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Toxic Effects of Low Concentration of Cyanotoxin (Microcystin-LR) on Mice and Study of Protective Efficacy of the Antioxidants Vitamins (C&E) and Capparis spinosa L. Root Extract

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Abstract
In this study the protective efficacy of vitamin C&E and ethanolic extract roots of *C.spinosa* against toxic effects of algal toxin (Microcystin –LR) in mice were done .Results showed significant decreasing $p \leq 0.05$ in values of hemoglobin (HB) and packed cell volume (PCV) reach to 7.77 and 27.23 g/l in blood of mice treated with low concentration of cyanotoxin (MC-LR = 0.15 ug/kg/day) compared with control group which reach to 12.21 and 40.22 respectively. Significant increasing were showed in some liver enzymes ALT,AST and ALP when exposed to toxin only which reach to 68.80 , 167.85 and 274 U/l respectively compared with control group , While groups treated with vitamins and extract showed non -significant differences with control group in values of blood parameters and enzymes. Histopathological changes in liver when group treated with toxin only represented by pyknotic hepatocyte , hypertrophy , binucleation , infiltration of lymphocyte, karyolysis. While kidney showed hypertrophy of cell glomerular arterioles , metaplasia of squamous epithelial tissue of bowman capsule wall in to cuboidal, hypertrophy of renal tubules cells , hyperplasia , also results showed the important role of vitamin C&E and root extract of *C. spinosa* to keep the normal values of blood parameters and prevent histological changes as control group.
Keywords: Algal toxin (Microcystin-LR) , protective efficacy of Vitamin C&E , *C.spinosa*

1. Introduction

Microcystin (MCs) are toxins produced by cyanobacteria (Blue- green algae)from water environment that can induce acute and chronic effects on humans and animals after ingestion /contact with contaminated water (Funari and Testai , 2008; Codd,1995;Dawson, 1998; Pouria *et al*, 1998), or concentration in plant tissue and inhibition of growth (Mmitrovic *et al*.2005) and can potentially transfer in to human and animals via food chains (Crush *et al*.2008). Microcystins are structurally drives cyclic heptopeptides that are primarily considered as heptotoxins (WHO,2008) although the gastrointestinal tract, kidney and other organs are also susceptible to mediated damage (Falconer,1996 ; Al-Sultan and Al-Ali , 2010 ; Al-Ali *et al*., 2011).

Microcystins have proved to be highly potent hepatotoxins in mammals and fish (Fisher and Dietrich,2000; Towner *et al*.,2002 ; Al-Sultan , 2007 ; Al-Aarajy and Al-Sultan 2008 ; Al-Ali *et al*., 2011). It is well recognized that among their toxigenic mechanisms they are potent inhibition of protein phosphatase 1 and 2A that cause increased protein phosphorylation which is directly related to their cytotoxic effects and tumor-promoting activity (Hosser *et al*, 1989; Carmichael,1994;Hooser, 2000) . The Liver play a pivotal role in metabolism,secretion and storage. Any injury to liver can result in many disorders ranging from transient elevation in liver enzyme to life threatening liver cirrhosis and hepatic failure. The common causative agent of liver injuries are toxic chemicals(Nasin Agh *et al* ., 2007) . Therapeutic drugs (Abd Al Majeed and Mustafa, 2010) alcohol, microbial agents(Subramonium and Pushangadan, 1999) metals (Almecola *et al*., 2002, Abd Al Majeed, 2010) and microcystin from cyanobacterial (Angeles *et al* 2003; Jos, 2005; Moreno, *et al*., 2004 ; Al-sultan , 2010 ; Al-Sultan and Al-Ali 2010 ;Al-Sultan *et al*., 2015)) and various pesticides (Sayeed *et al*., 2003).

Vitamin C also known as ascorbic acid (AA) and α-tocopherol (Vitamin E) are potent antioxidants and non-enzymatic defenses that capable to reduce oxidative damage by augmenting the function of endogenous free radical scavengers such as superoxide dismutase, catalase and glutathione peroxidase (Filho.,1996; Whitehead and Keller,2002; Son *et al*,2004; Ayo *et al* .,2006; Suteu *et al.,* 2007). A few papers have reported that a combination of vitamins E and C can reduce lipid peroxidation(LPO) caused by toxic substances (Appenroth *et al*.,1997; Gultekin *et al*., 2001; Irfan Altuntas *et al*.,2002).

