from *Anabaena* sp. had been extracted 3 times by solvent mixture of water:methanol:1-butanol 75:20:5 for 1 hr then sonication by path sonicator for 2 hr and the extracts were centrifuged at 15,000 rpm for 30 minutes at 20°C, and the supernatant has been combined. The combined of supernatants would be air-dried at 35°C to remove methanol and 1-butanol and to concentrate to 3 ml and MCs

in each extract detected using ultraviolet (UV)-spectrophotometer at 238 nm [11].

The purification of toxins has been performed according to Tredici [11] above extract was loaded on glass column (2 cm×15 cm) which contained silica gel (75-250 mesh), then the column washed by 120 ml of deionized water then followed by adding 20% of methanol and finally, the toxins had been eluted by adding 80% of methanol with flow rate 3 ml/minutes.

Analytical, purification and collection of MC-LR

The toxins fraction has been dissolved in absolute methanol specialized for preparative high-performance liquid chromatography (HPLC) and 0.25 ml was injected by microsyringe to PHPLC (type Shimadzu at Ministry of Science and Technology in the Department of Laboratory of Water and Environmental Analysis Test) have the following characters C18-Octanoldodecyl column with 25 cm×4.6 mm l.D and mobile phase (Methanol: H_2O) 20:80, flow rate (1 ml/minute) at wave length 238 nm and at 30°C of temperature [12]. The results compared with an absorbance and retention time of standard MC-LR was purchased from Sigma-Aldrich Company, then the peak of MC-LR was collected.

Experimental designs

The weight of male rats was 200±20 g purchased from Samarra province; the animals were handled following the guidelines in the Slovenian Law for Animal Health Protection and Instructions for Granting Permit for Animal Experimentation for Scientific Purposes. The rats were housed under controlled conditions of 12 hrs light/dark cycle, 50±5% humidity and 23±1°C, the animals were allowed for 1 week to acclimate and free access to food (pellet) and tap water. Number of rats for experimental were 40 individual, divide into two group, each group include 20 rats and subdividing into eight group, put in each groups six individuals of rats for acute and five individual for chronic exposures, the control group was intraperitoneal injected by distal water, and treatment groups were intraperitoneal injected to different concentration of MC-LR (3, 6, 9) µg/kg/day for 30 days as chronic exposure [13].

Antioxidants defense

Activity of SOD has been measured by autoxidation of pyrogallol according to Qiu *et al.* [14]. While CAT activities were determined according to the procedure of Marklund and Marklund [15] but activity of GSH was determined according to the method of Claiborne [16]. The acid soluble sulfhydryl groups form a yellow colored complex with dithio nitrobenzene.

Lipid peroxidation, ROS, and cytochrome P450 (Cyt P450)

Lipid peroxidation had been estimated by the assay of thiobarbituric acid for malondialdehyde (MDA) concentration according to Moreno *et al.* [17] and Aust [18]. Whereas ROS and Cyt P450 were determined according to ELISA kit of Elabscience, China.

DNA damage

Taken a small portion of liver tissue (50 mg) is serially washed and this piece of liver is placed in 2 ml microcentrifuge tube containing 1.5 ml of phosphate buffer solution then homogenization by homogenized (15-20 s) by pestle motor mixer, then added 40 µl of proteinase K liver tissue to remove protein and centrifugation at 13,000 rpm for 15 minutes, 4°C, taken 2-5 µl of suspension cell to a clean 1.5 ml tube and mix on comet slide with 40 µl of low melting agarose, then prepare lysis solution that consist of 2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base, and 8 g NaOH, all dissolved and complete to 700 ml deionized water, then added 110 ml from 55 ml 1% Triton X to 55 ml 10% dimethyl superoxide after that complete the volume to 100 ml by deionized water and before use, chill at 4°C or on ice for at least 20 minutes and combine 7.5 µl with 75 µl low melting agarose and immediately spread the mix onto the clear part of a comet slide, then warm comet slide on a heating plate at 42-50°C before application to prevent permit evenly spreading of the agarose and prevent the formation of air bubbles, slides may be stored in lysis solution at 4°C for 60 minutes after that removing the lysis solution and replace by alkaline solution contain 6 g NaOH and 500 µl 0.5% Na_2EDTA for 5-60 minutes at room temperature in dark, then removing slide from alkaline solution gently tap excess buffer from slide and washed by immersing in 1X TBE buffer for 5 minutes, after that, transferred slide from 1X TBE buffer to an horizontal electrophoresis apparatus and place slides on flat of gel tray and pour 1X TBE buffer until to cover the slides for 60 minutes vol 70, then very gently tap off excess TBE and added some drops of 70% ethanol on slides to remove the water, and stain the slides by ethidium bromide then leave the slides for 24 hrs and view slide by fluorescence microscope [19] with modification by Singh *et al*. [20].

