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Chemical and quantitive study of hepatotoxins from fresh water cyanobacteria on vertebrate animals

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Toxicity of the freeze-dried micro-aquatic planktonic cyanobacterium *Oscillatoria agardhii*, dominating and isolated from Makkah –KSA was studied. Microcystins were detected from freeze-dried cells using high pressure liquid chromatography HPLC. The histopathological examination of mice liver injected on week basis with diluted 1/5 of the lethal dose, (105 mg/ Kg body weight), of toxin extract revealed sever changes in liver histology and displayed apparent signs of degenerative hepatic structure. Cytoplasmic vacuolation and parenchyma exhibit hepatocytes degeneration. Hepatic vasculature and biliary system were blocked and severely damaged. The effect of the extract on blood contents and liver function of white mice was investigated. Mice were divided into three groups and injected intraperitoneally (i.p) with weekly reciprocal doses of toxin extract as 21, 42 and 63 mg/ mice, respectively, for seven weeks. Mice injected with 63 mg were the mostly affected and showed signs of acute cellular and physiological damage involving oligocythemia, leucoytosis, marked increase in serum urea, cholesterol, triglycerides, creatinine, Aspartate Aminotransferase (AST), alanine aminotransferase (ALT), hematocrit (HCT) and mean cell hemoglobin concentration (MCHC), while blood platelets showed abnormal increase which suggest an inhibitory action on haemopiesis. In addition, the abnormal pathophysiology observed here reflects the sever toxic effect from the crude extract of *O. agardhii* on both liver and kidney.

Key words: Cyanobacterium, blood, histopathology, mice.

INTRODUCTION

Many species of bacteria secret varity of toxins (Osman et al., 2007; El-Menofy et al., 2014; Osman et al., 2015). Only cyanobacteria produces cyanotoxins source of natural product of toxins known as cyanotoxins, which might occur in fresh, brackish and marine water bodies. Poisoning cases are attributed to cyanobacterial toxins known since the late 19th century (Francis, 1878). Fatalities were high among animal livestock, pets and

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Abbreviations: AST, Aminotransferase; **ALT,** alanine aminotransferase; **HCT,** hematocrit; **MCHC,** mean cell hemoglobin concentration; **HPLC,** high pressure liquid chromatography; **i.p,** intraperitoneally; **FS,** field stimulation; **SPE,** solid phase extraction; **IST,** international sorbent technology; **RBC′s,** red blood cell; **HB,** hemoglobin; **WBC′s,** white blood cell count.

wildlife following ingestion of water contaminated with toxic cyanobacterial cells or toxins. Toxins-producing cyanobacteria pose a worldwide health threat to both human and animal due to their presence in both drinking and recreational water (Mutwally and Jamel Al-Layl, 1992; 1993; Ismail and Jamel Al-Layl, 1995; Jamel Al-Layl, 1996; Dittmann and Wiegand, 2005; Boaru et al., 2006). Recently, studies on the classification and characterization of cyanobacteria strains have been increasing because these strains possess commercially valuable toxins. To date, several researchers have identified and characterized bacterial strains using phenotypic characteristics, DNA-DNA relatedness data, and analysis of the 16SrRNA sequence (Assaeedi et al., 2011; Osman, 2012; EL-Ghareeb et al., 2012; Abulreesh et al., 2012; Assaeedi and Osman, 2012; Osman et al., 2013; Organji et al., 2015). Human illness resulting from exposure to blue-green algal toxins is less common than poisonings of wild and domestic animals (Yoo et al., 1995; Frazier et al., 1998). A large number of reports of water blooms having toxic cyanobacteria in lakes, dams and rivers have appeared in the last decade (Dittmann and Wiegand, 2005; Boaru et al., 2006). On the other hand, cyanobacterial produce microcystins toxic materials, which affect either the liver or the nervous system (Lawton and Codd, 1988; Mutwally and Jamel Al-Layl, 1992; 1993; Berry et al., 2008).

