Identification and Hepatotoxicity of Microcystin-LR Isolated from *Microcystis aeruginosa* Kütz. in Huay Yuak Reservoir Chiang Mai Province

Soraya Aroonvilairat[1], Werawan Ruangyuttikarn[1*], Jeeraporn Pekkoh[2], Yuwadee Peerapornpisal[2], Xiaoyun Shen[3], Wasantha Wickramasinghe[3] and Glen Shaw[3]

[1]Department of Forensic Medicine, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand

[2]Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand

[3]School of Public Health, Griffith University, and CRC for Water Quality and Treatment, Meadowbrook Qld, 4031, Australia

**Corresponding author. E-mail: wruangyu@mail.med.cmu.ac.th*

ABSTRACT

Surface cyanobacterial blooms were collected from Huay Yuak reservoir in Chiang Mai, Thailand and extracted for microcystin identification and analysis. The hepatotoxicity of microcystin was investigated on primary cultured rat hepatocytes, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. M. aeruginosa was isolated and cultured in order to investigate the relationship between the intracellular and the extracellular microcystin-LR concentrations. The microcystin content was determined by high-performance liquid chromatography. The results showed that microcystin-LR was the main component of the surface cyanobacterial blooms and was quantitated as 0.14 mg/g of dried cells. The confirmatory identification of microcystin-LR was achieved, using an optimized high-performance liquid chromatography-tandem mass spectrometry system. Microcystin-LR exhibited toxic effects on primary cultured rat hepatocytes with an IC50 of 10.34 ng/mL at 24 h incubation. M. aeruginosa cultures showed that the intracellular microcystin-LR concentrations correlated negatively with the extracellular microcystin-LR concentrations. The intracellular microcystin-LR concentrations ranged between 120.09-458.59 µg/g of dried cells while the extracellular concentrations ranged between 0.67-10.46 µg/mL. This study indicated that microcystin-LR was the main toxic component found in cyanobacterial samples from Huay Yuak reservoir, using a variety of techniques.

Key words: Microcystin-LR, Identification, Hepatotoxicity, Huay Yuak Reservoir

INTRODUCTION

Massive surface accumulations of blue-green algae (cyanobacteria), resulting from eutrophication, are a serious water quality problem in many countries (Carmichael, 1989; Codd, 2000). Many common cyanobacteria genera, such as *Microcystis, Cylindrospermopsis, Anabaena, Oscillatoria* and *Nodularia* are capable of producing potent toxins which can cause domestic and wild animal death (Skulberg et al., 1984; Carmichael, 1994) and hazards to human health (Sivonen and Jones, 1999). *Microcystis aeruginosa* Kütz. is the dominant toxic cyanobacteria and the most widespread cyanobacteria in reservoirs in Thailand (Mahakhant et al., 1998; Peerapornpisal et al., 2002).

Microcystin-LR, primarily produced by *M. aeruginosa*, is a cyclic peptide hepatotoxin with LD50 i.p. or i.v. in mice and rats ranging from 36 to 122 μg/kg, while the inhalation toxicity (LCT50) in mice is 180 $mg.min.m^{-3}$, LD50 = 43 μg/kg (Dawson, 1998). Microcystins are a potent inhibitor of serine/threonine protein phosphatases type 1 and type 2A (Honkanen et al., 1990; MacKintosh et al., 1990; Toivola et al., 1994; Guzman et al., 2003). Consequently, hyperphosphorylation of cytoskeletal proteins leads to hepatic architecture disruption with hepatic haemorrhage and hepatocytes necrosis (Eriksson et al., 1987). Many studies have reported the carcinogenicity of microcystins in animals (Nishiwaki-Matsushima et al., 1992; Ito et al.,1997; Sekijima et al.,1999). Long-term exposure to low levels of microcystins in human has been reported to be linked to the development of primary liver cancer in China (Ueno et al., 1996). WHO (1998) has suggested a provisional guideline level of microcystin-LR at the concentration of 1 μg/L and a tolerable daily intake (TDI) value of 0.04 μg/kg body weight.

Huay Yuak reservoir, located in Chiang Mai province, Northern Thailand, was constructed in order to conserve water resources for recreational activities, e.g. swimming, boating and fishing. Recently, this reservoir has exhibited what appears to be eutrophic phenomena, such as cyanobacterial blooms (Kiatpradub, 2003; Ruangyuttikarn et al., 2004). The appearance of cyanobacterial blooms and the presence of their toxins, especially microcystins, have thus become a threat to human health.

Cytotoxicity of microcystins has been well documented both in vivo (Runnegar et al., 1993) and in vitro (Eriksson et al., 1987; Honkanen et al., 1990; MacKintosh et al., 1990; Henning et al., 1992). However, the cytotoxic effect of microcystins from *Microcystis aeruginosa* Kütz. found in several reservoirs in Thailand has not been reported.