Statistical analysis

Data of present study were analyzed according to the system of Statistical Package for the Social Sciences (SPSS) version 20 to found means, least significant differences by ANOVA.

RESULTS

Extraction and purification of MC-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 MC-LR and concentration of toxin was determined by comparing peak area and retention time of analytical standard of MC-LR with peak area and retention time of extraction of *Anabaena* sp., the retention time of analytical standard of MC-LR was 9.55 minutes (Fig. 1): And it's concentration was 10 µg/ml and *A. circinalis* retention time was 9.51 minutes (Fig. 2): And it's concentration was 74.832 µg/ml, then highly purified and collected of MC-LR by preparative HPLC.

Biochemical markers

The statistical analysis shown significant differences in all biochemical marker among control and treatments at $p<0.05$, the SOD activity in liver tissue control was 2.2 U/mg. While the activity of SOD in treated liver tissue was 6.1, 32.8, and 12.4 U/mg in doses 3, 6, and 9 µg/kg body weight (b.w.) In liver tissue control (Table 1 and Fig. 3): CAT activity was 28.8 U/mg, whereas in treated liver, the activity of CAT was reached to 132 and 40.1 U/mg in doses 3 and 6 µg/kg b.w., respectively, while CAT activity in doses 9 µg/kg b.w. has been decreased to 24.7 U/mg as compared with control groups (Table 1 and Fig. 4): GSH activity in control of liver tissue was 78.5 µmol/ml, while GSH activity in liver tissue was decreased 68.8, 72.7, and 75.4 µmol/ml in doses 3, 6, and 9 µg/kg b.w. as compared with control (Table 1 and Fig. 5).

MDA concentration of control was 0.86 µmol/ml in liver, while the MDA concentration significantly increased in three doses 6.19, 9.45, and 13.6 µmol/ml in liver tissue (Table 1 and Fig. 6). Whereas, the ROS concentration reached in control to 14.9 p/ml in liver tissue while ROS concentration in three doses were 206, 299.8, and 394.9 p/ml in treated liver (Table 1 and Fig. 7). The Cyt P450 concentration in control was 1013.5 p/ml in liver tissue, but it's concentration in the treatment was significantly decreased to 861 p/ml in dose 3 µg/kg, 614.8 p/ml in dose 6 µg/kg, and 434.4 p/ml in dose 9 µg/kg in treated liver tissue (Table 1 and Fig. 8).