Microcystins released by cyanobacteria have sever toxic effects on liver, kidney, blood and other organs in humans and animals (Milutinovic et al., 2003). This could be true for some countries but in many other countries the most dominant species are *Microcystis aeruginosa* (Namikoshi et al., 1992). Cyanobacteria, blue-green algae, are a distinctive group of prokaryotic microorganisms. There are two main types of toxins in cyanobacteria. These are neurotoxic alkaloids (anatoxins), saxitoxin, derivates and hepatotoxic peptides, microcystine, cyanoginosin, cyanoviridin and cyanogenosin (Anon, 1988). Mutwally and Jamel Al-Layl (1992; 1993) found that the extract of blue-algae (*Ossoillatoria agardhii*) (10^{-5}, 5×10^{-5} and 10^{-4} M), inhibited the spontaneous activity (Spt.act), 100 mM K+-contractures and field stimulation-(FS) responses of *Locusta migratoria* foregut and hindgut muscles in dose-dependent manner. They concluded that the neurotoxin effect was stronger on the tonic responses than on the phasic one and the site action of this extract affected the neuronal plexus driving both muscles. Mutwally (1993) reported the toxicity and liver tumor promotion of cyanotoxins microcystins. Jamel Al-Layl and Jamal Al-lail (1993) reported the first record on toxic cyanobacteria including *Oscillatoria* spp., Microcystis sp. and yellow Microcysits sp. from Makkah area, the west province of Saudi Arabia. In this study we isolated and purified cyanotoxins (Microcystin) from *O. agardhii* by means of High Pressure Liquid Chromatography (HPLC). The toxicity of different animal tissues after dosing with lethal and sub-lethal doses was investigated. Finally, the long term effect of sub-lethal doses on liver function and blood contents was measured.

MATERIALS AND METHODS

Isolation of cyanobacterial strain

Cyanobacterial strain *O. agardhii* was the most abundant cyanobacterial strain found in farm land under investigation in Makkah, KSA. The strain was purified using serial streaking techniques on Agar plates rich with CT medium (Watanabe and Ichimura, 1977) and allowed to grow in 10 L batch culture. The yielded cells mass were spin down and kept at -20°C.

Animals

White female Balb/c mice with average weight of 25 ± 2 g provided and delivered from the animal unit, Biology Department, Faculty of Applied Sciences, Umm Al-Qura University.

Toxicity assay

Preparation of cyanobacterial cells extract

Cyanobacterial cells were grown till late log phase (3 to 4 weeks), and then centrifuged at large scale centrifuge at 8000 rpm. Pellet was washed with 50 ml distilled water prior freeze-drying in Edward freeze dryer (modylyo, MKII, Edwards, UK.). The freeze dried cells approximately 5 to 10 g was homogenized and extracted with 250 ml sterilized distilled water for 2 h followed by centrifugation at 10000 rpm for 20 min. The supernatants were pooled and collected and kept at -20°C.

Mice bioassay

Each female mouse Balb/c weighing 20 to 25 ± 2.0 g received 1 ml of the aqueous cellular extract. The animals were injected intraperitoneally (i.p) and kept under continuous observation for 48 h. Control animals received 1 ml of sterilized distilled water. LD_{50}, (mg dry weight /kg of body weight), was calculated according to Keleti and Laderer (1974). The low value in LD_{50} is regarded as the higher toxin content within the cell extract.

Toxin extraction

The method of toxin extraction was done by using 1 to 5 g of dry homogenized extract, and then stirred in 75 ml of 100% methanol for overnight followed by centrifugation at 10000 rpm for 20 min. The supernatants were collected and kept at 4°C. The supernatants were dried under stream of air to dryness. The extract was re-suspended in distilled water (20 ml). Solid phase extraction (SPE) was achieved by passing the water suspension through methanol pre-activated C18 column (IST, International Sorbent Technology, Mid Glamorgan, U.K). The MCYSTs were eluted with 80% methanol in water (v/v). The methanol elution (10 ml) was then dried to complete dryness and re-suspended in 1 ml distilled water and kept at -20°C for further analysis.

