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Study of hematological and biochemical effects of sub-lethel doses of the blue green alga *Lyngbya aestuarii*

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Abstract

Cyanobacteria are photosynthetic prokaryotic organisms that colonize a wide variety of habitats. They are the source of more than 300 bioactive secondary compounds. Among those secondary metabolites, the cyanotoxins which have received most of the research attention therefore, the present work aimed to study the detrimental effects of methanolic extract of *Lyngbya aestuarii*on on some physiological aspects in female mice. Hematological parameters were analyzed with automated Hematological analyzer while other biochemical parameters were achieved with commercial kits using spectrophotometer apparatus according to manufacture instructions. The present results showed a significant increase in the red blood corpuscles' count in both low and high dose groups comparable with control group and there are a significant increase in both HGB and HCT in low dose group comparable with control. Also, there are significant increase in percentage of monocytes in high dose group comparable with control group. Liver enzymes showed significant differences in mice groups for AST only where the low dose treatment registered significant increase in concentrations comparable with control treatment. Concentration of both urea and creatinine showed significant increase in low dose group only comparable with control group which reflect kidney dysfunction. There are significant decreasing in concentration of HDL among mice groups comparable with control group. Finally, total protein concentrations showed significant increase in high dose treatment comparable with control treatment. In conclusion, the detrimental effect of the toxic extract of *L. aestuarii was* on kidney function.

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Introduction

Cyanobacteria are photosynthetic prokaryotic organisms that colonize a wide variety of habitats, from freshwater and marine environments to hydrothermal vents, from desert rocks to antarctic lakes (Testai *et al.*, 2016). Cyanobacteria are characterized by an active secondary metabolism, that is, a group of processes obviously not involved in primary metabolism (e.g., photosynthesis, respiration). They are the source of more than 300 bioactive compounds, many of which are yet of unknown function. Among those secondary metabolites, cyanotoxins have received most of the research attention so far (Cirés *et al.,* 2017).

Human deaths caused by acute exposure to cyanotoxins are limited to few cases so far. In 1996, several cases of liver failure and human death occurred in Brazilian dialysis clinic which named Caruaru syndrome, being caused by the exposure of the patients to microcystins-contaminated water when used for dialysis (Pouria *et al.*, 1998; Carmichael *et al.*, 2001). In 2002, a swimmer boy died after swallowing water with a blue-green algae scum at Wisconsin/USA. He suffered from a Seizure and died as a result of heart failure (Weirich and Miller, 2014). Food-supplements containing blue-green algae supplements (BGAS) are another route of chronic exposure to cyanotoxins. Dietrich *et al.* (2007) pointed that a woman died after its consumption of blue-green algae supplements (BGAS) for a long period.

Cyanobacteria species were investigated in Iraqi surface and drinking water (AL-Sultan, 2007; AL-Shaheen, 2011; Abed, 2013;Abed, 2015;Al Hassany *et al.*, 2014;Al- Hussieny *et al.*, 2015 ; Ali, 2016, abdulsalam *et al.*, 2016; Hassan *et al.*, 2017) but there is a limited ecological studies on the prevalence of cyanotoxins in Iraqi fresh waters (AL-Shaheen, 2002, Al-Sultan and Mahmood, 2016; AL-Sultan and Aubaed, 2017 and AL-Sultan, *et al.,* 2019).Also, there are few studies concerned with physiological and histopathological effects of toxic cyanobacteria on mice. Majority of them were conducted on male mice except few studies were conducted on both genders (AL-Sultan *et al.,* 2015; AL-Sultan and Abass, 2017; AL-Sultan and Aubaed, 2017; Kata *et al.,* 2017).Therefore, the present workaimed to study the detrimental effects of methanolic extract of *Lyngbya aestuarii* and their toxins on some physiological aspects in female mice.

Material and methods

Extraction of L. aestuarii

The toxic blue green alga "*L. aestuarii*" was collected from Shatt Al-Arab River as a unialgal bloom and its toxic compounds were detected with LC-tandem MS. They were neosaxitoxin, Cryptophycin C and Dudawalamide B and they previously published by Al-Sultan *et al.* (2019).

