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Toxic effects of cyanobacterial alga *N. muscurum* on some physiological parameters in blood of *Ctenopharyngodon idella* VaL.1844

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Abstract

Study included feeding juvenile grass carp *Ctenophartyngodon idella* on toxic alga *Nostoc muscurum* as solitary and mixture feeding (toxic alga and clover) as compared with fed on clover only (control group) with three feeding periods (24 h, 15 days and 35 days) to evaluated toxic effects of these types of food on some blood parameters and activity of some liver enzymes in blood of *C. idella* juvenile . Study showed significant decreasing in mean of red blood corpuscles (RBCs) , white blood corpuscles (WBCs), Haemoglobin (Hb), Packed cell volume (PCV) and total protein in blood plasma when *C. idella* juvenile fed on toxic alga and fed on mixture feeding compared with control group with increasing food periods . Also study showed highly significant increasing $P<0.05$ in activity of some liver enzymes represented by alkaline phosphatase (ALP) , alanine transaminase (ALT) and Aspartate transaminase (AST) in all feeding periods when *C. idella* juvenile fed on toxic alga and mixture feeding compared with control group with notes reduction in survival percentage and means weight of juvenile after fed on toxic alga and mixture feeding .

Key word: *Nostoc muscurum* ,*Blood parameter* , *Hepatotoxins* (Microcystin) ,Liver enzymes

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Introduction

Cyanobacteria are the dominant phytoplankton group in eutrophic fresh water bodies (Negri *et al.*, 1995) .They are prokaryotes possessing cell walls composed of peptidoglycan and lipopolysaccharide layers instead of cellulose of green algae (Skulberg , *et al*., 1993) . All cyanobacteria are photosynthetic and possess chlorophyll (a), morphological diversity rang from unicellular to small colonies of cells to simple branched filamentous forms (Weier *et al*.,1982) . Cyanobacteria are capable to producing two kinds of toxin cyclic peptide hepatotoxin and alkaloid neurotoxin. Serious illness such as hepatoenteritis, asymptomatic pneumonia and dermatitis may result from consumption of or contact with water contaminated with toxin producing cyanobacteria (Gupta and Guha, 2006). Microcystins are a family toxins produced by different species of fresh water cyanobacteria , namely *Microcystis* (order : Chroococcales), *Anabaena* , *Aphanizomenon* ,*Nostoc*(Order:Nostocales)and *Oscillatoria*(Order:Oscillatoriales)(Compos and Vasconcelos , 2010). There are over 80 different microsystins that differ primary in the two L-amino acids at position 2 and 4 , and methylation / demethylation on MeAsp and MDha . The unusual amino acid Adda is essential for the expression of biological activity .Other microcystins are characterized largely by variation in the degree of methylation; amino acid 3 has been found to be D-aspartic acid, replacing β-methylaspartic acid and amino acid 7 to be dehydroalanine, replacing N-methyldehydroalanine (An and Carmichael 1994) . The most common microcystin is microcystin-LR where the variable L-amino acids are leucine (L) and arginine (R)(Gupta and Guha , 2006) . The Adda moiety, Present in all variants, is critical to MC activity.also isomerization and / or oxidation dramatically reduces the toxicity (Song *et al.*, 2006) figure-1 .

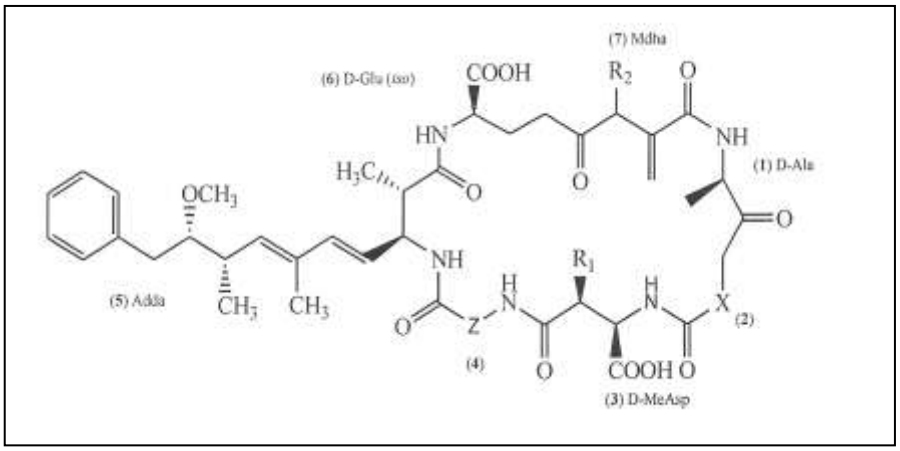


Figure (1): General structure of microcystin. In MC-LR X represents L- Leucine; Z L-Arginine, R1 and R2 CH3. McElhiney and Lawten (2005)

