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Research Article

Toxic effects of neurotoxins (Anatoxin-a) purified from blue-green algae *Pseudoanbaena limnetica* on some organs in laboratory mice (Mus musculus L.)

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ABSTRACT
Blue-green algae produce a wide range of toxic compounds, particularly neurotoxins, which effects have only been shown on the central and peripheral nervous system. The current study was conducted to show the histopathological effects of anatoxin-a purified from local isolates from Shutt Al-Arab river water in Basra governorate / Southern Iraq on some organs including testes, liver and kidneys. Results showed the ability of alga *P. limnetica* to produce anatoxin-a to reach 35 µg/g D.W. Three groups of laboratory mice (n=8) were injected intraperitonial daily for 15 days under laboratory conditions with (1)-Normal saline (Control group), (2) - low dose group with purified anatoxin-a = 0.5 µg/L and (3) - high dose group =1 µg/L). Purified toxin showed many histopathological effects at low and high doses and the effects increase with increasing dose. The testes showed a decreased number of elongated spermatids in central tubules in low dose group, while in high dose group, good evidence of the gross morphological effect of seminiferous tubules is seen which appear devoid of elongated spermatids in most tubules with predominant rounded spermatids. Liver cells in low dose appear more eosinophilic and without nuclei with fatty degeneration of sinusoids and mild inflammatory infiltrate. The appearance of large vacuoles within hepatocytes, necrotic cells, sinusoids poorly visualized with increased inflammatory cells in liver tissue under high dose. Kidneys at low dose showed increased eosinophilia of renal tubules cells, mild degenerative changes, hemorrhage and decrease in size of the urinary capsule. However, extravasation of red cells, congestion, dilatation of capillaries and chronic inflammatory cells were found under high dose exposure.

Keywords: Neurotoxins, Anatoxin-a, Blue-green alga *Pseudoanabaena limnetica*, Testis, Liver, Kidney

INTRODUCTION

The blue-green algae (Cyanobacteria) are widely distributed in aquatic, terrestrial habitats and capable to survive in a wide range of extreme environments [1, 2, and 3]. Cyanobacteria blooms may be a cause of toxicity to several organisms such as fishes, animals, invertebrates, plants, domestic animals and humans [4, 5, 6, 7, and 8]. Cyanotoxins are a diverse group of compounds, which include cyclic peptides (Microcystins and Nodularin), Alkaloids (Anatoxin-a, Antoxin-a(s), Saxitoxins) and non-protein amino acids [9]. Anatoxin-a is a similar structure analogue to acetylcholine (a neurotransmitter) with relatively small molecular weight (165 Da). This toxin is rapidly absorbed in the gastrointestinal tract. In mammalians the mode of action of anatoxin-a is a mimic to the action of acetylcholine; however, it could not be degraded by acetylcholine esterase [5, 10].

Anatoxin-a is a cyanotoxin produced by several genus and species particularly *Aphanizomenon*, *Microcystis*, *Anabaena flos-aque*, *Oscillatoria formosa* and *Pseudoanabaena limnetic* [11, 12, 13]. These types of algal toxins were termed as Very Fast Death Factor (VFDF), because it induces paralysis, convulsion, tremors and causes death after 2-7 minutes [14]. Several studies focused on the toxicity of anatoxin-a against animals such as the study of Claska and Gilbert [15] on the survival rate of *Daphnia pulex* and the study of Weigand and Pflugmacher [5] on reproductive rates of rotifers. A little information is known about the anatoxin-a toxicity against mammals, for example on rats, hamster and mice [16]. Algal neurotoxins (anatoxin-a, anatoxin-a(s) and homoanatoxins) do not completely comprehend upon their toxico-kinetic, except several emblems associated with oral exposure, which showed fast absorption of toxins from the gastrointestinal canal [17]. , while the study of Paschner et al., [18] revealed detected anatoxin-a in the urine of poisoned dogs and their bile acid without changing its composition.

The side effects of neurotoxin anatoxin-a in other organs except the nervous system are very rarely studied except the study of [19], which visualized

the toxic effects of this toxin on testes and sperm count. The present study aims to visualize the toxic effects (Histopathological effects) of purified anatoxin-a from local alga on some organs (Testes, Liver and kidney) in laboratory mice after experimental intraperitonial injections.