Twenty grams of lyophilized alga were weighed and extracted twice with 75 % methanol (1 liter) then it is re-extracted twice with 1 % acetic acid (40 mililiter), then both extractions were mixed together before desiccation with Freeze dryer (Rangel *et al.*, 2013).

Experimental design

The LD_{50} of algal crude extract was 560 mg/kg according to AL-Sultan *et al.* (2019).Twenty four adult female mice weighing about 23-26 gram were divided into three groups, each group comprised of eight mice and the groups were injected with two doses derived from the LD_{50} value for fifteen days. The highest dose represented 1/7 LD_{50} and it was 80 mg/kg b.w. while the lowest dose represented 1/14 LD_{50} and it was 40 mg/kg b.w. At the end of treatment period, mice were anesthetized by chloroform, and sacrificed for blood collection by heart puncture.

Hematological aspects

Blood samples used for hematological analysis were collected into lavender EDTA-containing tubes. Erythrocytes (RBCs), total leucocytes count (WBCs), hemoglobin (HGB), hematocrit value (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and WBC differential were

analyzed with automated Hematological analyzer.

Biochemical aspects

Blood samples were transferred to serum separating tubes, these tubes with golden cap contain particles that cause blood to clot quickly, as well as a gel to separate blood cells from serum. Blood samples were left to clot and then centrifuged at 3000 r.p.m for 10 minutes at room temperature to separate the serum and finally the serum is stored at -20°C until assay. The biochemical tests includes ALT, AST, ALP, urea, creatinine, glucose, total protein, total cholesterol, triglycerides, HDL and LDL which achieved with commercial kits using spectrophotometer apparatus according to manufacture instructions.

Statistical analysis

Results are expressed as mean ± standard deviation. The difference between the means was determined by one-way analysis of variance (ANOVA). In all statistical tests, a value of $P \leq 0.05$ was considered significant. All analysis was performed using statistical package for social sciences (SPSS) for Windows, Version 21.

Results

Hematological aspects

Effect of methanolic extract of L. aestuarii on Red Blood Corpuscles' parameters

The present results showed a significant increase ($p \leq 0.05$) in the red blood corpuscles 'count in both low and high dose groups comparable with control group.

While there are a significant increase ($p \leq 0.05$) in both HGB and HCT in low dose group comparable with control group but there is no significant changes ($p > 0.05$) observed in MCV, MCH and MCHC (Table 1).

Table 1. Hematological effects of methanolic extract of *L. aetuarii* on red blood corpuscles' parameters for female mice (n=8) after 15 days of i.p. injection.

Parameters / Treatments	RBC (10^6/mm^3)	HGB (g/dL)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)
Control	b 7.29±0.47	b 10.46±0.67	b 31.98±2.83	43.80±1.22	14.28±0.25	32.74±1.14
Low dose (40 mg/kg)	a 8.58±0.41	a 12.30±0.73	a 39.86±1.96	46.52±0.97	14.30±0.51	30.82±0.90
High dose (80 mg/kg)	a 7.99±0.58	ab 10.98±1.72	ab 35.64±4.24	44.52±3.02	13.72±2.12	30.84±4.05
R.L.S.D.	0.44	1.61	4.11	N.S.	N.S.	N.S.

a,b indicate the presence of significant difference ($p \leq 0.05$) among treatment groups.

Effect of methanolic extract of L. aestuarii on White Blood Cells parameters

The present results show a significant increases ($p \leq 0.05$) in percentage of monocytes in high dose group comparable with control group (Table 2).

Table 2. Hematological effects of methanolic extract of *L.aestuarii* on white blood cells parameters for female mice (n=8) after 15 days of i.p. injection.

Parameters / Treatments	WBC (10^3/mm^3)	Lymphocytes (%)	Monocytes (%)	Granulocytes (%)
Control	9.62±2.58	6.36±1.90	b 0.18±0.08	3.08±0.64
Low dose (40 mg/kg)	8.48±2.48	5.66±1.88	b 0.20±0.00	2.62±0.71
High dose (80 mg/kg)	11.94±10.00	8.16±8.14	a 0.54±0.27	3.24±2.04
R.L.S.D.	N.S.	N.S.	0.21	N.S.