Capparis spinosa L. (caper) (family Capparidaceae) is plant from dry regions in the west and central Asia and widely grown particularly in Mediterranean basin. From ancient time Greeks and the Romans were used for medicinal purposes. Capers were employed as a flavorings in cooking, also used in traditional medicine for their diuretic, antihypertensive poultice and tonic properties (Baytop, 1984; Calis*, et al*., 1999;Eddouks, *et al* 2004) . Capers are said to reduce flatulence and to be anti-rheumatic in effect. In Ayurvedeic medicine capers are recorded as hepatic stimulants and protectors, improving liver function, hepatotoxic activity and potential antioxidant (Moghaddasian, *et al*.,2012; Baijal, 2004; Sandhir and Gill,1999;Mishra,*et al*.,2007; Abd Al Majeed and Mustafa.2010;Gadgoli and Mish ,1999). Capers have reported uses for arteriosclerosis, kidney sources of novel antimicrobial compounds especially against bacterial pathogens and new research suggest a possible use of

C. spinosa source of natural disinfections, vermifuges. Infusions and decoctions from caper root bark have been traditionally used for dropsy, anemia, arthritis and gout. Capers contain considerable amounts of the anti-oxidant, alkaloids, glycosides, trepans, sterols and bioflavonoid (Bonina *et al*., 2002, Chaudhari *et al*., 2004). The present study was undertaken to evaluate the protective efficacy of the vitamins E&C and *Capparis spinosa* ethanolic root extract against toxicity of algal toxin hepatotoxin (MC-LR) after acute exposure.

2.Material and methods

2.1.Animals: Adult male 12-weeks old albino mice (*Mus musculus* L) weighing 25-30g. were used in the present study. They were housed and maintained in controlled condition animal house (25±2c°) and 50% relative humidity with 12:12 light- dark cycle. Food and water were given *ad labium.*

2.2 Plant material: The root bark of *Capparis spinosa* was collected from Abu- AL Kaseeb and Karma Ali, Basrah southern of Iraq , during July 2014, and were identified and authenticated by Proof. Dr. Abd AL Retha A.A. Toxonomist, Biology Department, Science College, University of Basrah.

2.3 Preparation of extract: The root bark of *C. spinosa* L. was washed thoroughly in tap water and shade-dried, the plant material was coarsely powdered and 100g of the powdered were extracted exhaustively with 90% ethanol . After filtration with using Whatman No.1 filter paper, the extract was concentrated and dried using Rotary flask Evaporator, which stored in refrigerator for further use.

2.4 Chemicals: Cyanotoxin (Microcystin-LR) were purchased from Alexis company (USA) to prepare (0.15µg/ml). Vitamin C (ascorbic acid) were obtained from the Department of Biology/college Education/University of Basrah . Vitamin E acetate(α-tocopherol) with purity > 96% was obtained from Sigma-Aldrich Co, USA. The Kit, for estimation of enzymes aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) were purchased from biondox, Biomerieux were used to calculate enzyme concentration in blood of mice**.**

2.5 Treatments: Thirty two mice were equally divided into four experimental groups. They were treated as follows: Group (1) received 0.1ml normal saline and considered as control , group(2) received 0.1ml normal saline , the toxic group , group (3) received 0.1 ml alcohol extract of *C.spinosa* root bark (200mg/kg BW), extract group , and group (4) received (Vit. C&E), Vitamin C 60mg/kg/0.1ml(in normal saline) were treated intraperitoneally (ip), and Vitamin E 75mg/kg BW/0.1ml(in corn oil) were given orally by gastric intubation using a once daily, the Vitamins. Group. All (treatment were given daily for one week . After one week the groups (2,3,4) were received 0.15µg/kg/BW/0.1ml of algal toxins cyanotoxin (MC-LR) . All treatment were given (ip) injection.

