Bioseparation and Purification of Hepatotoxins Microcystin–LR from Cyanobacteria species *Westiellopsis prolifica*.

M. M. Khadairi¹; A. I. H. Al-janabi²; M. j. y.Al-amari³ and A.M. Almamoori³.

¹Teaching hospital of Marjan, Directorate of Babylon Health, Ministry of Health, Iraq.
²Department of Biotechnology, College of Biotechnology, University of Al-Qasim Green, Babylon, Iraq
³Department of Biology, College of Science, University of Babylon, Iraq

Mahir.aljuboori@gmail.com

**Abstracts**

The present study included bioseparation and purification of hepatotoxins microcystin-LR from cyanobacteria species *westiellopsis prolifica* the concentration of toxin was determined by preparative high performance liquid chromatography by comparing peak area and retention time of analytical standard of microcystin-LR with peak area and retention time of extraction of each species of cyanobacteria, the retention time of analytical standard of microcystin-LR were 9.55 min and it’s concentration was 10μg/ml, *W. prolifica* retention time was 9.5 min, it’s concentration was 28.385 μg/ml.

**Keywords : Biological toxins , Cyanobacteria , Microcystin-L**

**Introduction**

1-Cyanobacterial toxins

Cyanobacteria are prokaryotic, autotrophic microorganisms, gram-negative bacteria with thick peptidoglycan layer and lipopolysaccharide, are present in
fresh and brackish waters, cyanobacteria can be produced secondary metabolites which are toxic to other organism called cyanotoxins [2] [1]

Cyanotoxins are chemical substance which is produced at stationary phase as secondary metabolite by cyanobacteria that can induce the toxic effects or a diverse groups with natural substance both from toxicological , chemical properties and cause direct intoxication to aquatic and terrestrial organism due to their ability to accumulate in tissue of organism and transfer through food chain. Cyanotoxins like microcystins can produce via decrease of Phosphate, nitrate, ferric Fe^{+2} and zinc Zn^{+2} [1] [3].

Cyanobacteria can be released several toxins at the same time, while some species of cyanobacteria does not produce toxins at all. The major cyanobacteria producer of cyanotoxins are *Anabaena, Microcystis, Aphanizomenon, Nostoc, Cylindrospermopsis, Lyngbya, and Oscillatoria (Planktothrix)* [4]

2-Material and methods

2-1-Sterilization and Preparation of BG11 medium

All glassware and BG11 medium had been sterilized in autoclave at 121°C, 15J for 15 min and the BG11 medium was utilized for cyanobacterial growth [5] [6]

2-2-Culturing of Cyanobacteria Species

Unicellular algae of Cyanobacteria was taken of 10 ml of isolate of Cyanobacteria in log phase which added to a flask contained 90ml of BG11 media and incubated at 27±2°C with a photo period of 8 hour darks:16 hour lights for 14 days, this flask that contained 100ml growth of cyanobacteria would transport to flask contained 900 ml of media and incubated for 14 days and ultimately the growth of cyanobacteria in the flask that contained 1000ml, would be transported to pools 20 liter and harvested after 5-6 days at stationary phase (Cyanotoxin was
formed in this phase) and concentrated by centrifugation at 3000rpm for 15min and 
lyophilized by Oven at 35°C for 48h, repeated culturing of each Species of 
cyanobacteria four time to obtain large amount of biomass[7] (Tredici,2004)

2-3-Extraction and Purification of microcystin-LR

The cyanobacterial cell are freeze-thaw, three time before extraction to disrupt 
the cell wall lead to easy release of microcystin from cell and lyophilized cell of 
cyanobacteria (2g) from *W. prolifica* had been extracted three time by solvent 
mixture of water:methanol:1-butanol 75:20:5 for one hour then sonication by path 
sonicator for two hour and the extracts were centrifuged at 15000 rpm for 30 min 
at 20 °C and the supernatant combined, the combined supernatants would be air-
dried at 35°C to remove methanol and 1-butanol and to concentrate to 3ml and 
microcystins in each extract detected by using Ultraviolet-Spectrophotometer at 
238nm [8]

The purification of toxins has been performed according to Namikoshi *et al.*, (9) 
above extract was loaded on glass column (2 × 15cm) which contained Silica gel 
(75-250 mesh), then the column washed by 120 ml of Deionized water followed by 
20% methanol (20 ml methanol : 80 ml Deionized water) and finally, the toxins 
was eluted by 80% methanol with flow rate 3ml/min.