High pressure liquid chromatography (HPLC) analysis

Solid phase extraction (SPE) fractions were further analyzed using HPLC. The elute fractions were dissolved in 15% methanol /ddH_2O

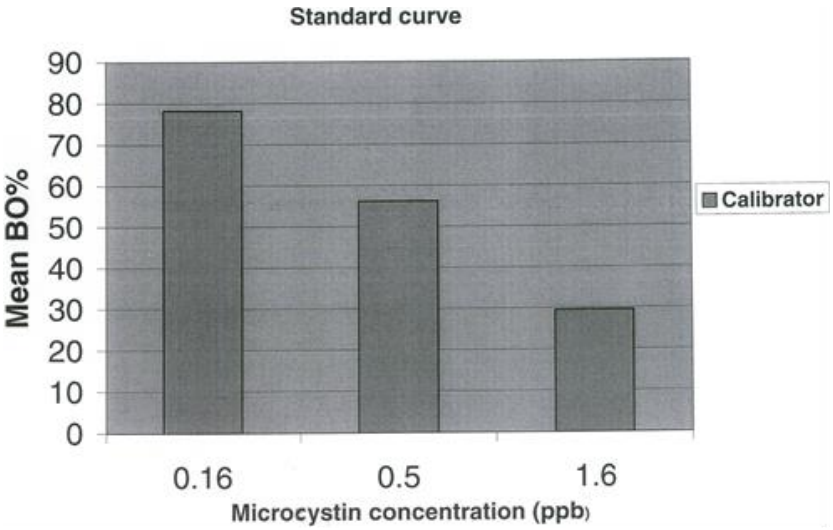

Figure 1. .Standard curve for cyanobacterial toxins determination in partial cells extract.

(v/v). 20 µl of the SPE fraction was injected onto a monochrome (5 µm 50 × 2.1 mm) C18 column (Metachema Technologies, Inc, Torrance, CA). The flow rate was adjusted to 0.25 ml/min. Separation was achieved by gradient elution. The mobile phase (A: ddH_2O/0.1% formic acid and B: acetonitrile / 0.1% formic acid) regimes were as followed: 0.00 to 5.0 min 70 to 30% A - 5.00 to 8.0 min 30 to 7% A.

ELISA technique

Enzyme linked Immunosorbent assay (ELISA) was carried out according to the method of Carmichael et al., (1999). The suspension extract from SPE step was tested for containing MCYSTs by using commercially available ELISA kit. Direct inhibition assays was done using polyclonal antibodies (Envirologix Inc., Portland, ME, and USA). Microtiter plate precoated with MCYST-LR was used for the quantitative amount of MCYST present in the cells extract. 125 µl of microcystin assay diluent were added to each well. 20 µl of NC (Blank without MCYST), MCYST-LR different calibrators concentrations (0.16, 0.5 and 1.6 ng/ ml^{-1}) and the unknown samples, were added to their designated wells. The plate was then covered with parafilm and allowed to be incubated for 30 min at room temperature on orbital shaker at 200 rpm. 100 µl of microcystin enzyme conjugate were added to each well, mixed thoroughly and incubated for 30 min. The incubated plate was then washed using washing solution (phosphate-buffer saline-Tween 20, pH 7.4). 100 µl of substrate were added to each well and incubated for 30 min at ambient temperature. Finally, 100 µl of (1.0 N HCl) were added to stop the reaction. A color of the wells contents will turn to yellow. The plate was read on semi-log scale at 450 nm. Optical density (OD) of each well contents were measured and standard curve graph of the microcystin concentration vs. BO% (maximum amount of microcystin-enzyme conjugate that was bounded by the antibodies in the absence of any microcystin in the sample).

Liver histopathology examination

Liver samples from injected animals were excised at the end of the experiment and fixed in Boiun solution for 24 h, followed by passage through dehydration series (v/v) of 70% ethanol for 24 h, 80% ethanol for 1 h, 90% ethanol for 2 h and finally in 100% ethanol for 2 h. The liver tissues were then replaced in histoclear agents (xylene) for 15 min. The samples were impregnated in embedding soft wax (melting at 54°C) for 1 h and then embedded in hard wax (melting at 60°C). Sectioning of the fixed liver tissues was achieved by cutting 5 µm slices using type 1512 microtome (litz, Wetzlar, GMBH, West Germany). The sections were stained with eosin and hematoxlyin and viewed under light microscope.