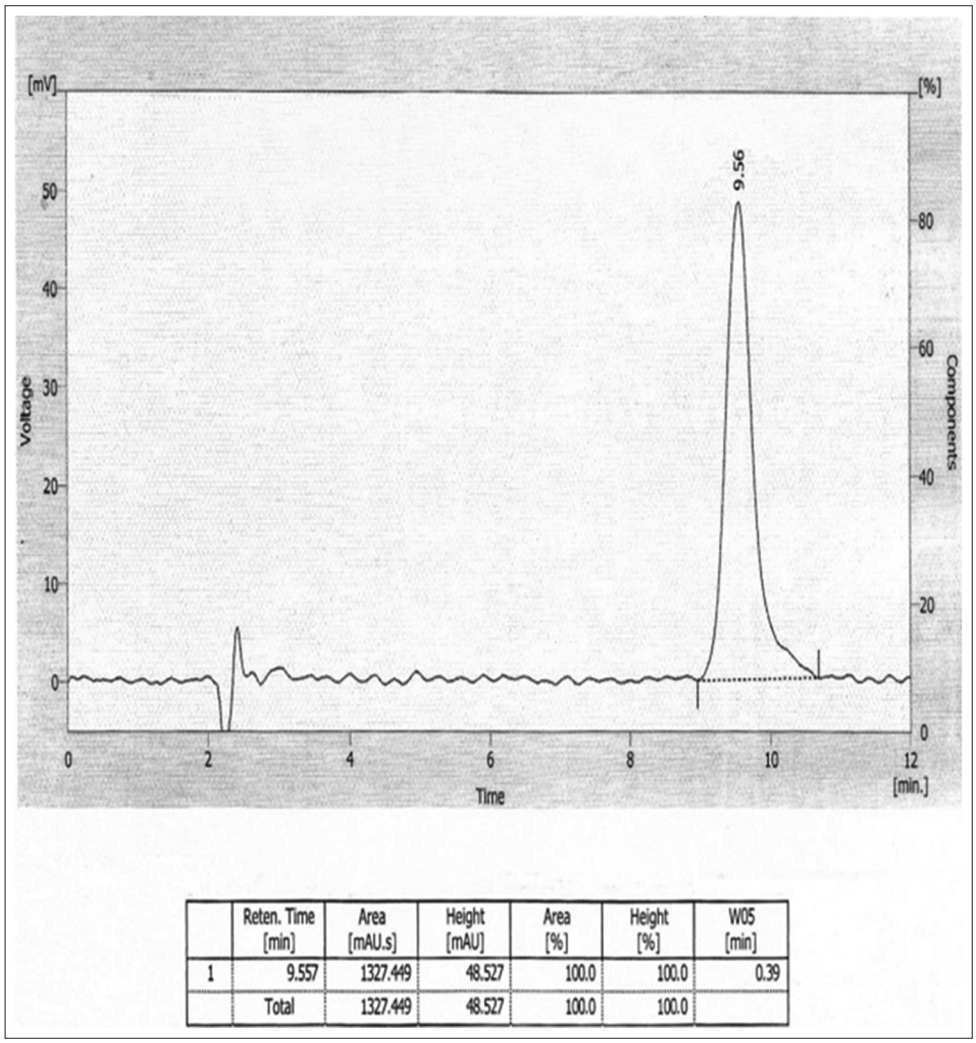
DNA damage markers

The DNA damage markers were showed significant differences between control and treatments according to statistical analysis at $p<0.05$. The comet length scored highest levels in rats at dose 9 µg/kg b.w. that are 312.5 µm (Fig. 9). Whereas tail length was recorded the highest levels at dose 9 µg/kg b.w. that have reached to 97.42 µm (Fig. 9). While the highest levels that were observed in tail moments in rats was 85.07 µm at dose 9 µg/kg b.w. (Fig. 9). According to pictures were taken, the DNA damage can be classified into three class of damage that is Class 1: Low damage, Class 2: Medium damage, and Class 3: High damage in liver of rats (Fig. 10).

Table 1: The biochemical markers in the liver of rat during chromic exposure period to MC-LR mean±SD

Biochemical markers	Control	3 µg/kg b.w.	6 µg/kg b.w.	6 µg/kg b.w.
SOD U/mg	2.2±0.043	6.1±1.033	23.75±3.782	12.35±1.132
CAT U/mg	28.8±2.453	132.48±21.760	40.08±1.320	24.72±3.672
GSH µmol/ml	78.46±3.440	68.76±5.401	72.66±10.234	75.4±12.675
MDA µmol/ml	0.86±0.001	6.19±0.532	9.45±0.895	13.6±1.006
ROS p/ml	14.85±0.456	206.34±17.120	298.85±22.136	393.57±11.289
Cyt p450 p/ml	1035.13±34.78	861±110.32	614.84±5.435	434.36±6.98