Biochemical aspects

Effect of methanolic extract of L.aestuarii on liver parameters

The present results showed significant differences in mice groups for AST only where the low dose treatment registered significant increase in concentrations comparable with control treatment (Table 3).

Effect of methanolic extract of L.aestuarii on kidney parameters

Concentration of both urea and creatinine showed significant increase ($p \leq 0.05$) in low dose group only comparable with control group (Table 4).

Effect of methanolic Extract of L.aestuarii on lipid profile

There are no significant changes in concentration of total cholesterol (TC), triglycerides (TG) and low-density lipoprotein-cholesterol (LDL) among female mice groups but there are significant decreasing ($p \leq 0.05$) in concentration of high-density lipoprotein-cholesterol (HDL) among mice groups comparable with control group (Table 5).

Table 3. Effect of methanolic extract *of L.aestuarii* on hepatic enzymes for female mice (n=8) after 15 days of i.p. injection.

Parameters / Treatments	ALP (U/l)	ALT (U/l)	AST (U/l)
Control	8.05±4.35	21.94±1.16	c 5.51±1.96
Low dose (40 mg/kg)	9.86±2.35	37.47±14.18	a 126.61±19.27
High dose (80 mg/kg)	6.89±2.56	32.54±9.08	b 77.78±19.87
R.L.S.D.	N.S.	N.S.	14.50

Effect of methanolic extract of L.aestuarii on glucose and total proteins

Glucose concentrations did not show any significant difference ($p > 0.05$) between control group and other treated groups. While protein concentrations showed significant increase ($p \leq 0.05$ in high dose treatment comparable with control treatment (Table 6).

Discussion

Hematological aspects

Effect of Methanolic Extract of L. aestuarii on Red Blood Corpuscles (RBC)

The hematological indices for female mice exhibited a significant increase in the RBC count in both low and high dose groups comparable with control group while both HGB and HCT exhibited a significant increase in low dose group comparable with control group but there are no significant changes observed in both MCH and MCHC. In the present study, the increase in RBC count are related to increase in erythropoiesis (Lai *et al.,* 2006). The reduction in RBC count and/or their hemoglobin content yield a hypoxia which is occurred as a result of exposure to neosaxitoxin in the methanolic extract of *L. aestuarii* (Silva de Assis *et al.,* 2013). According to hospital case reports, Patients intoxification with saxitoxins in Chile after their eating poisonous mussels suffered from hypoxia with low arterial oxygen pressure concomitant with bradycardia as a consequence of respiratory failure (Carcía *et al.,* 2005). In the present study, hypoxia gives rise to increased erythropoiesis as evidence by increasing in RBC count, HGB and HCT (Vargas *et al.,* 2011). Under normal conditions, expression of erythropoietin in kidneys is responsible for erythrocytes production. Erythropoietin is secreted to the plasma and in the bone marrow it binds to erythropoietin receptors on the surface of erythroid progenitor cells. Both erythropoietin

concentration and erythropoietin receptor activation are important for triggering erythrocyte production by erythroblastic islands (Srbová, 2011). The process of stimulation an erythropoiesis known as stress erythropoiesis. Stress erythropoiesis has been studied mainly in mice models of acute anaemia (Riley *et al.,* 2001; Vignjević *et al.,* 2014).In addition to erythropoietin role, several additional growth factors have been implicated in stress erythropoiesis. Our findings are in agreement with Ramamurthy *et al* (2013) when they fed the fish *Platycephalus gibbosus* with the cyanobacterium Lyngbya *hieronymusii* and found that both RBC count and their hemoglobins content were significantly higher in all fish groups fed with *L. hieronymusii* compared with the control group for all doses and periods. Also, WBC count was significantly higher in some of fish groups after feeding them with *L. hieronymusii* at different doses and for different periods. So, in the present study, the stimulation of erythropoiesis in female mice may be due to an increase in percentage of monocytes in blood stream (Miller, 1989; Moldawer *et al.*, 1989).