MATERIAL AND METHODS

Collection, isolation, purification and cultivation of blue-green alga *P. limnetica.*

The alga was collected from Shutt AL-Arab river water by using phytoplankton net (Mesh size = 20 μm). Dilution method was used to isolate this alga as unialgal culture and cultivated in Cu-10 liquid medium (As of patch culture 1500 ml media in 2l conical flasks) according to [20]. The purification method of alga was done according to [21] to get axenic cultures. Algal identification was made according to [22].

Purification of anatoxin-a

Anatoxin-a was extracted and purified depending on [23]. With some modifications by use of 20 ml of trichloroacetic acid methanol (0.01% V/V). Fifty milligrams of algal dry weight was used to extract the purified anatoxin-a [13]. The purified toxin was preserved in dark glass containers to prevent degradation by the light and kept in the refrigerator at (-18 C◦).

Quantitative determination of Anatoxin-a

The Enzyme-linked immunosorbent assay (ELISA) technique was used to determine the quantity of toxin in algal cells after purification above according to the method of [24]. The kit was purchase from Abraxis Company.

Preparation of Laboratory mice

In the present study 24 male mice, each of 25 gm. in weight were obtained from the animal house of Biology department / College of Education for pure sciences at Basra University/Iraq. Male mice were kept under laboratory conditions at 25±1 C◦ and constant light periods 12/12 light and dark respectively according to [25]. Mice were divided into three groups (8 mice for each group) and were in three middle-size plastic cages separately. All cage floors were replaced weekly by new sawdust.

Preparation of purified anatoxin-a doses and design of experiment

Two doses were made from purified anatoxin-a represented by Low dose = 0.5 μg/l and high dose =1 μg/l, also, to control = normal saline. Each experimental group of mice were injected daily with 0.1 ml of each dose intraperitonial (i.p. injection) for 15 days.

Histological sections

At the end of experiments, all mice were sacrificed and organs including liver, kidneys and testes were dissected and processed for pathological procedures. After that fixation in 10% formalin solution followed by dehydration in ascending concentrations of ethanol and clearing in xylene followed by impregnation in paraffin wax and preparing wax cubes to be cut by the microtome into appropriate 4-5 micrometer sections which were then stained by hematoxylin & eosin stains according to the standard method of [26]. Light microscope with a digital camera (Type Genex USA) was used to take photos at 100X and 400X magnification.

RESULTS

The findings showed that puri-fied neurotoxin (Anatoxin-a) from alga *P.limnetica* has caused many histopathological changes on various important organs represented by the liver, testes and kidneys of laboratory mice under the two doses used in the study. The results revealed the following:

Liver:

A: low dose

The hepatocytes appear more eosinophilic and some cells without nuclei. Most cells show early changes of fatty degeneration indicating reversible hepatocellular injury, while the sinusoids are less clear with mild inflammatory infiltrate of the portal area under low dose compared with the control group as shown in Fig-1, 2.

B: High dose

A toxic effect appears more severe with a marked degree of fatty degeneration (large vacuoles within hepatocytes). Liver sinusoids could not be visualized with inflammatory cells infiltration of hepatocytes. Markedly dilated and congested venules with extravasation of red blood corpuscles. Many necrotic cells are found with chronic inflammatory cells at the portal region, Fig-3, 4.

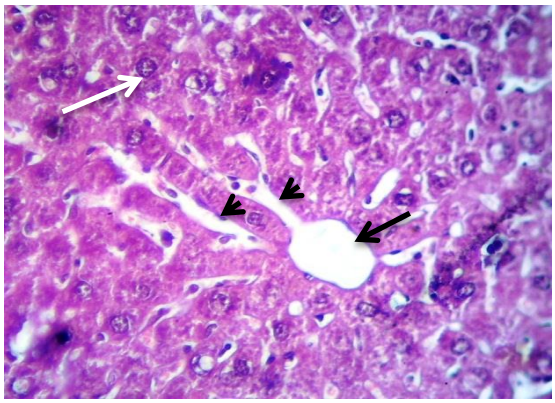

Fig.1: Liver (control) show central vein (Long arrow, Sinusoid (Head arrows), hepatocytes (White arrow).