Microcystins inhibit serine / threonine -specific protein phosphatases (PPS) such as PP1 and PP2A through the binding to these enzymes (Gulledgea *et al*., 2002). The acute toxicity of MC can be explained by this phosphatase inhibition which leads to an excessive phosphorylation of proteins and of alternation in cytoskeleton, change of cell shape with subsequent destruction of liver cells causing intrahepatic haemorrhage or hepatic insufficiency (Van Apeldoorn *et al*., 2007). Microcystins are also responsible for the increase of oxidative stress in cells which subsequently can trigger apoptotic processes (Prieto *et al*., 2010 ; Zhang *et al*., 2008) .These molecules have been regarded also as tumor promoters (Lankoff *et al*., 2008) .

In aquatic systems, fish stand at the top of the aquatic food chain, and are possibly affected by exposure to toxic cyanobacteria. Many experimental studies have been conducted to document the toxicity of microcystin exposure through gastrointestinal or blood circular systems on common carp (Wu *et al*., 2002) and gold fish (Xu *et al*., 1998), and grass carp(Chen *et al*., 1995). However, all these studies were limited to acute experiments, and they were based on either oral gavaging, or intraperitoneal injection, or administration via the dorsal aorta of the toxin, which can not reflect the uptake route under natural environments. Additionally, the toxins used in these experiments were either purified MC or dried algae containing MC. Therefore, toxic effects of fish from MC exposure through natural food uptake need to be evaluated experimentally, especially if there is long term frequent exposure in natural environment (Xie *et al* , 2004).

In the last few years , evidence has been accumulated pointing to the fact that not only mammals but also fishes are susceptible to this class of natural toxins - fish kills involving algae-feeders such as carp (*Cyprinus carpio*) (Viala *et al.*, 1986) as well as omnivores stages of carnivorous fish strictly carnivorous fish , have been reported in conjunction with toxic cyanobacterial blooms (Andersen *et al*., 1993 ; Rodger *et al*., 1994) . Its needed surprising that carp can be affected by toxic cyanobacterial blooms, as algae feeders such as carp and tilapia (*Oreochromis niloticus*) are known to graze on non-toxic *Microcystis aeruginosa* strains in environmental conditions (Northcott *et al*., 1991) and have been showed to avoid ingestion of cyanobacteria (Dewan *et al*., 1991) as well as to decrease grazing activity in conjunction with increasing percentage of toxic cyanobacteria (Keshavanath *et al*., 1994).

This study may be considered the first in Iraq to evaluate toxicity of toxic alga *N. muscurum* as force feeding and mixture feeding (toxic alga and clover plant) on some blood parameters and important liver enzymes in blood of economic fish juvenile grass carp (*Ctenopharyngodon idella*) and compared with normal food (Clover plants) only .

Material and Methods

Isolation and purification of Toxic alga *Nostoc muscurum*

The isolate of toxic cyanobacterial alga *N. muscurum* was obtained from Algal laboratory (College of Education / Basrah university).this cyanobacterial species which has ability to produce toxins namely microcystins exactly MC-LR reach to 27.57 µg / ml (Al-Sultan , 2011).

Toxic alga was cultivated in modified Chu-10 liquid medium according to (Al-Aarajy -1996). Conical flasks 3 L in volume were used in cultivation of toxic alga as batch cultures filling with 2L of

liquid medium , cultures were incubated in growth chamber with temperature 23±2 and continuous illumination to get unialgal culture of toxic alga . Axenic culture were made according (Weidman *et al*., 1984). algal isolate was classified according to (Desikachary , 1959 ; Prescott , 1975).

Source of fish juvenile: Grass carp *Ctenopharyngodon idella* were obtained from vertebrate departments / marine science center / Basrah university with mean weight 5.44 g . Fishes were acclimation and feeding experments were done in plastic cages volume 30 L with using clover only as food during acclimate period. Each cage contain 5 fishes Juvenile. Quarter volume of each water cages were removed daily to save the quality of water.