Macrophages play an important role in supporting stress erythropoiesis by increasing erythropoietic activity (Heideveld *et al.,* 2018).

Table 4. Effect of methanolic extract of *L. aestuarii* on kidney parameters for female mice (n=8) after 15 days of i.p. injection.

Parameters / Treatments	Urea (mg/dL)	Creatinine (mg/dL)
Control	b 27.14±1.90	b 0.44±0.22
Low dose (40 mg/kg)	a 32.59±3.25	a 0.98±0.51
High dose (80 mg/kg)	b 27.57±1.25	ab 0.83±0.34
R.L.S.D.	6.62	0.39

Effect of methanolic extract of Lyngbya aestuarii on White Blood Cells (WBC)

Monocytes percentages were increased significantly in high dose group comparable with control group. Our present findings are in agreement with findings of Esmaeel (2017) in that the increasing of monocytes percentage were in female mice only after injection them with crude extract of *Oscillatorialimosa*. Monocytes are precursors of macrophages which represent the body's second line of defense against infection (Lu and Kacew, 2002; Kemal, 2014). Therefore an increasing in percentage of monocytes, in the present study, is due to immunological response of female mice to the methanolic extract of *L. aestuarii* which contains lipopolysaccharides (LPS) of the cell walls and neosaxitoxin. This response is similar to human response after exposure to cyanobacteria during recreation on beaches. The sequential exposure of recreational people to low levels of the cyanotoxins such as MC-LR and LPS leads to a hypo-responsiveness condition known as cyanotoxin tolerance. This is an important and active immune response to chronic exposure to cyanotoxins that prevents excessive inflammatory responses (Pandey and Prasad, 2016). It initiated by the first exposure, mediates by a short-term memory macrophages and low level of liberated TNF-α. Then gradually resulting in a complete canceling of TNF-α release (Stewart, 2004). It had been found that fishermen who ate shellfish containing low concentrations of saxitoxins were less susceptible for harmful effects of these toxins. Also, tolerance of saxitoxins had been induced in experimental rodents where their injection with sub-lethal doses made them not affected by lethal doses (Alexander *et al.* 2009).

Table 5. Effect of methanolic extract of L. *aestuarii* on lipid profiles for female mice (n=8) after 15 days of i.p. injection.

Parameters / Treatments	TC (g/dL)	TG (mg/dL)	HDL (mg/dL)	LDL (mg/dL)
Control	126.67±45.45	104.80±27.17	a 11.05±4.50	94.65±50.12
Low dose (40 mg/kg)	147.98±32.42	96.69±21.51	b 0.02±0.00	128.64±30.79
High dose (80 mg/kg)	129.14±38.55	94.11±16.72	b 0.92±0.43	109.39±37.57
R.L.S.D.	N.S.	N.S.	7.42	N.S.

Biochemical aspects

Liver parameters

The present results do not show any significant alteration in both ALT and ALP concentrations among treatment but they showed significant increase in AST for low dose treatment comparable with control treatment. Our findings are in agreement with those of Zepeda *et al.* (2014) in that intraperitoneal injection of rats with 6 µg/kg neosaxitoxin produced increased in concentrations of the hepatic AST enzyme only. The estimation ALT is a more specific test for detecting liver necrosis since it is primarily found in the liver. While AST is considered a less specific biomarker enzyme for hepatocellular injury because it is also found in heart, skeletal muscles, brain, kidney and in erythrocytes. Injury to any of these tissues can cause an elevated concentration of AST in serum (Nsiah *et al.*, 2011; Woreta and Alqahtani, 2014). Several cyanotoxins are biotransformed in the liver of vertebrates through phase I and phase II reactions. García *et al.* (2010) confirmed the metabolism of some saxitoxins in human using human liver microsomes. Also, Ramos *et al.* (2014) have observed alterations in the antioxidant machinery in rat liver after oral exposure to sub-lethal doses of saxitoxin which indicated that the liver is susceptible to oxidative stress.