SOD: Superoxide dismutase, CAT: Catalase, GSH: Glutathione, MDA: Malondialdehyde, ROS: Reactive oxygen species, SD: Standard deviation, Cyt p450: Cytochrome p450, MC-LR: Microcystin-leucine arginine, b.w.: Body weight



	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-leucine arginine standard that used in study, analyzed by preparative high-performance liquid chromatography

DISCUSSION

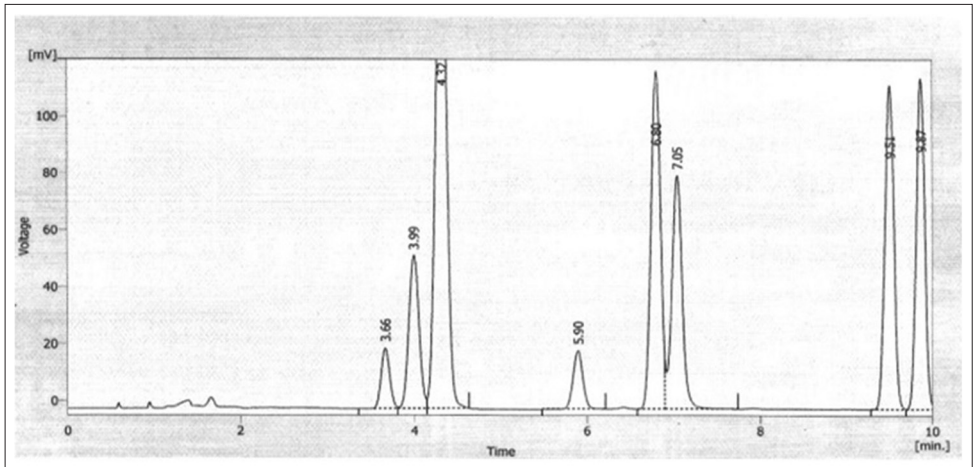
Extraction, separation, and purification of MC-LR

Cyanobacterial toxins are intracellular toxins that are released to medium by breaking *Cyanobacterial* cell or die so that *Cyanobacteria* was needed solvents for extraction Cyanotoxins. In this study, the solvent used in extraction of MC-LR was water:methanol:butanol (75:20:5) because of efficiencies of extraction of different structure variant of MC were increased with their hydrophobicity ,[21,22] shown that the polar extracts (water: methanol:butanol) have higher contents of MC because of MC polarity, therefore, MCs are soluble in water:methanol:butanol.

MCs variants require high resolution for separation and purification, which depends on the mobile phase composition and stationary phase that is used in analysis [12]. In this study, silica gel column was used and elution by 80% methanol to partial separation of MC and then highly purified and collected MC-LR from *A. circinalis* by PHPLC at 238 nm absorbance of MCs because of strong absorbance of MC at 238 nm enabling sensitive UV detection due to the main chromophore of the toxins can absorb at 238 nm is the conjugated diene in the adda residue [23].

Biochemical markers

The results of presents were showed that the SOD activities significantly increased with increasing dose of MC-LR, SOD plays essential role in converting superoxide anion radical produced in body to hydrogen peroxide [24]. This result agreed with study by Sicinska *et al.* [25] reported increased SOD activity induces by



	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 high-performance liquid chromatography at absorbance 238 nm for *Anabaena circinalis*