Feeding Experiments

C. idella juvenile were fed on three types of food (three treatment) 1- toxic alga *N. muscurum* only in portion (1m/L from axenic culture) 2- mixture feeding 1ml /L (Toxic alga and clover in portion 1:1 v/v) and 3- clover only as control group . Toxic alga were used as food after cultures reached to early stationary phase i.e after 14 days (Al-sultan, 2011). Fish juvenile were fed daily on those three type of food separately until reach to saturated status. Feeding experiments were extended to 35 days. Three replicate were done for each treatments (each replicate contain 5 fish juvenile). Three time periods were selective to get fishes sample (three fishes) for all physiological paramerters after 24 h , 15 days and 35 days of feeding.The temperature of aquarium water range between 22-24 C° and dissolved oxygen between 7.5-8.3 mg /L . All blood samples were getting from cutting the caudal fin of *C. idella* juvenile.

Physiological study

A- Blood parameters: Red and white blood corpuscles (RBCs, WBC) were counted according to Lucky (1977) by using haemocytometer type (improved Neubaure).Haemoglobin concentrations were measured according Sahli method depending on Hesser (1960). The Percentage of packed cell volume were measured by using microheamatocrite method. The blood were taken from caudal area by micropipette (1.1 – 1 × 75) mm filling with 90 % from fish juvenile blood. Micropipette centrifuge for 2-3 min. at 10000 rpm to separate blood cells from plasma. The volume of blood cells were measured by special ruler microcapillary reader type DAMON/ IES and Total protein in blood were measured according to the method of Bioret (Henry *et al* ., 1974) by using bioret Kit produce from RANDOX company , France under wave length 560 nm .

B-Enzymes activity : Kind and King (1954)colorimetric method was used to measure activity of enzyme Alkaline phosphatase (ALP) activity in plasma according alkaline phosphatase kit(Biomerieux company) under wave length 510 nm . The activity of enzyme Aspartate transaminases (AST) was measured according colorimetric method of Doumas and Briggs (1969) by using Aspartate transaminase kit produce from RANDOX company under wave length 546 nm and By using colorimetric method of Reitman and Frankel (1957), activity of enzyme Alanine transaminase (ALT) was measured by using Alanine transaminase kit improved from RANDOX company under wave length 546 nm.Statistical analysis by using anova test(Two way anova) by using program spss version11

Results

A- Effect of toxic alga *N. muscurum* on several blood parameters of *C. idella* juvenile

Result revealed significant decreasing $p<0.05$ in the mean number of red blood

corpuscles (RBCs) of *C. idella* juvenile for all feeding periods after 24 h , 15 and 35 days when fed on toxic alga *N. muscurum* only and mixture feeding these values reached to(2.18, 2.93) × 10^3 cell / cm^3 after 24hrs , (3.17, 2.49) × 10^3 cell/cm^3 and later (1.10 , 1.57) × 10^3 cell / cm^3 after 35 days respectively compared with fed on clover only figure (2). Also fed on toxic alga led to significant decreasing P< 0.05 in white blood corpuscles (WBCs) reached to 10.2 × 10^3 cell / cm^3 compared with control group 10.5 × 10^3 cell / cm^3 after 24 h . Also this decreasing were found after 15 and 35 days of feeding when juvenile fishes fed on toxic alga and mixture feeding reached to (10.7 , 12.9)× 10^3 cell / cm^3 and (10.4 , 10.03) × 10^3 cell / cm^3 for two type of food and food period respectively figure (3)

Mixture feeding showed significant decreasing P<0.05 of haemoglobin concentration in blood of *C. idella* juvenile after 24 h reach to 47 % and 52% after fed on toxic alga . Also decreasing in Hb concentreations were showed with increase of feeding periods 15 and 35 days on toxic and mixture feeding reached to (40, 50) % and (40 , 42.7) % for two food type and feeding period respectively compared with control group figure (4) . The packed cell volume was showed non significant decreasing p<0.05 after 24 h for three type of food and non significant differences in PCV after 15 days followed by significant decreasing in this value after 35 days of feeding especially when fed on toxic alga *N. muscurum* reached to 26% compared with control group figure (5).