RESULTS

Toxins from cyanobacteria were determined at the beginning using mouse- bioassay. The strain under this investigation namely *O. agardhii* has an estimated LD_{50} of 105 mg dry weight of cyanobacterial cells / kg body weight. ElISA has successfully be employed for quantitation of MCYTS in drinking water (Ueno et al., 1996). The ELISA results can be related to toxicity (Harada et al., 1996). However, this method is currently considered to be the most quick and reliable used method for toxicity level evaluation and early predication. It had showed that cell free extract of *O. agardhii* cross-react with microcystin antibodies in the microtiter plate. Standard curve was drowned and the total BO% was calculated (Figure 1). 3.8 ng/mg^{-1} of MCYTS was detected from cells extract of *O. agardhii*. Figure 2 shows the HPLC profile of *O. agardhii* cells extract. Major product ions of MCYST-LR standard are also observed and compared with that of *O. agardhii*. It was concluded that *O. agardhii* cells extract contain MCYST-LR; chromatographic properties of toxin were compared with that of standard MCYST-LR toxin. These results were confirmed by collecting the separated peaks produced by HPLC analysis method, evaporated to dryness and

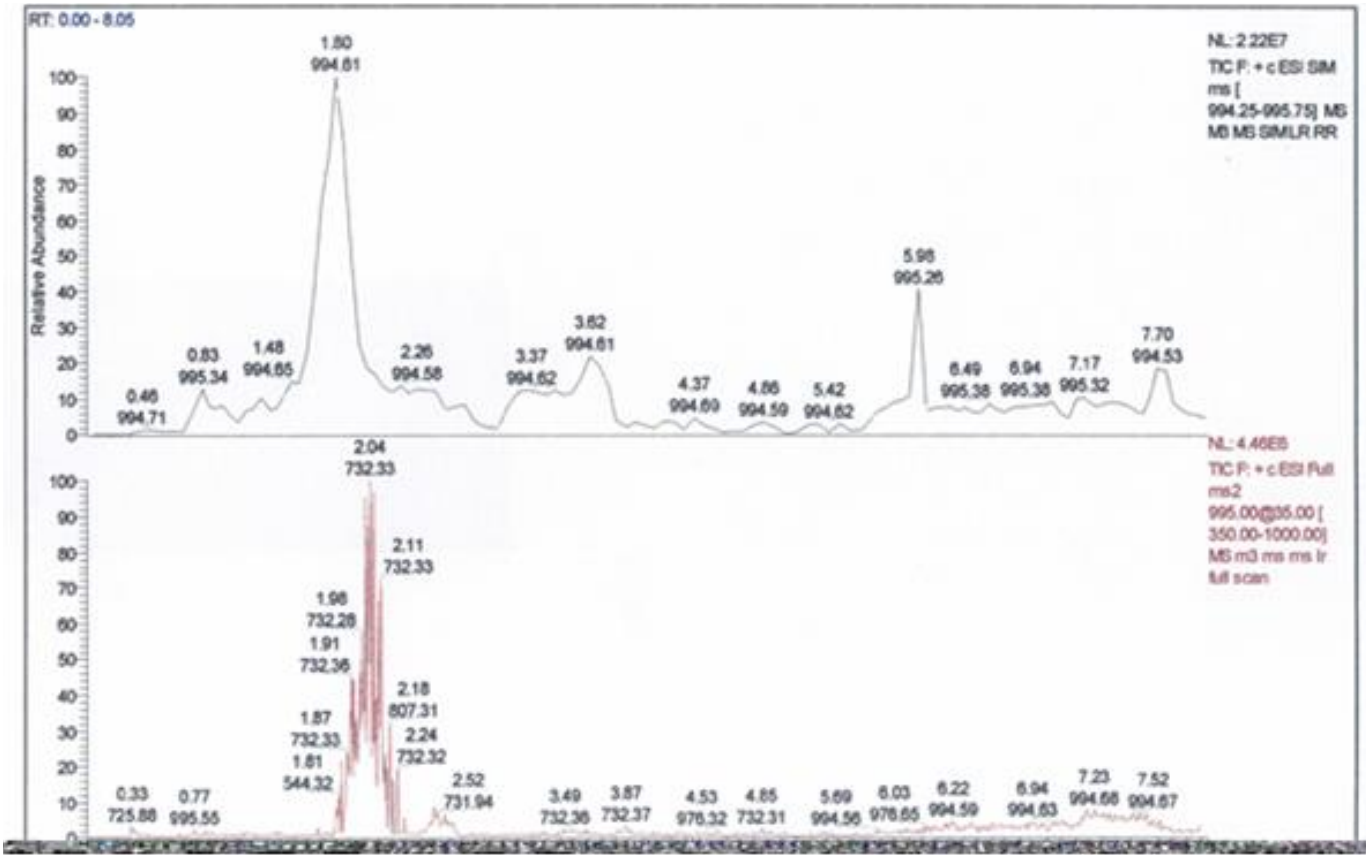

Figure 2. HPLC typical profile of microcystin –LR eluted from cyanobacterium *Oscillatoriaa agardhii* cell free extract. Peak at 2.0 min; retention time with MW at 994.