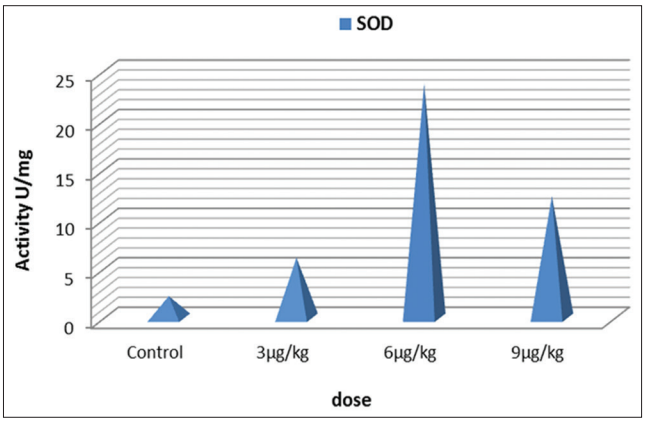


Fig. 3: Superoxide dismutase activities in liver of rats after chronic exposure period by purified microcystin-leucine arginine

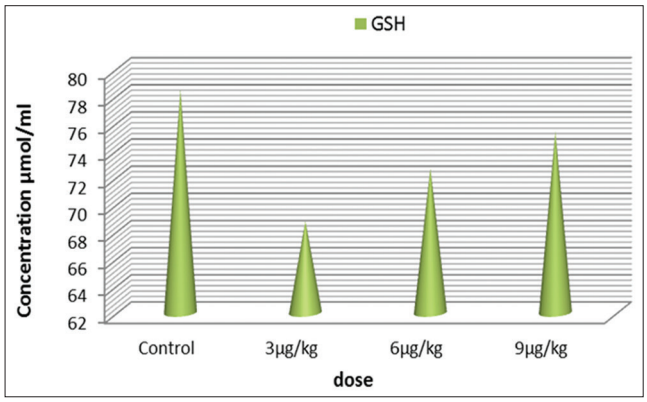


Fig. 5: Glutathione concentration in liver of rats after chronic exposure period by purified microcystin-leucine arginine

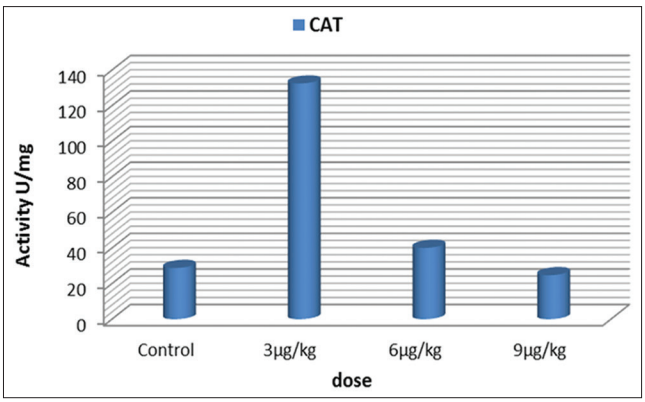


Fig. 4: Catalase activities in liver of rats after chronic exposure period by purified microcystin-leucine arginine

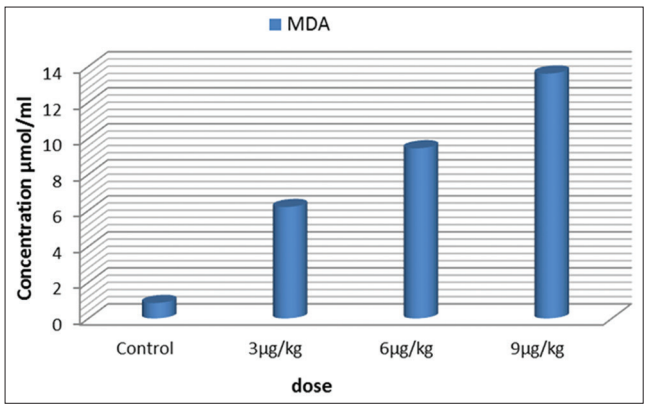


Fig. 6: Malondialdehyde concentration in liver of rats after chronic exposure period by purified microcystin-leucine arginine