2-4-Analytical, Purification and collection of microcystin-LR

The toxins fraction has been dissolvd in absolute methanol specialized for 
preparative high performance liquid chromatography (PHPLC) and 0.25ml was 
injected by microsyring to PHPLC type (Shimadzu in ministry of Science and 
technology in Lab. Of water and environmental analysis test) have the following 
characters C18-Octanoldodecyl column with 25cm×4.6mm I.D. and mobile phase( 
Methanol : H₂O ) 20:80 , flow rate (1ml / min) at wave length 238 nm and at 30C
of temperature [10]. The results compared with an absorbance and retention time of standard Microcystin - LR was purchased from sigma Aldrich Company, then peak of microcystin-LR was collected and put in oven at 35°C for two weeks to remove methanol.

3-Results

3-1-Culturing of Cyanobacteria Species

Cyanobacteria were obtained during the period of this study from scientific centers Iraqi Universities, Which is *Westiellopsis prolifica* that is growing on BG11 medium was the best media for obtaining biomass. *W. Prolifica* was identified as producer of microcystin-LR for the first time in Iraq. Figure (1-1). The microcystin-LR of *W. Prolifica* was extracted at stationary phase and its entered stationary phase at nine day figures (1-2).
3-2-Analysis and Purification of Microcystin-LR by Preparative HPLC

Extraction Crude of each species of cyanobacteria were occurred by utilizing water: methanol: butanol and partially purified by silica gel column, then analyzed by preparative HPLC to detect the present of microcystin-LR, the concentration of toxin was determined by comparing peak area and retention time of analytical standard of microcystin-LR with peak area and retention time of extraction of each species of cyanobacteria, the retention time of analytical standard of microcystin-LR were 9.55 min figure (1-3) and it’s concentration was 10µg/ml, *W. prolifica* retention time was 9.5 min figure (1-4), it’s concentration was 28.385 µg/ml.
Figure (1-3) HPLC analysis of standard Microcystin-LR
Figure (1-4) Preparative HPLC Analysis of *W. prolifica* microcystin-LR toxin
4-Discussion

The Peptide toxins are intracellular toxins that are released to medium by breaking cells, the microcystins have been not actively secreted to the surrounding water, Studies with laboratory cultures of cyanobacterial strain demonstrated that most (<80%) of the toxin is intracellular in healthy growing cells and that the releasing of toxin occurs during the cultures senescence, and the shift from growth to stationary phase and cell death [11] [3].

Toxin levels were expressed in volumetric units that are more suitable for the risk estimation for aquatic organisms, wildlife, and humans [11]. The microcystin-LR guideline value in drinking water was (1 µg/ml) and tolerance daily intake of microcystin in human was 0.04µg/kg bodyweight per day [12].

The results of present study were shown the toxin concentration were (28.385) µg/ml in W. prolifica, Zhang et al., (13) observed highest concentration of microcystin-LR was 7300 µg/g in microcystis.sp. and Lindholm and Meriluoto,(2004) shown the highest concentration 40 µg/l of demethylmicrocystin-RR in O. agardhii and in microcystis bloom, the microcystin concentration were 2500 µg/l in lake water of Ostera in Aland, Al-Aarajg and Al-Sultan (14) shown that species Hapalosiphon Welwitschii contain the highest concentration of microcystin-LR(44.415) µg/ml While Lalita et al .,(15) recorded the highest concentration of microcystin-RR and was 732 µg/ml in strain of microcystis, the differences in variant of microcystins concentration between different or the same species of cyanobacteria can be caused by intra- and interspecific variability, as well as by regulation of microcystin synthesis under different condition, differences in toxin gene expression, growth phase and environmental factor such as temperature, pH and nutrients [3] [16] [17]
5-References