After 48 h., from last treatment, the mice of all groups , were scarified by cervical dislocation under ether anesthesia and venous blood samples were collected by direct heart puncture. The blood samples were divided into two portions, one was collected in clean tubes with few drops of EDTA for measurement of some blood parameters (HB and PCV) , and the other was centrifuged, serum was recovered, and diagnostic Kits were used to determine the serum activity of AST, ALT, and ALP, in addition, the organs liver and kidney were removed for histopathological examinations.

2.6 Histopathological Examination: For light microscopical examination, tissue specimens from liver and kidneys tissue were obtained from each groups and fixed in 10% formalin, embedded with paraffin. After routine processing, paraffin sections were cut into 5µm thickness and stained with haematoxylin and eosin (Humason , 1972).

2.7 Statistical analysis: The results was analyzed statististicaly by using statistical program (SPSS version-19) . Analysis of variance One Way ANOVA were done to evaluate the significant difference between treatments under propability $p \leq 0.05$ and calculate the revised least significant difference (R.L.S.D) .

3. Results and Discussion

The phisyological parameters of the present study are found in the Table 1. The hemoglobin (Hb) and (PCV) in cyanotoxin treated mice (toxic group) was significantly decreased ($P \leq 0.05$) compared to the control mice,(9.75g/L), (29.25g/L) respectively. Also, it showed that treatment the mice with vitamins (C&E),7 days before cyanotoxin administration (vitamins group) reversed the changes in Hb and PCV to the normal control, non-significant differences($P \leq 0.05$) compared to the control mice (13.61g/L), (40.87g/L), respectively. Also the treatment of mice with *C. spinosa* root extract, (extract group) were showed non- significant differences of Hb and PCV compared with control group (13.12g/L), (39.37g/L), respectively.

The decrease in Hb and PCV in the toxic group was indicating anemia. A study by (Sukenik *et al*.,2006) reveals that the anemia produced by the cyanobacteria is due to deformation of red blood cell, which transform in to acanthocytes. This transformation of the red blood cell structure is caused by liver dysfunction which affects heam synthesis. This also agrees with finding by(Zhou *et al*., 2013, John.*et al* 2014) who also found significant decrease of RBC count, Hb, and hemacrit in mice 30 day exposure to microcystin which they attributed to disturbed hematopoictic growth factors and bone marrow cell apoptosis. Some studies showed that reduction of (RBCs, WBC, and Hb) count in response of fed the juvenile grass carp on toxic alga (AL-Sultan *et al* .,2011), also (Shalaby 2001) cytotoxicity test on human blood showed that human cells can effected by the cyanotoxins (Microcystins) because the ability of latest to analyze the membranes of RBCs especially at high concentration.

When vitamins C&E were given before toxin, (vitamin group), showed no significant changes as compared with control group . Vitamins C&E defend the integrity of the cell membrane against oxidant agents as antioxidants (Scbaia CM,1989). In the studies curried out , it was determined that vitamin E& C stimulated the prostaglandin synthesis (Hirata,1981, Clara,1986). Vitamin C is a well- known cell protective natural antioxidant. Also the protective effects of vitamin C are observed in oxygen- dependent pathophysiological condition (Jacob ,2002). These results were agree with present study.

From the results the Hb & PCV in the group treated with *C.spinosa* root bark extract showed non-significantly differences (P≤0.05) when compared with control, The mechanism by which *C. spinosa* extract its protective action against cyanotoxin induced disorders in the liver may be attributed due to the antioxidant effect of plant extract. *C. spinosa* was reported to have a number of potentially useful medicinal attributes including anti-oxidative (Germano. *et al* 2002), antihepatotoxic (Gadgoli, and Mishra,1999), antifungal (Ali- Shtaych,and Abu-Ghdeib,1999),anti-worm activities (Mustafa, 2011),dropsy, anemia, arthritis and gout(Bown.1995) and anti-inflammatory (Al-Said, *et al* 1988). Various biochemical compounds, alkaloids, lipids, polyprenol, flavanoids inodols, phenols, sterols and glycosides present in *Capparis* sp. might be medicinally important (Mishra, *et al* 2007).