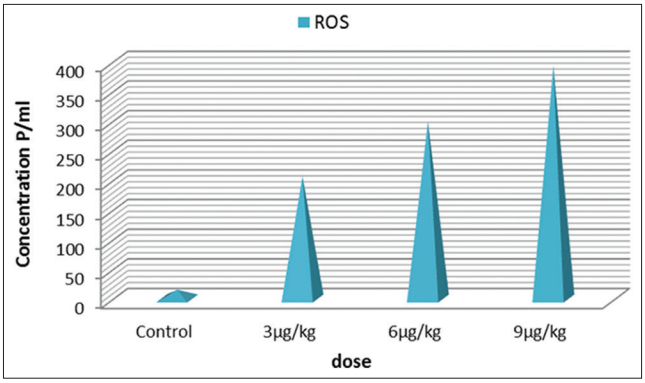


Fig. 7: Reactive oxygen species concentration in liver of rats after chronic exposure period by purified microcystin-leucine arginine

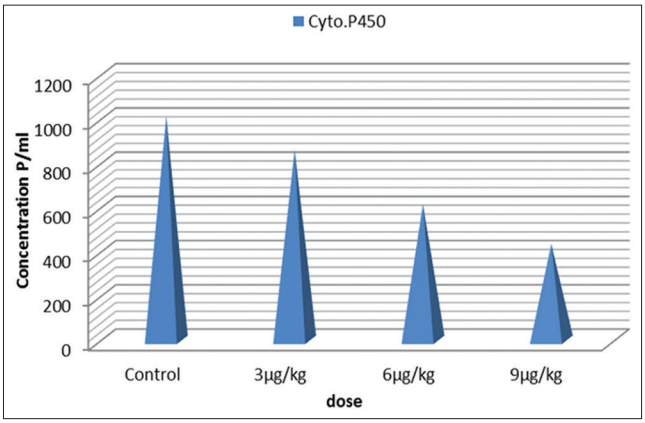


Fig. 8: Cytochrome p450 concentration in liver of rats after chronic exposure period by purified microcystin-leucine arginine

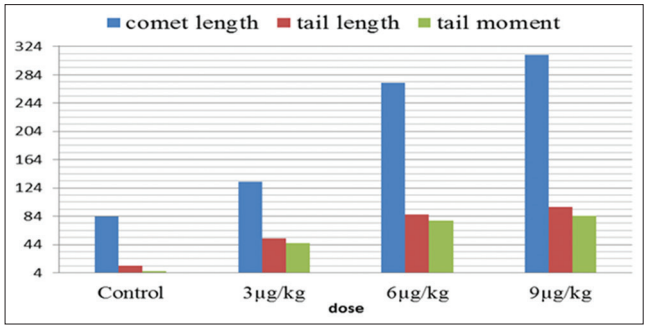


Fig. 9: The criteria of DNA damage in liver of rats after chronic exposure period by purified microcystin-leucine arginine according to comet assay

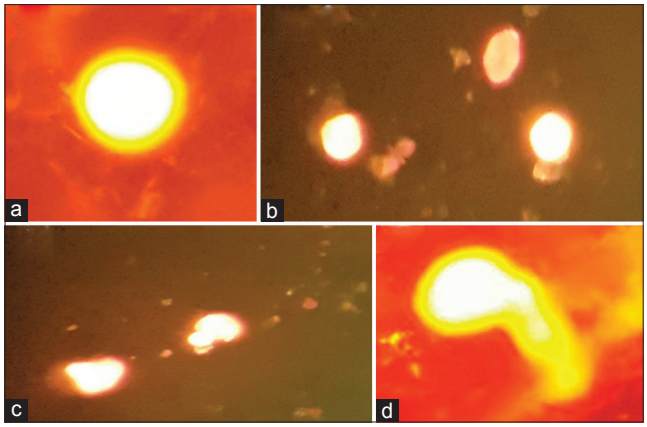


Fig. 10: DNA damage in the liver of rats according to comet assays. (a): Control, (b): Low damage, (c): Medium damage, and (d): High damage (×40)