Total protein in plasma of *C. idella* juvenile after 24 hrs showed non significant differences under three type of food types , while significant decreasing after 15 and 35 days of feeding on toxic alga and mixture feeding reach to (4.15 , 4.34) g/100 cm^3 and (3.67 , 3.98) g / 100 cm^3 respectively compared with fed on clover only figure (6).Results also showed decreasing in survival percentage of *C. idella* juvenile after fed on toxic alga and mixture feeding reached to (85, 90)% and (80, 85)% at feeding periods 15 and 35 days respectively compared with control group figure (7).Mixture feeding showed significant increasing in weight of *C. idella* juvenile after 15 days(5.45 g) , but after the end feeding periods (35 days) significant decreasing p< 0.05 in mean weight of juvenile when fed on toxic alga 3.23 g and mixture feeding 2.96 g compared with fed on clover only figure (8) .

Effect of toxic alga *N. muscurum* on activity of some liver enzymes of *C. idella* juvenile

Figure (9) showed significant increasing P<0.05 in the activity of enzyme alkaline phosphatase (ALT) after 24h when *C. idella* juvenile fed on toxic alga *N. muscurum* and mixture feeding reach to (3.17, 3.23) IU/100 ml.Also after 15 days highly significant increasing in activity of this enzyme when *C. idella* juvenile fed on mixture and toxic alga reach to(4.16 , 3.7)IU/100 ml respectively compared with fed on clover only,but non significant differences found after 35 days of feeding in all food type figure (9) .

The activity of enzyme AST after 24h showed significant decreasing p<0.05 when *C. idella* juvenile fed on toxic alga and mixture feeding reach to(49.45 , 50.12) IU/100 ml , while significant increasing in activity of this enzyme was showed after 15 and 35 days of feeding on toxic alga and mixture feeding reach to (65.73 , 56.65) IU / 100ml and (67.93 , 64.63) IU / 100ml for two type of food and two periods of feeding respectively compared with feeding on clover only figure (10).

The activity of enzyme (ALT) in blood of grass carp juvenile were showed significant increasing p < 0.05 at all periods

of feeding when fed on toxic alga and mixture feeding reach to (4.49, 4.63) IU/100 ml after 24h and (8.63,7.93)and (10.03 , 9.65) IU/100ml after 15 and 35 days of feeding for two type of food respectively compared with fed on clover only figure(11)

Discussion

In present study it is importance of hematology in diagnosis of fish diseases and assessment of the effect of hepatotoxins (Microcystins) exactly has been widely accepted . The reduction of erythrocyte count (RBCs), (WBCs) and haemoglobin count (Hb) in response to fed on toxic alga *N.muscurum* and mixture feeding respectively. These results are in agreement with those of Ramadevi *et al*., (1998) who found a decrease in RBCs, Hb and haematocrite value Hct in the blood of broiler chicks after ochratoxin intoxication (ochratoxin is hepatotoxin produce from fungus *Aspergillus niger*), also Shalaby (2001) found that the RBCs, Hb and packed cell volume (PCV) were decreased significantly in Nile tilapia poisoned by mercury. In addition Mousa and Khattab (2003) their found a decrease in RBCs, Hb and Hct in blood of the African catfish (*Clarias gartiepinus*) after ochratoxin intoxication. The perturbation in these blood indices may be attributed to defenses reaction against toxicity of hepatotoxins (ochratoxin) through the stimulation of erythropiosis or may be related to the decrease in RBCs , Hb , and Hct due to the exaggerated disturbances that occurred in both metabolic and hemopoitic activities of fish exposed to sub lethal concentration of pollutant (Mousa , 1999) . One of the most important functions of serum protein is the maintains of osmotic balance between the circulating blood and the tissue fluids (Harper *et al*.,1977). The influence of toxicants on the total protein concentration of fish has been also taken into consideration in evaluation the response to stressors and consequently the increasing demand for energy (Shalaby, 2001). Cytotoxicity test on human blood showed that human cells can affected by the cyanotoxins (Microcystins) because the ability of latest to analyze the membranes of the RBCs especially at hight concentration. Grabow *et al.,* (1982) referred to a trypan blue staining test showed that the toxin hepatotoxins (Microcystins) disrupted cell membrane permeability with in a few minutes , also he found that erythrocytes of human , mouse , rat , sheep and Muscovy duck were also lysed within a few minutes and hemolysis was temperature dependent . Sicinska *et al.,* (2005) showed that lysis RBCs memberanes may be results as conjugate between microcystins and free radicals of proteins (SH). Cyanotoxin especially MC-LR reduced the activity of spleen cell after 24h to 23% and cause death especially B-lymphocyte and the smash of cell memberane of erythrocyte may be tutor to inhibition in the activity of enzyme protein phosphatase and then protein synthesis such as plasma memberane and cytoskeletal cells (Teneva *et al.,* 2005) .