The serum biochemical analysis in this study presented in (Table -1), the activity of enzyme ALT , AST and ALP of toxic group showed significantly increasing (P≤0.05) as compared with control,(70.75 U/L) (169.75 U/I) and (276U/L) respectively. While the activity of enzymes ALT, AST in vitamins C&E group and extract group, were not affected significantly as compared with control, (53.75 U/L), (48.00 U/L) and (80.75 U/L), (66.00 U/L) respectively. But the activity of enzyme alkaline phosphatase (ALP) in Vitamins C&E group showed significantly increasing (P≤0.05) as compared with control (167.5 U/L), and showed significantly decreasing (P≤0.05) as compared with toxic group, (276 U/L), but extract group not effective significantly as compared with control (141U/L).

The increasing activity of enzymes ALT, AST, and ALP may be return to the toxic effect of MC-LR. and could be attributed to the hepatic damage necrosis and cell membranes in liver loss of activities, resulting increased release and leakage out of these enzymes from the liver cytosol into the blood stream which gives an indication on the hepatotoxic effect of MC-LR, (AL-Sultan *et al*., 2015, Mazorra *et al*,.2002).Toxic drug, alcohol, virus, and heavy metals(Sherlok and Dooly,2002, Abd Al Majeed, 2010).

The activity of enzyme (ALT), (AST) in vitamins C&E group and extract group, were not affected significantly (P≤0.05) as compared with control, (Table 1). The pretreatment of Vit. C&E were reduced the oxidative damage by augmenting the function of endogenous free radical scavenger such as superoxide dismutase, catalase and glutathione peroxidase, this results agree with Gehringer *et al*., (2003), study the protective efficacy of both, Vit E and Trolox against MC-LR toxicity is common to both mice and *A. Franciscar nauplii*. This suggests that cellular enzymatic defense mechanisms were supported by the antioxidant action of vitamin E pre- treatment, and agreement with (Ayo *et al* 2006, Son *et al* 2004, Suten *et al* 2007, and White head and Keller 2002).

The pretreatment of *C. spinosa* extract for 7 days before MC-LR administration result in significant protection of MC-LR-induced elevation of hepatic serum markers ALT, AST, this is also comparable to the effect of the control. *C. spinosa* has been reported to have hepatoprotective activity due to the presence of flavonoid like quercetin and quercitoside p-methoxybenzoic acid and rutin, it was found to exhibit antihepatotoxic activity against MC-LR. This result agreement with (Pradhan and Girih 2006,and Abd AL Majeed and Mustafa 2010), against paracetamol.

3.1 Histopathology

The histopathological examination of mice liver treated with cyanotoxin after 48 hrs showed the multifocal areas of necrotic hepatocytes and pyknotic nuclei were seen in some hepatocytes (chromatin condensation and nucleoli disappearance) . The cytoplasm undergo hydropic degeneration. The degenerated cells show vacuoles in the cytoplasm with no distinct borders. The cytoplasm is diluted, and dispersed . In addition to degeneration the hypertrophy of hepatocytes as nuclear enlargement and Some hepatocytes showed

binucleate(binucleation) (figure-1-B), and (Plate 1-C) showed increase of necrotic hepatocytes that associate with karyolysis . In addition the infiltration of lymphocytes are present near the areas of necrosis . The histopathological changes in liver were reduced to near normal in the mice treated with vitamins C&E supplements, (Plate-1-D). Mice treatment with *C.spinosa* extract showing mild changes in the liver cell such as hypertrophy of hepatocytes , (Plate-1E&F) .