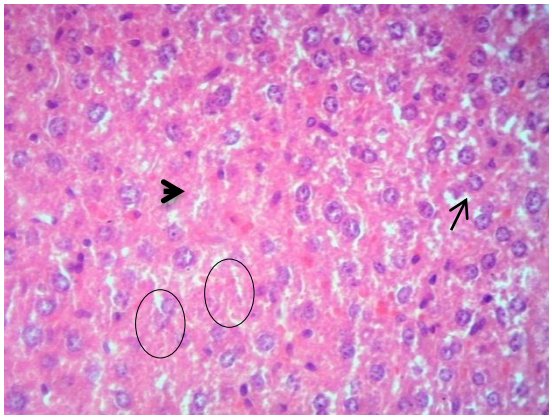

Fig.2: Liver (Low dose): Eosinophilia of hepatocytes and some cells without nuclei (Head arrow), Early fatty degeneration (Thin arrow), Sinusoids less clear (Circles).

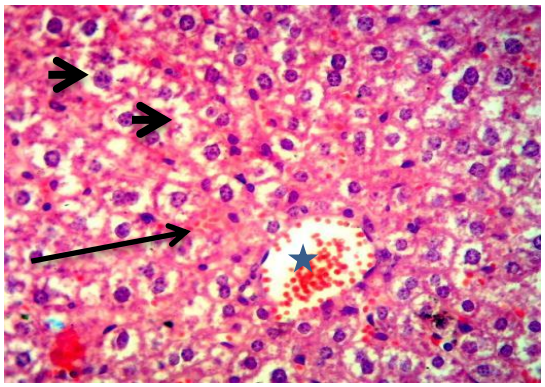

Fig.3: Liver (High dose): Fatty degeneration of hepatocytes (Short arrows), Extravagated red corpuscles (Long arrow) & Congested vessel (Star).

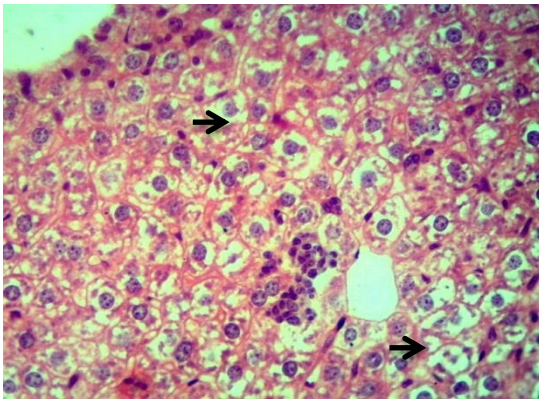

Fig.4: Liver (High dose): Fatty degeneration of hepatocytes (Short arrow).

Kidneys

A: low dose

The results showed increased eosinophilia of cells of renal tubules with mild degenerative changes of many tubules, especially the proximal tubules. Some of the glomeruli appear hypercellular and hemorrhagic, with increasing size of the urinary capsule, Fig-5, 6.

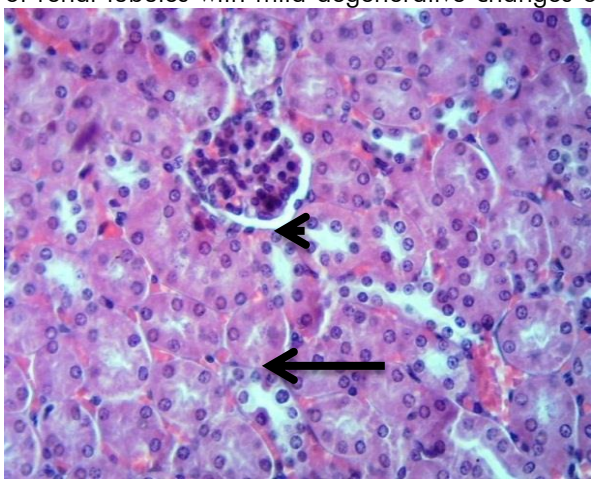

Fig.5: Kidney (Control): Renal corpuscle (Head arrow), Renal tubules (Long arrow).