resuspended in water. Similar properties of the eluted toxins were compared with that of standard MCYST-LR toxin. Results from blood samples were collected by means of cardiac puncture weekly according to Waynforth (1980). Treated mice exposed to different doses of cells extract of *O. agardhii* were studied. The haematological parameters investigated were red blood cell (RBC's) count, hemoglobin content (HB), haematocrite value (HCT), mean cell hemoglobin concentration (MCHC), white blood cell count (WBC's), platelets, serum glucose, albumin, cholesterol, triglycerides, urea, creatinine, lymphocyte; AST, and ALT were measured to evaluate the pathophysiological changes induced by MCYST-LR. Histopathological results of mice liver exposed for prolonged duration of exposure of sub-lethal doses are presented in Figures 3 to 7. The data in these plates show sever liver damage and abnormalities in the structures followed by disappearance of normal organization of liver tissue.

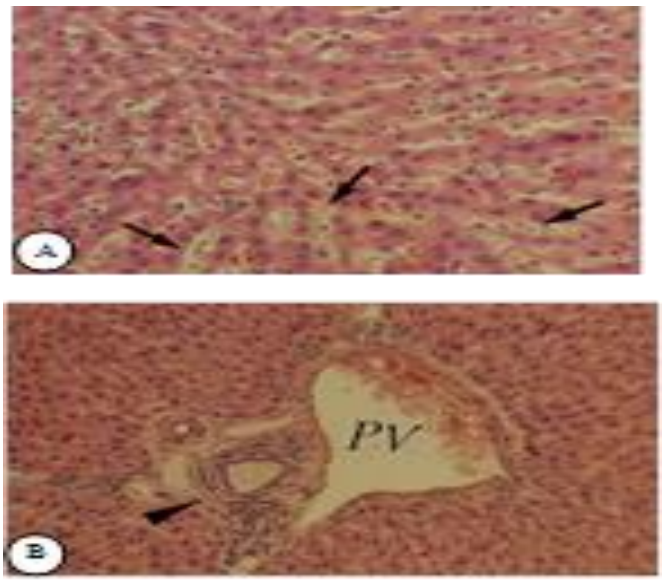

Figure 3. A. A photomicrograph revaeling marked dilatation in blood sinusoids arrows with conomitant hepatic cords atrophy (X100). **B.** A photomicrograph showing mild eosinophilic infiltration and collagenous fibers deposition head of arrows with conomitant hepatic cords atrophy (X40).