The mice kidney treated with MC-LR after 48h showing hypertrophy of endothelial cells of glomerular arterioles. These cells had dark nuclei with dense chromatin, in addition to the single cell layer of the parietal epithelium of bowman capsule can undergo metaplastic changes from the normal simple squamous epithelial tissue to simple cuboidal epithelial tissue, These cells had light rounded nuclei with fine chromatin, (Plate-2-B), and hypertrophy of renal tubules cells as apparent increase in size of individual tubule cells . these appeared to have a lining that number lead to disappear or narrow its cavities and atrophy of haemopoieatic tissue, (Plate- 2-C) ,the interstitial tissue and blood vessels were normal with no inflammatory changes, and Kidney treated with vitamin E&C showing normal bowman capsule, glomerulus and renal tubule (Plate 2-D).While Kidney treated with *C. spinosa*. extracts showing normal bowman capsule, glomerulus mild hyperplasia of renal tubules (Plate 2-E). Some studies showed necrosis and degeneration in liver and kidney in rainbow trout treated with algal toxin MC-LR (kotak *et al*.,1996), in carp (Rabergh *et al*.,1991) and in mice (AL-Sultan *et al* ,2015). In mice renal damages are seldom observed (Kotak *et al*.,1993). In acute experiment of fish ,microcystins induces dilation of Bowman's capsule in glomeruli and necrotic tubular cell with pycnotic nuclei (Rabergh *et al*.,1991, Kotak *et al*., 1996). These results were agree with present study. Whereas the treated with root extract of *C. spinosa* can inhibited these lesion. Yu *et al*.,(2008) showed that the alkaloids of *C. spinosa* can inhibit the *C. spinosa* possess considerable inhibition of AMN3 cell, and has ability to reduce the tumor volume in vivo (Al-Assady,2007). Abd AL Majeed and Mustafa (2010) showed that the supplementation of *C. spinosa* stem extract to paracetamol- intoxicated mice were reduced paracetamol-induced tissue damage. Similar finding were reported by (Girish *et al*., 2009) on the mice used pretreatment (Liv52, contains 24% *C.spinosa*) for heapatoprotective activity in CCl4-induced liver toxicity. Studies have reported that Caper bud extracts exhibit strong anti-oxidant and free radical scavenging effect. These activities have been attributed to the presence of phenolic compounds, palmitic acid, Y-linoleic acid, rutin, and flavonol glucosides identified in *C. spinosa* (Mishra, 2007). The histopathological of liver and kidney of Vitamins (C&E) treated mice showed a comparable to normal control mice. This is agree with some previous studies which have reported the beneficial role of vitamins in scavenging free radicals and enhancement of cellular antioxidant defense(Ghosh *et al*., 2002, Dahdouh, *et al*., 2013, Mohammed, *et al*., 2014).

Table-1: physiological parameters values in mice blood after expose of hepatotoxins (MC-LR) and treatment with Vitamins (E&C) and *Capparis spinosa* L. root extract. N=8

Physiological parameter	**Control**	**Toxin after 48 hours**	**Vitamins(C&E)**	**Extract of *C. s***	**R.L.S.D**
HB (g/L)	12.21 ^{a}	7.77 ^{b}	13.61 ^{a}	13.12 ^{a}	1.01
PCV (g/L)	40.22 ^{a}	27.33 ^{b}	40.87 ^{a}	39.37 ^{a}	3.04
ALT(U/I)	38.23 ^{b}	68.80 ^{a}	53.75 ^{b}	48.00 ^{b}	14.75
AST(U/I)	95.00 ^{b}	167.85 ^{a}	80.75 ^{b}	66.00 ^{b}	31.2
ALP(U/I)	140.2 ^{c}	274^{a}	167.5 ^{b}	141.00 ^{c}	23.2

Similar letters: non-significant differ P≤ 0.05. Different letters: significant differ P≤ 0.05.