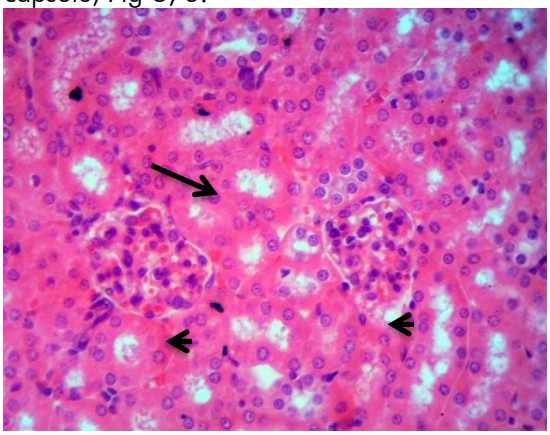

Fig.6: Kidney (Low dose): Increased eosinophilia of renal tubules with mild degenerative changes (arrow), Hemorrhage of glomeruli with increased size of urinary capsule (Head arrow).

B: High dose

Many gross morphological effects appear in the kidneys exposed to the high dose including extravasation of red corpuscles with clear degeneration of many renal tubules and congestion and dilation of capillaries and venules. Loss of many glomeruli is noticed with the presence of a few collections of chronic inflammatory cells, Fig-7.

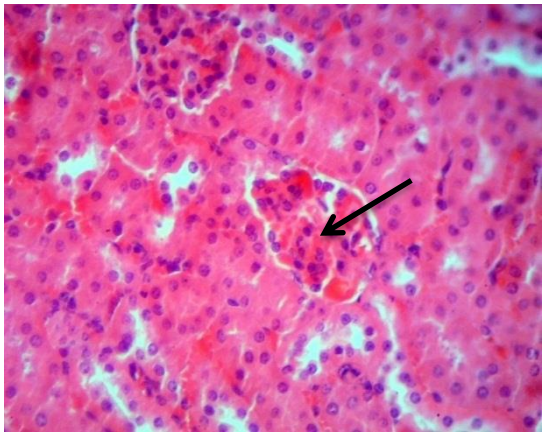

Fig.7: Kidney (High dose): Congested and hyper-cellular glomerulus (Long arrow), degenerated tubules (Head arrow).

Testes

A: Low dose

The exposure to this concentration of anatoxin did not induce marked gross morphological effect on mouse seminiferous tubules. However, there appears a loss of organization and decrease in the numbers of elongated spermatids in the center of tubules. Leydig and Sertoli cells appear unaffected by the toxin, Fig-8, 9

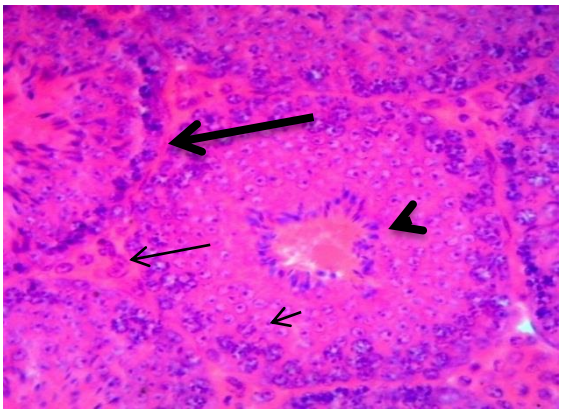

Fig.8: Testis (Control): Spermatogonia (Thick and long arrow), Elongated spermatids (Head arrow), leyding cells (Thin and long arrow, Round spermatocytes (Thin and short arrows).

B-High dose

The higher dose revealed good evidence of the gross morphological effect on seminiferous tubules, which appear devoid of elongated spermatids in most tubules with predominant round spermatids Fig- 10-13.

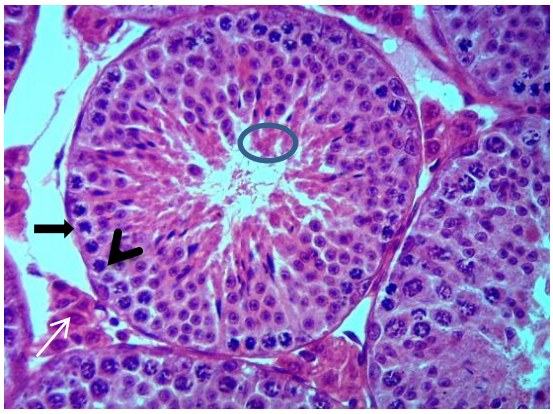

Fig.9: Testis (Low dose): Elongated spermatids (Circle), Sertoli cells (White arrow), Round spermatogonia (Head arrow), basement membrane (arrow) and Leydig cells (Star).