DISCUSSION

In the present study, we successfully isolated a dominate

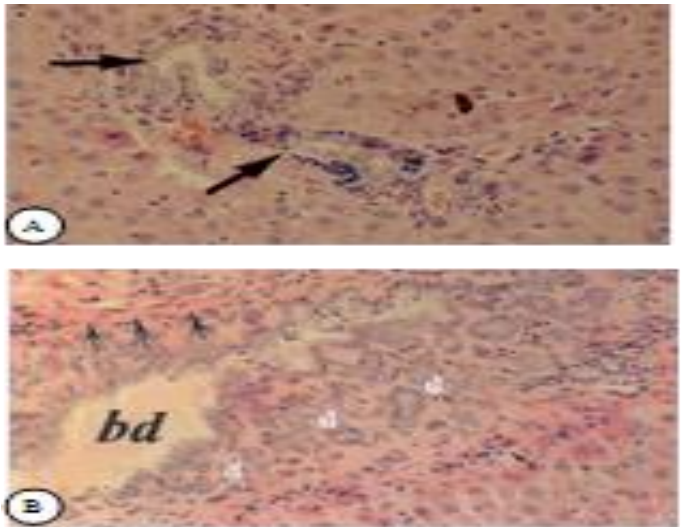

Figure 4. A. A photomicrograph showing deformity of biliary architecture with mild eosinophilic infiltration arrows (X100). **B.** A photomicrograph showing sever construction in bile duct (bd) interior wall with a well marked disarrangement of lining cell. Arrows are pointed to thick fibrotic layer surround with degenerating of hepatic tissue (X164).

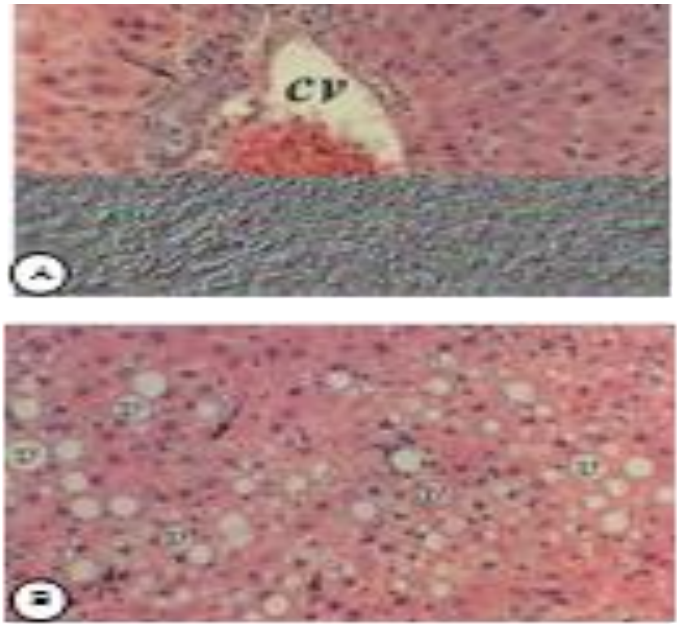

Figure 5. A. A photomicrograph showing intial fibrosis in the area surounded the central vein (CV). Head of arrowspointed to thick fibers with mild eosinophilic infilteration (X164). **B.** A photomicrograph showing several vacoulation (V) distributed in parenchyma. Arrows are pointed to foci of fat cells, with substituted the hepatocytes (X164).

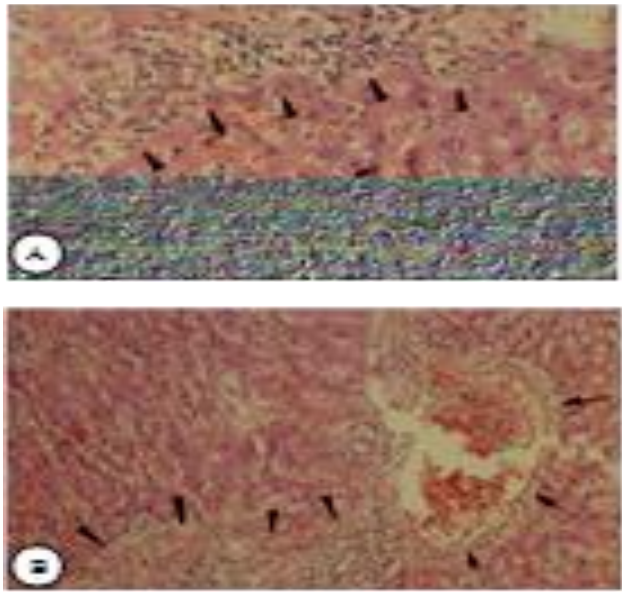

Figure 6. A*l* A photomicrograph reveling marked dilatation in a portal vein (head of arrows) by mean of liver congestion. Note also the sinusoiddal dilation (arrows). X100. **B.** A micrograph showing area of premalignant changes (heads of arrows note the disappearnce of hepatic architecture. Note the portal vein is congested and the bile ductible are surrounded with heavy infilteration of inflammatory cells (arrows) 40X.