The quantitative determination of the total protein in plasma, muscle and liver reflects the liver capacity of protein synthesis and denotes the osmolarity of the blood and renal impairments. So it is of a few valuable factor in the diagnosis of toxicity in fish. In the present study the total protein in plasma of *C. idella* juvenile decreasing significantly when fed on toxic cyanobacterium alga *N. muscurum* and mixture feeding, this decrease might have been attributed to several pathological process including plasma dissolution, renal damage and protein elimination in the urine, decrease in liver protein synthesis, alternation in hepatic blood flow and / or

hemorrhage into the peritoneal cavity and intestine (Salah El-deen *et al*., 1996). These results agree with those of Saleem and Khafajii (2001) who found a significant decrease in serum protein in the rabitt after toxication with mycotoxin. Kopp and Hetesa (2000) found that total protein in common carp (*Cyprinus carpio*) was significantly reduced after exposure to the cyanobacteria microcystis aeruginosa and *Anabaena flos-aque*.Also Mousa and Khattab (2003) showed that the plasma protein and liver protein were decreased significantly in ochratoxin poisoned fish . The decrease in plasma and tissue protein may occur due to the increase of protein breakdown as a result of stimulated corticosteroid hormones which to provide amino acids for enhance the breakdown of proteins and gluconeogenesis to provide glucose to compensate for increase in energy demands under stressful condition.

In present study *C. idella* juvenile showed increase significantly in some important enzymes after fed on toxic alga *N. muscurum* and mixture feeding. So this increasing in activity of some important enzymes such as alkaline phosphatase (ALP),aspartate aminotransferase(AST)and alanine transaminase (ALT) reflect injury in some tissues in fish juvenile represented by blood , liver , kidney tissue do to the toxicity of microcystins, this results agree with AL-Sultan (2011) who found the ability of this cyanobacterial species *N.muscurum* to produced microcystin - LR at concentration 27.57 µg /ml and agree with study of Gupta and Guha (2006) their found increasing in the activity of these enzymes ALP , AST and ALT in blood of fresh water fish (*Heteropneuster fossilis*(Bloch))compared with control group after itraperitoneal injuction of microcystin after 1h from injection and maximum activity was observed after 24h of treatment .

Vajcova *et al*., (1998) showed significant increase in some enzymes ALT , AST and lactate dehydrogenase LDH in silver carp (*Hypophthalmicthyes molitrix*) after intraperitoneal application of pure microcystin , Also Rabergh *et al.,* (1991) reported that blood plasma enzymes (ALT , AST and LDH) increase two hours after intraperitoneal injection of toxins(microcystins)as a consequence of hepatocyte necrosis . In other wise Kopp and Hetesa (2000) have reported on increase in ALT and AST in the juvenile carp (*Cyprinus carpuio* L.) on exposure of fish to different natural populations of cyanovbacterial water blooms . After 35 days of feeding *C. idella* juvenile on toxic alga *N. muscurum* and mixture feeding non significant differences were found in the activity of Alkaline phosphatase between two type of food so as fed on clover only, this result also agree with report of Li *et al.,* (2004) who found that ALP and LDH activity remained unchanged in sub chronic oral toxicity of microcystin in common carp.