Kidney function

Urea and creatinine are general markers of renal function both in man and rodents. In injured kidney, these substances are not excreted normally, therefore their levels have increased in blood. The present results show highly significant increasing in urea concentration which indicate renal failure. Urea is derived from metabolism of protein and is excreted in the urine. High level of urea in serum usually indicates of acute renal dysfunction due to glomerular damage (Mounira, 2015). Creatinine is a metabolite of creatine degradation and is excreted completely in the urine via glomerular filtration. The amount of creatine per unit of skeletal muscle mass is consistent and the breakdown rate of creatinine is also consistent (Knaack *et al.*, 2016; Coleman *et al.*, 2018). The present results show a significant increasing in creatinine concentrations in treated mice comparable with the control ones. Our findings are in agreement with those of Pan *et al.* (2008) who study the toxicity of crude extract of *Anabaena flos-aquae* in mice and they found an elevation in both urea and creatinine levels in mice serum. Also, an elevation in creatinine concentrations in serum may be indicated to both kidney dysfunction and muscular dystrophy (Lu and Kacew, 2002; Martin and Sheaff, 2007; Basten, 2010; Kopp *et al.*, 2010).

Lipid profile

The present results did not show any significant alteration in total cholesterol, triglycerides and LDL but they showed decreasing in HDL in treated mice comparable with control ones.High- density lipoproteins (HDL) involved in VLDL metabolism and also in cholesterol transport (Murray *et al.*, 2008). HDL serve as acceptor of excess cholesterol from

various tissues and as transporter of unused cholesterol from the tissue back to the liver where it is broken down to bile acids and then excreted or re-utilized (Al-hassan, 2012). They promote the removal of cholesterol from tissues and its secretion into the bile by the liver. It is known to be the good cholesterol in the body as it decreases the occurrence of cardiovascular diseases (Haghpanah *et al.,* 2010; Seixas *et al.,* 2010). So, the decline in HDL concentration for female mice would subject them to cardiovascular disorders which have been provoke by exposure to saxitoxin as proved by experimental animals after their intravenously injection (i.v.) with 1 µg saxitoxin/kg body weight (Alexander *et al.,* 2009).

Table 6. Effect of *L. aestuarii* on glucose, total proteins and total bilirubins for female mice (n=8) after 15 days of i.p. injection.

Parameters / Treatments	Glucose (mg/dL)	Total proteins (g/dL)
Control	112.88±55.41	b 4.98±1.06
Low dose (40 mg/kg)	103.80±24.31	ab 5.55±0.64
High dose (80 mg/kg)	105.97±9.27	a 6.51±1.20
R.L.S.D.	N.S.	1.05

Glucose and total proteins

The present results did not show any significant differences in glucose concentrationsamong female mice treatments but the results show significant increase in concentration of total protein in high dose for female mice comparable with control mice only which reflects the enhancement role of algal extract on stimulation of protein synthesis by liver wheremajority of plasma proteins like albumins and globulins are produced in the liver (Al-Hassan, 2012).

Our present findings are in agreement with those of Palikova *et al.* (2004) in that total protein concentrations were increased after exposure of the carp fish (*Cyprinus carpio L.*) to stress caused by toxic cyanobacterial extract containing microcystins and the biomarkers of this stress represented by increasing in RBC count, HGB and HCT in the exposed fish groups comparable with control group.

Also, the stimulation of stress erythropoiesis in carp fish are in agreement with our findings for female mice as previously discussed in hematological aspects. Similar findings were also found by the study of Ramamurthy *et al.* (2013) when they registered an elevation in serum total protein in the fish *P. gibbosus* after feeding them with the cyanobacterium *L. hieronymusii* and these higher concentrations of total protein were associated with stimulation of both erythropoiesis and non-specific immunity response in fish blood.

Conclusion

The present study showed the detrimental effect of the toxic extract of *L. aestuarii* on kidney function while less effect was on liver functions.

Akcnowlegment

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