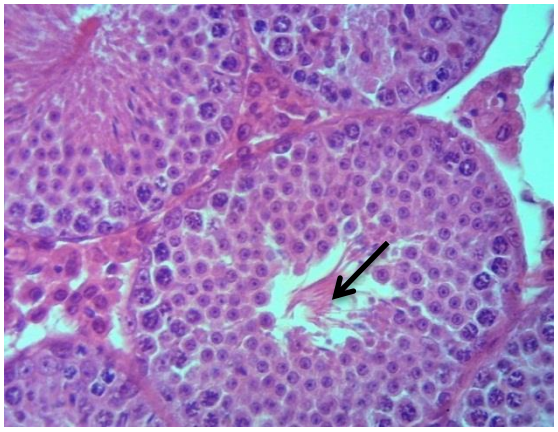

Fig.10: Testis (High dose): Loss of organization and decrease in the number of elongated spermatids in the center of tubules (arrow).

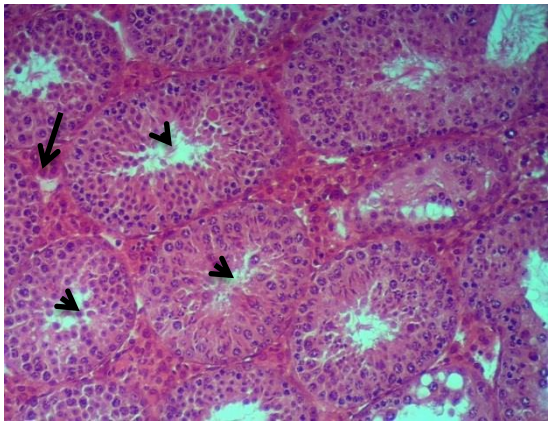

Fig.11: Testis (High dose): Absence of elongated spermatids (Head arrow), Leyding cells (Arrow).

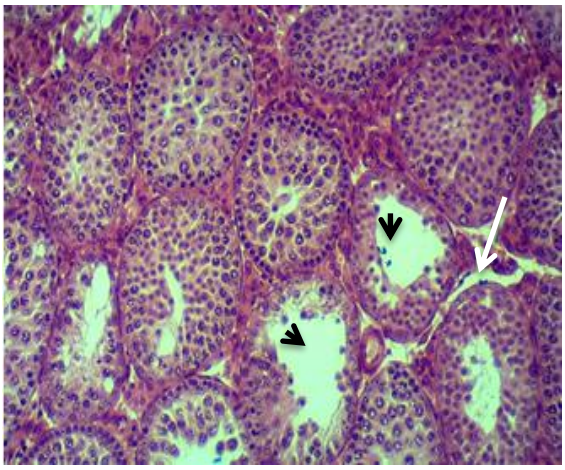

Fig.12: Testis (High dose): Absence of elongated spermatids (Head arrow) and Leyding cells (White Arrow)

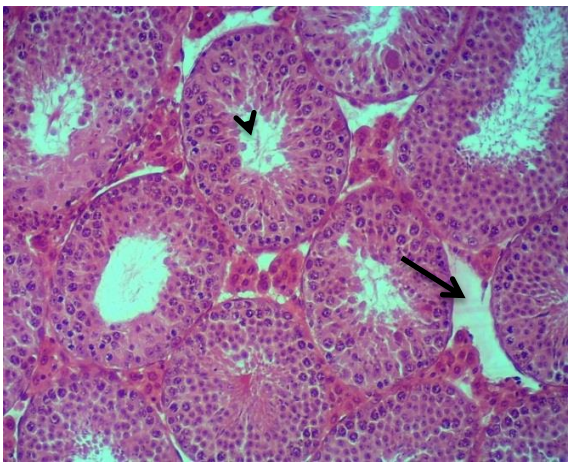

Fig.13: Testis (High dose): Absence of elongated spermatids (Head arrow) and Leyding cells (Arrow).