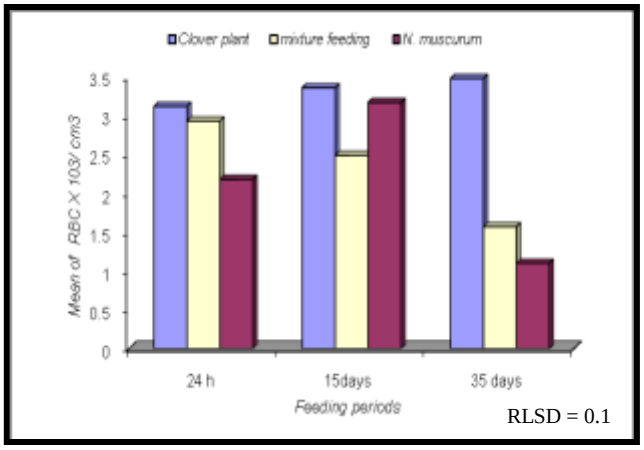
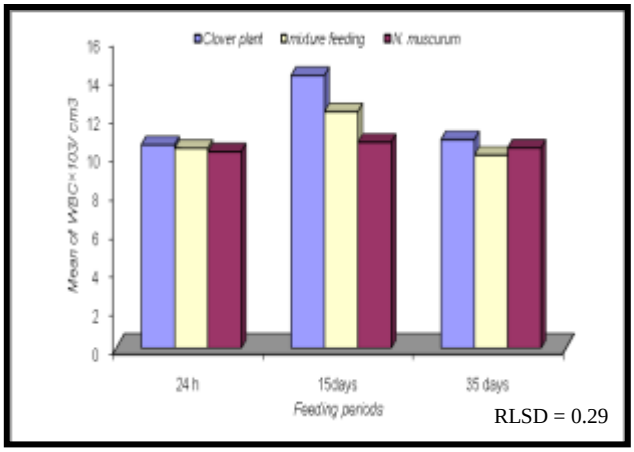
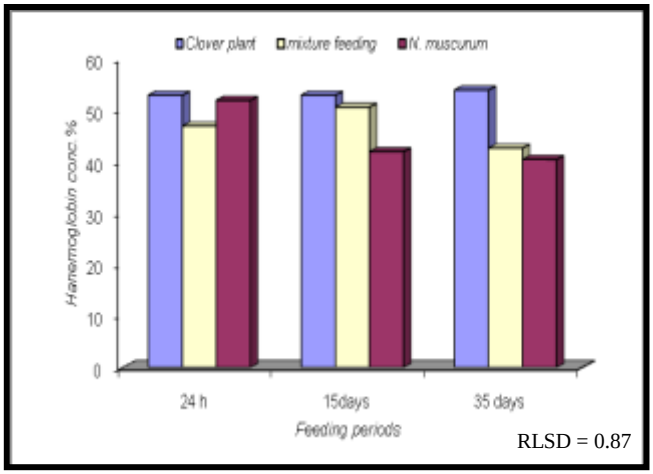


Figure (2): Mean numbers of RBC in blood of *C. idella* juvenile under three type of food (Toxic alga only, Mixture feeding and clover only at three time periods.



Figure(3) : Mean numbers of WBC in blood of *C. idella* juvenile under three type of food (Toxic alga only , Mixture feeding and clover only at three time periods .



Figure(4) : Mean numbers of haemoglobin in blood of *C. idella* juvenile under three type of food (Toxic alga only , Mixture feeding and clover only at three time periods .

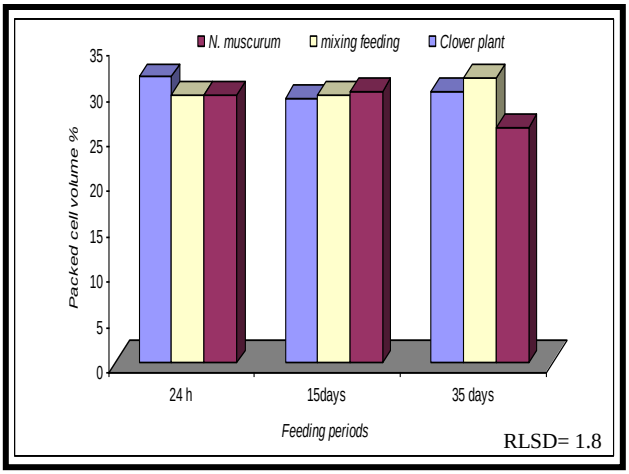


Figure (°): Packed cell volume (PCV) in *C. idella* juvenile under three type of food (Toxic alga only, Mixture feeding and clover only) at three time periods.

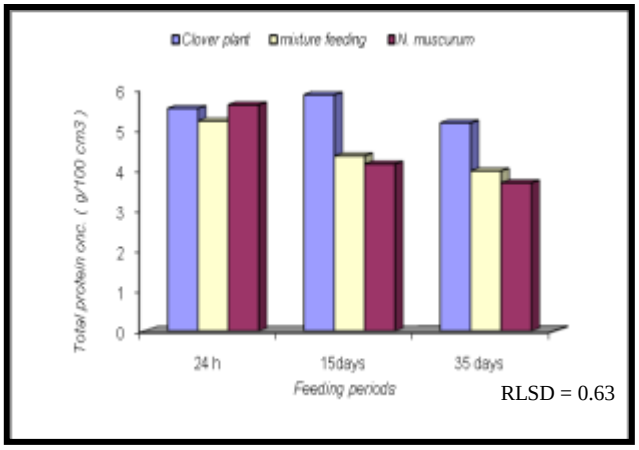


Figure (6): Total protein in blood of *C. idella* juvenile under three type of food (Toxic alga only, Mixture feeding and clover only) at three time periods.