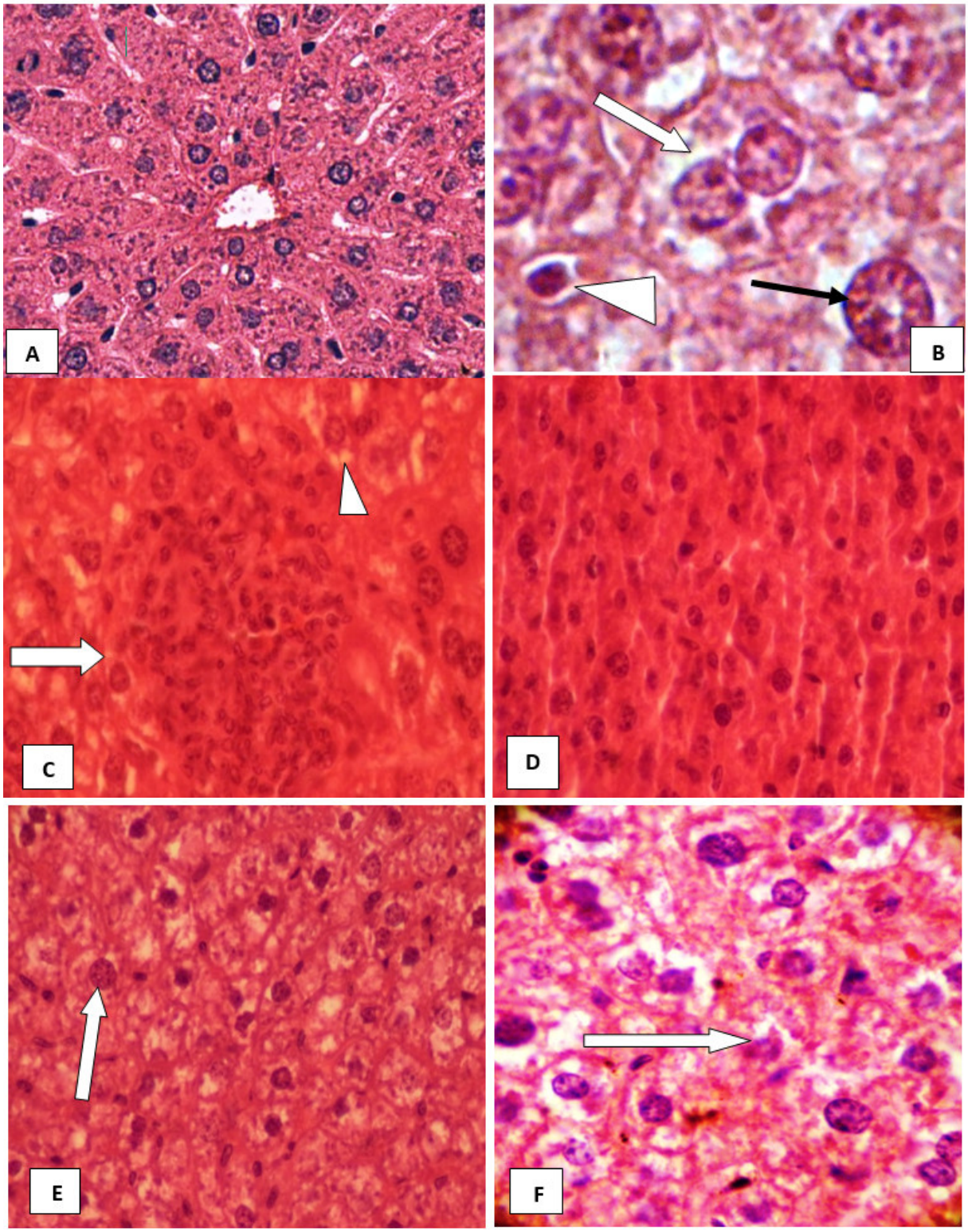

Plate-1-**(A)** control liver showing normal hepatocytes and sinusoids. 400x. **(B)** liver treated with cyanotoxin after 48 hrs (acute exposure) showing pycnotic hepatocytes (head arrow). hydropic degeneration with hypertrophy (black arrow) and binucleation(short arrow),400x. **(C)** : liver treated with cyanotoxin after 48 hrs showing infiltration of lymphocytes (arrow) and karyolysis of necrotic hepatocytes (head arrow) . 400x. **(D)** :Liver treatment with vitamin (E&C) showing normal hepatocytes.400x **.(E&F)**Liver treated with extract of *C. spinosa* showing mild hypertrophy of hepatocytes (arrow) 400x.