MC-LR. In contrary with Harada [24] study demonstrated MC-LR can decrease the SOD activity and increase lipid peroxidation. While CAT activity significantly decreased with increasing concentration of MC-LR, CAT is localized in subcellular peroxisomes where convert hydrogen peroxide to water and molecular oxygen, CAT activity decreased may be due to either direct damage to structure of protein or increased the level of superoxide anion radical [26]. GSH which is the main compound in the intracellular redox status regulation and it's considered as essential cofactor in many metabolic reactions [27]. The results of this study, observed significant decrease of GSH in liver of rats may be due to a compensatory response induce by imbalance in the cell redox state as the result of excessive H_2O_2 production or may be related to its involvements in detoxification of deleterious effects of the increase free radical produce within cells [17,28,29]. Demonstrated that exposure of rats to MC-LR resulted in an increase of the endogenous antioxidant defense system together with an increase of lipid peroxidation in liver and in the kidney. The MDA and ROS concentration significantly elevated with increasing concentration of MC-LR due to MC-LR induced oxidative stress that occurs through an imbalances between the rate of production of ROS and the rate of removal of these ROS by antioxidants defense systems [30]. The studies that are reported the MC-LR ability to induce the ROS formation that lead to oxidative stress and lipid peroxidation in many organisms such as rats [16].

Cyt P450 belong to a superfamily of heme monooxygenase that is catalyzed oxidation of lipid, steroidal hormones and numerous xenobiotic chemicals such as drug, carcinogen and environmental pollutants, and the Cyt P450 that are involved in metabolizing endogenous substance and biotransformation of MCs through converting from lipophilic into less toxic hydrophilic [31]. These results of presents study were showed in chronic period, the Cyt P450 concentration in liver of rats was significantly decreased due to Cyt P450 activity was changed can reflect the presence of MC-LR lead to alter the Cyt P450 expression or enzyme activity in various organism and Cyt P450 activity decreased via increasing the ROS formation [32-34]. The study reported by Zhang *et al.* [35] showed that MC-LR induced decreased the level of cytochrome b5 and Cyt P450 liver of rats during period. While in another study was showed that Cyt P450 activity change that induces by MC-LR in zebrafish and mice [36,37]. This study corresponding with [38] testing the adverse effect of purified MC on mouse liver have found that MC-LR is 30-100 times less toxic via oral ingestion than via intraperitoneal injection, also antioxidant such as SOD, CAT and ROS and MDA increased with increasing period of exposure, and concentration of MC-LR, while GSH and Cyt P450 decrease due to oxidative stress.

DNA damage

DAN damage occurred by MC-LR through the involvement of ROS that leads to oxidative stress, via mitochondrial permeability transition and cytoskeleton disruption DNA strand breaks and DNA oxidized bases products of free radical attack that are stimulating by MCs [39].

MC-LR had been induced DNA damage in liver cell *in vivo* and *in vitro* through the formation 8-oxo-dG, and DNA lesions are used as markers for oxidative DNA damage [40]. The DNA damage that induces by MC-LR can be related with apoptosis rather than with genotoxicity [41] while Lankoff [42] also demonstrate MC-LR induce DNA damage and genotoxicity through its ability to cause genetic instability in erythrocytes in bone marrow of mice injected 45 µg MCLR/kg bw for 24 hrs and MC-LR stimulate a 2-fold increase

in the level of DNA damage in blood cell after 30 minutes of i.p. injection (37.5 µg MCLR/kg bw) of rats.

The result of presents study had been showed, the parameters of comet assay were significantly differences between treatment and control group, the highest values of comet length µm, tail length µm, and tail moment µm in liver of rats were recorded at dose 9 µg/kg b.w. as compared with control group, the statistical analysis appeared positive significant correlation between DNA damage markers and the dose of MC-LR, biochemical markers. The comet length was positively correlated with the level of DNA breakage in cell because the distribution the comet was heterogeneous [19]. The highest value of the markers of DNA damage would be increased with increasing the toxicant dose this may be due to insufficient produce of antioxidant defense systems to scavenge the ROS that is generated by MC-LR which lead to finding their way across nuclear membrane indicating DNA strand breakage [42-46].

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