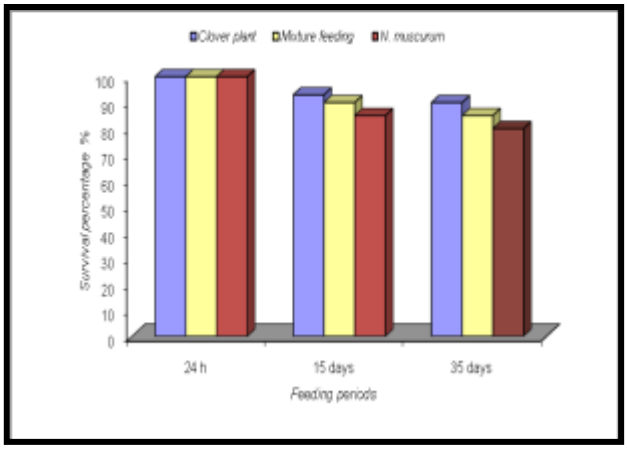


Figure (7): Survival percentage of *C. idella* juvenile after fed on toxic alga *N. muscurum*, mixture feeding and clover only.

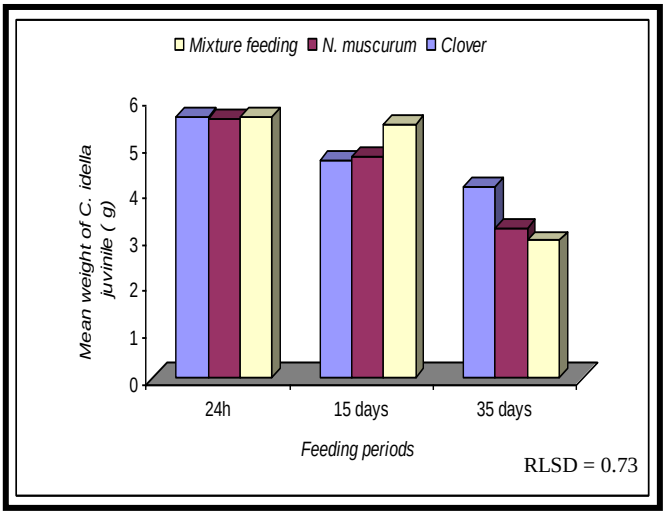


Figure (8): Mean weight of *C. idella* juvenile under three type of food Toxic alga, Mixture feeding and clover only at three time periods.

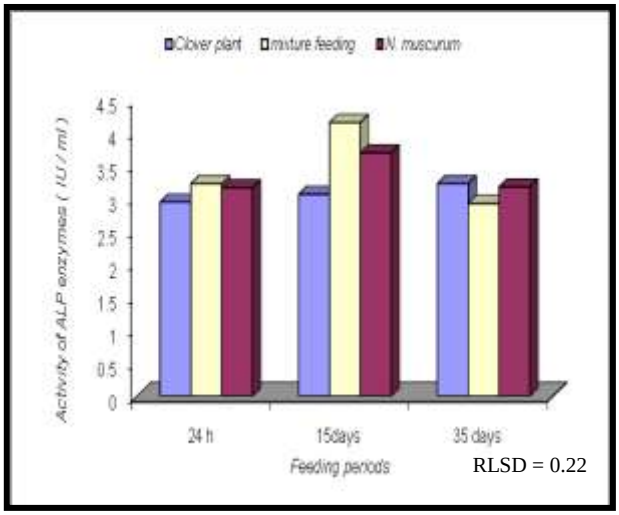


Figure (9): Activity of ALP enzyme in *C. idella* juvenile after feeding on toxic alga *N. muscurum*, mixture feeding and clover only at three time periods.