[1] Almamoori A et al 2017 The effects of purified hepatotoxins, microcystin-RL from Anabaena cicinalis on some biochemical markers of common (cyprinus carpio). *J. Glob. Pharm. Techn.* 10(9):40-46.

[2] Campos A and Vasconcelos V 2010 Molecular Mechanisms of Microcystin Toxicity in Animal Cells, *Int. J. Mol. Sci.* 11, 268-287;

[3] Khadairi M et al 2017 The biochemical alteration and DNA damage in rats rattus rattus after chronic intraperitoneally injection to purified microcystin-LR from Anabaena cicinalis. *As. J. Pharma. and Clin. Rese.* 10(11):277

[4] Adamovsky O 2010 Bioaccumulation and effects of cyanotoxins in the aquatic environment. *J. En Ch.* 4: .223-227.

[5] Aderson R A 2005 Algal culturing techniques. Physiological Society of America, *El. A. p.*, 589P.

[6] Alghanmi H and AbdulWahid M 2014. Investigation of geosmin and 2-Methylisoborneol produce by some species of Cyanophyta under different condition. University of AlQadisiya, Doctor of philosophy in Biology.

[7] Tredici M R 2004 Mass production of microalgae: photobioreactors. In: Richmond A, ede. Handbook of microalgae culture: *Biotech. and Appl. phyc.*. Oxford. Bla. Sci. 2004.

[8] Fastner, J et al 2006 Optimized extraction of microcystins from field samples - a comparison of different solvents and procedures. *Water Res.* 32, 3177-3181.

[9] Namikoshi M M et al 1995 Phytochemistry 31 Rinehart, L. Rouhiainen, F. Sun, S. Brittain, A. Otsuki, 1247. *Chem. Res. Toxicol.* 11 143.

[10] Purdie E L et al 2009 Toxicity of the cyanobacterial neurotoxin β-N-methylamino-L-alanine to three aquatic animals species. *Amyo. Lat. Scler.* 10 : 67-70
[11] Meriluoto J et al 2005 Isolation and detection of microcystins and nodularins, cyanobacterial peptide hepatotoxins. In: Holst, O. (Ed.), Methods in Molecular Biology. *Humana Press Inc., New Jersey*, pp. 65–87.

[12] WHO 2003 Guidelines for Drinking-water Quality. Second edition, Addendum to Volume 2, Health Criteria and Other Supporting Information, *World Health Organization, Geneva*.

[13] Zhang Q.-X et al 1991 Cyclic peptide hepatotoxins from freshwater cyanobacterial (blue-green algae) waterblooms collected in central China. *Environ. Toxicol. Chem.* **10**, 313-321

[14] AL-Aarajya M J and Al-Sultan E Y A 2008 Isolation and purification of Hepatotoxin (Microcystin-LR) from some Blue–green algae of sweage water in Basrah. *Mar. Bull.* **3(1)** 1-16

[15] Lalita N S et al 2009 Isolation and characterization of microcystin producing Microcystis from a Central Indian water bloom. *Harl Alg.* (8) :674–684

[16] Marten J C and Vasconcelos V 2011 *Differential protein expression in Corbicula fluminea upon exposure to a Microcystis aeruginosa toxic strain*. *Toxicon.*, **53**: 409–416.

[17] Lindholm T and Meriluoto J A O 2004 Recurrent depth maxima of the hepatotoxic cyanobacterium Oscillatoria agardhii. *Can. J. Fish. Aquat. Sci.* **48**, 1629-1634.