DISCUSSION

In previous studies, it was revealed that cyanobacterial alkaloids, especially anatoxins and saxitoxins on exposure to mice by oral administration or intraperitoneal injection have caused many symptoms represented by paralysis, cyanosis, respiratory arrest and death within few minutes depending on the dose of exposure [27]. Also, they caused many pathological changes in brain tissue such as decay in the thickness of grey matter, karyopycknosis of nuclei of glial cells, shrinkage of neurons, congestion of capillaries, the disappearance of endothelial cells lining the blood vessels and white matter appear as spongiform when mice were injected with sub lethal doses of purified anatoxin-a (0.5 and 1μ/L) from alga *P. limnetica* [13]. The present study aims to show if the purified anatoxin-a at sub-lethal doses (0.5 and 1μ/L) from alga *P. limentica* can cause toxic pathological effects in vital organs of male mice including the liver, kidneys and testes or not.

The finding in the present study showed that dose-dependent histopathological changes were detected in experimental animals compared to the control group. The liver showing an increased degree of fatty degeneration, congestion, inflammation and necrosis which is more prominent in high dose group (1 μg/L), as seen in Fig. (3, 4), Similar findings were noted in liver tissue of mice after exposure to toxic effects of insecticide agents like dimethoate (an organophosphorus compound with anti-acetylcholine esterase like activity) which revealed more toxic dose-dependent changes [28]. Similarly, the kidneys produced morphological alterations that varied according to the concentrations of the anatoxin-a administrated, so that more severe pathological effects on renal tubules, glomeruli and vasculature were noticed in the high dose group. Consistent results were mentioned by other researchers[29,30], which showed many histopathological effects on liver, kidneys and lungs of mice when they used acetic acids extract (AEs) of two cyanobacterial species *Pseudoanabaena galeata* and *Geitlerinema splendidum*. In their study, they concluded that the two species produced unknown anti-acetylcholine esterase toxic effect on mammals after oral administration. Another study [31] revealed that the extract of alga *P. galeata* which contains three types of cyanotoxins (Microcystins, saxitoxins and anatoxins) that were detected by two detection techniques HPLC and ELISA was caused the hepatic injury in mice after intraperitoneal injection and also a reduction in body weight and observed tumor promotion in the liver after one week of treatment. The results of our study are consistent with several studies which revealed that neurotoxins may produce hepatic hemorrhage and different symptoms [32, 33]. The histopathological effects in this study on some important organs (liver, kidney and testis) revealed that exposure to low concentrations of anatoxin-a (1 μg/l) for a short period (15 days) it's harmful and influential for the integrity of living organisms [34]. The histopathological examination of testes exposed to anatoxin-a at low dose showed minor changes Fig.9 which were more severe in the high dose testes. These findings indicate the arrest of spermatogenesis and delay in the production of spermatozoa which might lead to infertility. Microscopic examination of germ cells nuclei shows condensed chromatin with hyperchromasia and prominent nucleoli that may be due to DNA damage induced by the toxins compared with control, Fig-8. These results agree with [19], whose study was conducted to evaluate the effects of neurotoxin (standard pure of anatoxin-a) on testes, however, the dose that was used in that study was much higher than this research which reaches to

50, 100 and 150 μg/kg/day i.e. (1.25, 2.5 and 3.75 μg/25g/day.

CONCLUSION

The present study showed that the purified neurotoxin (Anatoxin-a) from toxic alga *P. limnetica* under low concentrations (0.5 and 1 μg/l) led to many histopathological effects, especially on the liver and kidneys, as well as its significant impact on the testes in male laboratory mice, which may cause infertility. It can be said that exposure to water contains these concentrations of toxin for at least two weeks can cause significant harm to live creatures in aquatic and terrestrial environments.

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CONFLICT OF INTEREST

The authors declare no conflict of interests, associated with the present study.