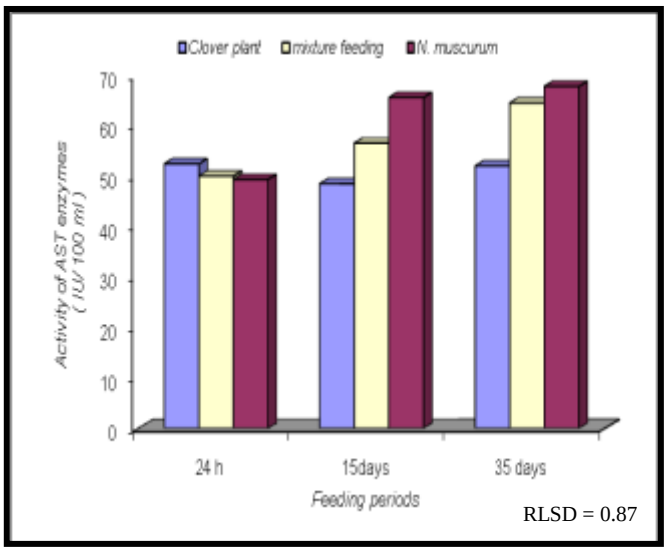


Figure (10): Activity of AST enzyme in blood of *C. idella* juvenile under three type of food (Toxic alga, Mixture feeding and clover only at three time periods.

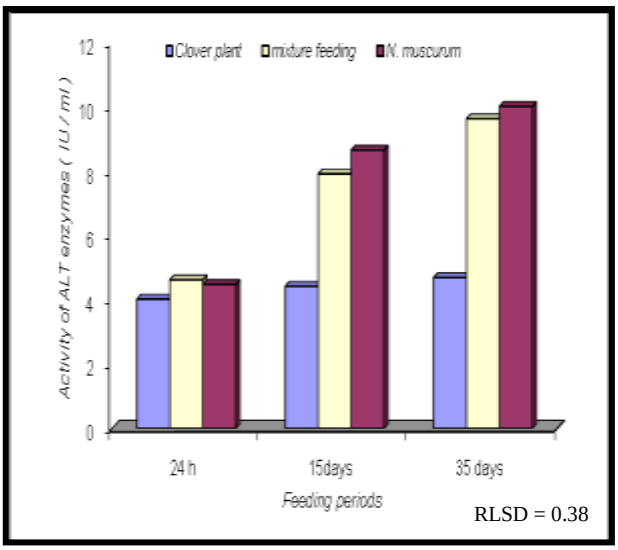


Figure (11): Activity of ALT enzyme in blood of *C. idella* juvenile under three type of food (Toxic alga, Mixture feeding and clover only at three time periods.

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التأثيرات السمية للطحلب الأخضر-المزرق *Nostoc muscurum* على بعض المعايير الفسيولوجية في دم اسماك الكارب العشبي *Ctenopharyngodon idella* VaL. 1844

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الخلاصة

تضمنت الدراسة تغذية يافعات أسماك الكارب العشبي *C. idella* على الطحلب السام *Nostoc muscurum* بصورة مفردة والتغذية المختلطة (الطحلب السام + البرسيم) والتغذية على البرسيم فقط كمجموعة سيطرة ولثلاث فترات زمنية (٢٤ ساعة ، ١٥ يوم و٣٥ يوم) و بيان تأثير تلك التغذية على بعض المعايير الدموية وفعالية بعض الانزيمات الكبدية في دم تلك الأسماك . أظهرت الدراسة انخفاضا معنويا $P<0.05$ في معدل أعداد كريات الدم الحمرRBCs والبيض WBCs والهيموكلوبين Hb وحجم الدم المضغوط PCV والبروتين الكلي Total protein عند التغذية على الطحلب السام والتغذية المختلطة بأزدياد فترة التغذية مقارنة مع التغذية على البرسيم فقط ، كما بينت الدراسة أيضاً ارتفاع معنوي كبير$P<0.05$ في فعالية بعض الانزيمات الكبدية المتمثلة بإنزيمات Alkaline phosphatase (ALP) و(ALT) Alanine transaminase وأنزيم Aspartate transaminase (AST) عند التغذية على الطحلب السام والتغذية المختلطة مقارنة مع مجموعة السيطرة و في جميع فترات التغذية مع ملاحظة انخفاض معنوي في معدل أوزان ونسب البقاء ليافعات الكارب العشبي عند التغذية على الطحلب السام وكذلك التغذية المختلطة مقارنة بمجموعة السيطرة.

كلمة المفتاح : الطحالب السام *Nostoc muscurum* ، السموم الكبدية (المايكروسستينات) ، الانزيمات الكبدية ، المعايير الدموية