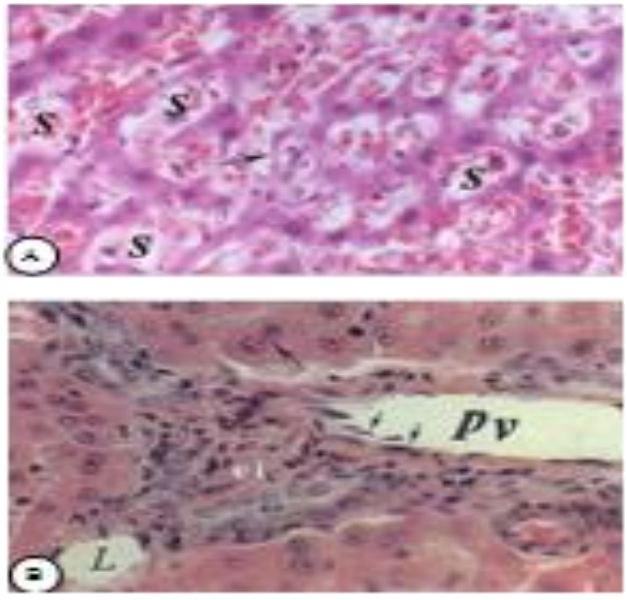

Figure 7. A. A photomicrograph showing well marked dilatation of blood sinusoids (S) accompanied by atrophy of hepatic cords (X330). **B.** A photomicrograph showing damage of endothelial lining of portal vein (Pv). The adjacent area shows eosinophilic infiltration with sever damage to bile ductile (BD) (X330).

species of *Oscillatoria* sp. from Makkah Al-Mukarmah area. The identification was confirmed by morphological observation. Microcystins (MCYTS) was detected from the crude cells extract by ElISA and HPLC. HPLC

technique was applied to isolate and characterize different forms of Microcystin. HPLC profile was typical of MYCTS-LR produced by different strains of MCYTS producing cyanobacteria. The major product ions peak were found in MCYSt-LR at 2.0 min retention time. This confirmed the presence of MCYST-LR in *O. agardhii* cells extract. Efficacy of water treatment procedures of recent evidences suggest that parts per billion levels of certain cyanobacterial toxins (microcystins and nodularins) may be associated with non-lethal acute chronic health effects and should be monitored and controlled in domestic water supplies. Several nations, including Canada, Australia and the United Kingdom are currently moving towards the development of health guidance levels for microcystins in drinking water. When evaluating water treatment procedures for the removal of cyanobacterial toxins, one is faced with problems regarding soluble and suspended substances. Extensive growth of cyanobacteria as indicators of polluted waters and their presence in the food chain have been recognized. The reason could be lesser awareness of problem and need for more surveys in the third world countries. This study gives an account of the toxic potential of these cyanobacteria, which cause hepatotoxicity.

Cyanobacterial toxins involve in food supplements may lead to greater health hazards. It is of great demand to test all potential sources of cyanobacterial contamination such as water storage reservoirs, drinking water and food supplements. The problems of toxic cyanobacteria in environment also have other serious implications. For instance, Non-toxic cyanobacteria that regarded as safe and promising sources of food dietary. Cyanobacteria are currently regarded as bio-fertilizer and the practice of Anabaena– Azolla symbiotic system has been followed in wetland rice farming for ages. For their success, it is essential to remove the undesirable effects such as toxins, odour and taste, filter clogging and water flow blocking. Cyanobacteria have been preferential proli-feration in wetlands waters. This is even more serious with water bodies subjected to thermal pollution through heated effluents, such as ones from cooling towers of thermal power plants. Care has to be taken in avoiding toxic cyanobacteria blooms in such cases. It is suggested that cyanobacteria species and their toxicity, should also be among regulatory parameters for environmental monitoring, impact assessment and eco-epidemiological surveys along with drinking water related activities and over all water resources management.

Conflict of interests

The author(s) did not declare any conflict of interest.

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