REFERENCES

1. Friedmann EI. Endolithic microbial life in hot and cold deserts. In Limits of Life; Springer: Berlin, Germany, 1980, pp. 33–45.
2. Rogers EH, Hunter ES, Moser VC. Potential developmental toxicity of anatoxin-a, a cyanobacterial toxin. J. Applied Toxicology, 2005, 25(6):527-534.
3. Wierzchos J, Ascaso C, McKay CP. Endolithic cyanobacteria in halite rocks from the hyperarid core of the Atacama Desert. Astrobiology, 2006. 6, 415-422.
4. Fawell JK, Mitchell RE, Hill RE, Everett DJ, 1999. The toxicity of cyanobacterial toxins in mouse: II Anatoxin-a .J. of Human and Experimental Toxicology, 2005, 18(3):168-73 https://doi.org/10.1177%2F096032719901800306
5. Weigand C, Pflugmacher S. Ecotoxicological effects of selected cyanobacterial secondary metabolites: a short review. Toxicol Applied Pharmacology, 2005, 203:201-18.
6. AL-Sultan EYA .Isolation, Purification and Identification of Blue-green alga *Hapalosiphon aureus* and evaluation of its histopathological effects on fresh water snail *Lymnaea aurecularia*. Journal Applied Sciences, 2017.17(2):61-71.
7. AL-Sultan EYA, Hatem MT .Isolate and Cultivate Three Species of Blue-Green Algae from Soil Southern of Iraq and Study the Effect of Purified Microcystins from Alga Oscillatoria pseudogeminata on Seed Germination of Tomato Plant Lycopersicon Esculentum. Journal of Biology, Agriculture and Healthcare, 2018, 8(16):27-36.
8. AL-Sultan EYA, Hatem MT.Toxic effects of purified Microcystins from soil Blue-green alga Oscillatoria pseudogeminata on tomato plant Lycopersicon esculentum .J.of Baghdad Science Journal ,2019,16(1): 196- 177.
9. Metcalf JS, Codd GA. Cyanotoxins. In Ecology of Cyanobacteria II; Springer: Berlin, Germany, 2012, pp. 651–675.
10. Whitton BA, Potts M. The ecology of Cyanobacteria. Their diversity in time and space. Dordrecht, Boston, London: Kluwer Academic Publishers. II; Springer: Berlin, Germany, 2000, pp. 1-13.
11. Skulberg OM, Carmichael WW. Investigations of a neurotoxic oscillatorialean strain (Cyanophyceae) and its toxin – isolation and characterization of homoanatoxin-A. J. of Environmental and Toxicological Chemistry, 1992, 11: 321-329.
12. Viaggiu E, Melchiorre S, Volpi F, Di Corcia A, Mancini R, Garibaldi L. Anatoxin-a toxin in the cyanobacterium *Planktothrix rubescense* from a fishing pond in northern Italy. J. of Environmental Toxicology, 2004, 19:191–7.
13. Al-Sultan EY, Aubaed, AMA. Extraction and purification of neurotoxin(Anatoxin-a) from blue-green alga *Pseudoanaaena limnetica* and indicating its histopathological effects on the rain of male laboratory mice (Mus Musculus L.). J.of Biology, Agriculture and Healthcare, 2017, 7(18):77-93.
14. NCEAToxicological reviews of cyanobacterial toxins: Anatoxin-a .National Center for Environment, 2006, pp. 1-34.
15. Claska ME, Gilbert JJ. The effect of temperature on the response of Daphnia to toxic cyanobacteria. J.of Freshwater Biology, 1998, 39:221-32.
16. MacPhail RC, Jarema KA. Prospects on behavioral studies of marine and freshwater toxins. Neurotoxicol Teratology, 2005, 27:695-9.
17. Patoka J, Ramesh C, Gupta C, Kamil K. Anatoxin-a(s): Natural organophosphorous antcholinestrase agent. J. of Mil. Med. Sci. Lett. , 2011, 80.p:129-139.
18. Puschner B, Pratt C, Tor ER. Treatment and diagnosis of a dog with fulminant neurological deterioration due to anatoxin-a intoxication. J. Vet Emerg Crit Care; 2010, 20: 518-22.
19. Yavasoglu A, Karaaslan MA, Uyanikgil Y, Sayim F, Ates U, Yavasoglu NUK. Toxic effects of anatoxin-a on testes and.sperm counts of male mice. J. of Experimental Toxicologic Pathology, 2008, 60(4-5):391-6 Doi:10.1016/j.etp.2008.04.001.
20. Stein, JR. Handbook of phycological method. Cambridge University press. Cambridge, 1975, 445 pp.
21. Weideman VE, Walne PR, Tainor FR. A new technique for obtaining axenic cultures of algae. Can. J. Bot., 1984, 42: 958 -959.
22. Gongliang Y, Meng ling Z, Youxin C, Qianqi an P, Weno C, Renhui . LPolyphasic characterization of four species of *Pseudoanabaena* (Oscillatoriales, Cyanobacteria) from China and insight into