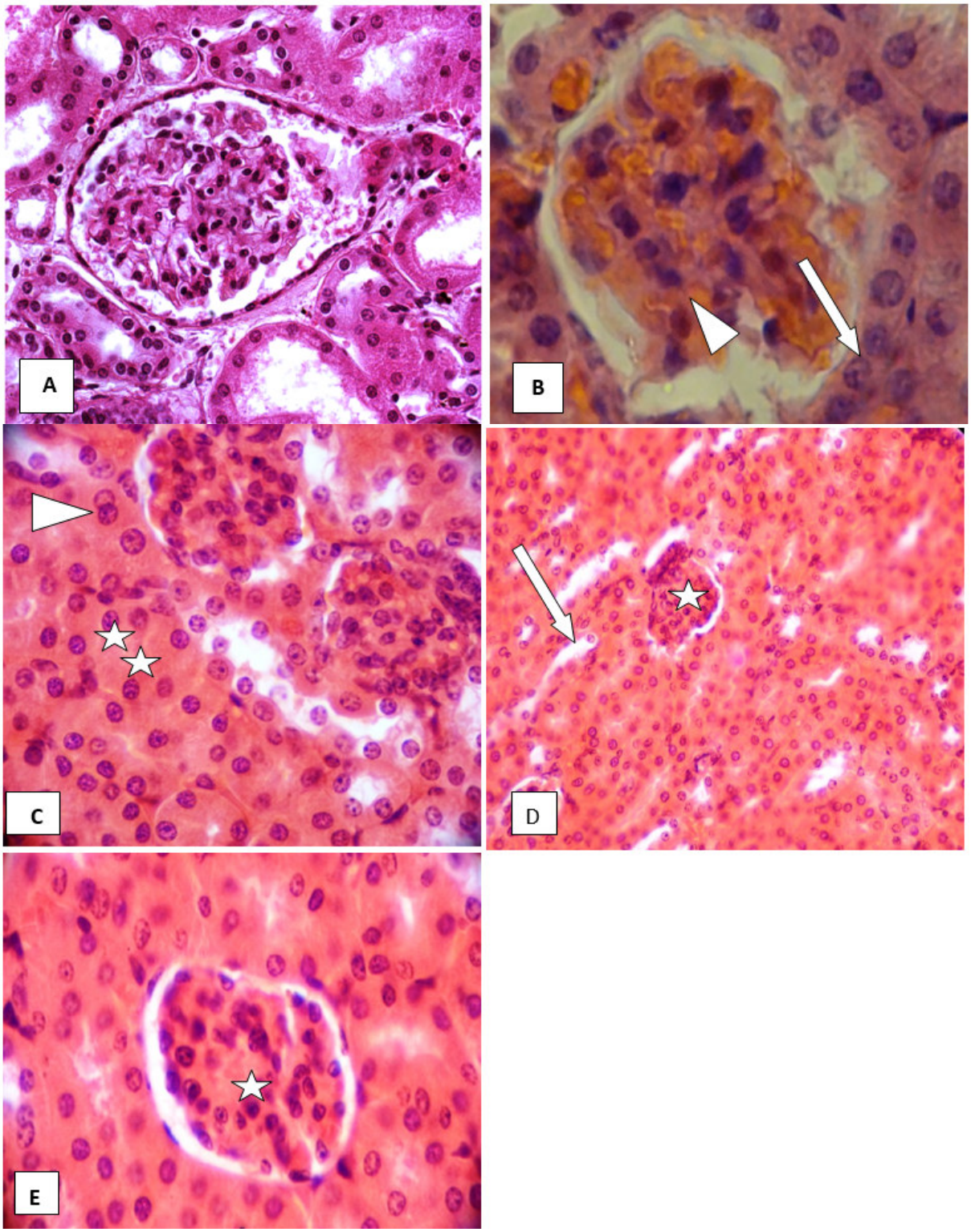

plate-2: (A)- control kidney showing normal bowman capsule ,glomerulus and renal tubules. **(B)-** kidney treated with cyanotoxin 48 hrs. (Acute exposure) showing hypertrophy of cells of glomerular arterioles (head arrow) and metaplasia of squamous epithelial tissue of bowman capsule wall (parietal wall) into cuboidal epithelial tissue(arrow). **(C)-** kidney treated with cyanotoxin after 48 hr showing hypertrophy of renal tubules cells (star) and hyperplasia (head arrow) lead to disappear or narrow its cavities. **(D)** Kidney treated with vitamin E&C showing normal bowman capsule(star) ,glomerulus and renal tubules (Arrow) . **(E)** Kidney treated *C. spinosa* extracts showing normal bowman capsule ,glomerulus and mild hyperplasia of renal tubules (Star) 400x**.**

4. Conclusion

The results showed the important role of vitamin C&E and ethanolic extract of *C. spinosa* roots to protective efficacy against algal toxin MC-LR and keep the normal values of blood parameters and liver enzymes in normal levels as well as keep normal tissues of liver and kidneys especially when mice treated by vitamins and extracts before one week of exposure to algal toxins .

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