polyphyletic divergence within the *Pseudoanabaena* genus .J. of Phytotaxa, 2015, 192 (1):1-12.
23. Harada K, Nagai H, Kimura Y, Suzuki M, Park HD, Watanabe MF, Luukkainen R, Sivonen K , Carmichael WW. Liquid chromatography/mass spectrometric detection of anatoxin-a, a neurotoxin from cyanobacteria. J.of Tetrahedron, 1993, 49:9251.
24. Fischer WJ, Garthwaite I, Miles CO, Ross KM, Aggen JB, Chamberlin AR, Towers NA , Dietrich DR. Congener-Independent Immunoassay for Microcystins and Nodularins. J.of Environmental Science Technology. 35, 2001, 4849-4858.
25. Al-Maliki SJ. A behavioral and some physiological effect of (*Apium graveolens)* seeds in albino mice. J.of Science. Basrah, 2000, 2:77-88.
26. Humason GL. Animal tissue techniques. Freeman, W.H. (3th ed.), San Francisco press. UAS, 1972, PP.641.
27. Harada K, Kondo F, Lawton L. Laboratory Analysis of Cyanotoxins. In *Toxic Cyanobacteria in Water: A Guide to Their Public Health Consequences, Monitoring and Management*, 1st ed.; Chorus, I., Bartram, J., Eds.; E & FN Spon: New York, NY, USA, Volume 1, 1999, pp. 369-405.
28. Alarami AMJ. Histopathological changes in liver and kidney of albino mice on exposure to insecticides, Dimethoate .Int.J.Curr. Microbiology and Applied Sciences, 2015, 4 (7):287-300.
29. Rangel M, Brunetti RL, Garcia, AN, Cambu CCN, Conserva GAA, Neves AC, Sant'Anna CL, Carvalho LR. Acute effects of three *Geitlerinema* spp. (Cyanobacteria) extracts administrated in mice: Symptoms and histopathological aspects. Phytochemical Review, 2012, 11, 1-11; Doi: 10.1007/s11101–012–9240-x.
30. Rangel M, Martins JCG, Garcia AN, Conserva GAA, Costa-NA, Anna, LCS, Decarvalho LR. Analysis of the toxicity and histopathology induced by the oral administration of *Pseudoanaaena galeata* and *Geitlerinena splededidum* (Cyanobacteria) extract to mice .J. of Mar. Drug, 2014, 12(508-524).Do: 10.3390/md12010508.
31. Brunetti RL, Campos-Junior AG, Garcia AN, Dogo CR, Mattos LFA, Carvalho LR,Rangel, M. Histopathological evaluation of injected mice with extracts of two toxic cyanobacteria strains. In Proceedings of the XI Reunião Cientifica Anual do Instituto Butantan, Sao Paulo, Brazil, 2–4 December 2009; Memórias do Instituto Butantan: Butantã, Brazil, 2009, Volume 66, pp. 267–267.
32. Conserva GAA, Sant'Anna CL, Cambui CCN, Brunetti RL, Rangel M, Torres LMB, Young MCM, Carvalho LR. Prospecção de atividades toxicológicas e farmacológicas em cepas de cianobactérias da Coleção de Culturas do Instituto de Botânica (Screening of toxicological and pharmacological activities in cyanobacteria strains of the Culture Collection of the Institute of Botanyll). In Proceedings of the 18ª Reunião Cientifica Anual do Instituto de Botânica, Sao Paulo, Brazil, 2011, 21-25, pp. 1-4.
33. Carvalho LR, Neves AC, Conserva GAA, Brunetti RL, Hentschke G S, Malone CFS, Torres LMB , Sant'Anna CL, Rangel M. Biologically active compounds from cyanobacteria extracts: *in vivo* and *in vitro* aspects. Braz. J. Pharmacognoncy., 2013, 23, 471-480.
34. Fawell JK, Mitchell RE, Hill RE, Everett DJ. The toxicity of cyanobacterial toxins in mouse: II Anatoxin-a .J. of Human Experimental Toxicology, 1999, 18: 162–167. doi.org/10.1177%2F096032719901800305.