The main objective of this study was to identify and isolate microcystin from *M. aeruginosa* collected from Huay Yuak reservoir, Chiang Mai province. The correlation between microcystin concentrations in *M. aeruginosa* cells and the culture media was also investigated. The MTT assay was chosen to investigate the cytotoxicity of microcystins on the primary rat hepatocytes in this study, and high-performance liquid chromatography and tandem mass spectrometry were used to determine the microcystin composition.

MATERIALS AND METHODS

1. Apparatus

High-performance liquid chromatography (Varian, U.S.A.), consisting of Varian 9012Q solvent delivery system, Varian Prostar 310 UV-visible absorbance detector, and Varian Polychrome 9065 diode array detector with preparative Luna C18 5 µm, 10 x 250 mm (Phenomenex, U.S.A.) column and analytical Mightysil RP-18GP C18 5 µm, 4.6 x 150 mm (Kanto Chemicals, Japan) column were used.

High performance liquid chromatography tandem mass spectrometry (HPLC-MS-MS, Perkin-Elmer Sciex Instruments, Canada), consisting of HPLC-200 series system, high flow electrospray (TurboIonspray) interface, API 300 mass spectrometer, and analytical HPLC Altima C18, 5 µm, 4.6 x 150 mm (Alltech Associates, Australia) column were used to confirm the identity of the toxin.

2. Chemicals

Microcystin-LR standard (10 µg/mL in 20% methanol) was purchased from Kanto chemical (Tokyo, Japan). Fetal Calf Serum was purchased from Gibco (U.S.A). Dulbecco's Modified Eagle Medium, bovine serum albumin and collagenase were purchased from Sigma (St. Louis, MO, USA). Solvents used were of HPLC grade. Other chemicals and reagents were of analytical grade, ordered from local agency.

3. Surface cyanobacterial bloom sampling

Samples of surface cyanobacterial bloom were collected, using a 10 µm mesh size plankton net in April, 2003 from Huay Yuak reservoir, Chiang Mai province, Thailand. The cyanobacteria were preserved in lugol's solution and the species was identified under light microscope.

4. Identification and isolation of *M. aeruginosa* Kütz.

The identification of *M. aeruginosa* was carried out according to the textbooks (Prescott, 1970; Komarex and Anagnostidis, 1999), using microscopy. Then *M. aeruginosa* cells were isolated and transferred into the culture flask for cultivation.

5. Cultivation of M. aeruginosa Kütz.

M. aeruginosa cultivation method was slightly modified from that of Otsuka et al., (1999). Briefly, isolated *M. aeruginosa* cells were cultured in medium containing 50 mg $Ca(NO_3)_2.4H_2O$, 100 mg KNO_3, 50 mg $NaNO_3$, 40 mg Na_2SO_4, 50 mg $MgCl_2.6H_2O$, 100 mg beta-Na_2glyerophosphate.$5H_2O$, 5 mg Na_2EDTA, 0.5 mg $FeCl_3.6\ H_2O$, 0.5 mg $ZnCl_2$, 5 mg $CoCl_2.6\ H_2O$, 0.8 mg $Na_2MoO4.2\ H_2O$, 20 mg H_3BO_3, 500 mg Bicine, and 5 mg $MnCl_2.4\ H_2O$ in 1 litre of distilled water, pH8.6 at 25°C under a 14:10 h light:dark cycle, with a photon flux density of about 30 $\mu mol.m^{-2}.s^{-1}$ provided by the daylight fluorescent lamps. The *M. aeruginosa* cells were cultured in 10 L batch-culture vessels and harvested for one month. The algae cells were separated from the culture media by centrifugation, followed by freeze-

drying and stored at 4°C for microcystin analysis. Microcystin concentrations in the algae cells and also in the culture media in each sample, were determined at every 2 days by HPLC.

6. Extraction of microcystins

Microcystins extraction was modified from the method of Aguete et al., (2003). Lyophilized algae cells, 0.5 g, were extracted with 10 mL of absolute methanol and homogenized for 10 min in a variable-speed reversible Glas-Col homogenizer. The mixture was then centrifuged at 6,000 rpm (11,000x g) for 10 min at room temperature. The supernatant was concentrated to 1 mL as a final volume under a nitrogen stream.

The lyophilized culture media was directly injected into the HPLC after being reconstituted with methanol and filtered through a 0.45 μm nylon syringe filter.

7. Analysis of microcystins

Microcystins in the cyanobacterial extract and culture media were analyzed by using a linear gradient elution of 30-70% acetonitrile with 0.05% trifluoroacetic acid (TFA) in 40 min according to Lawton et al., (1994) with a flow rate of 1 mL/min at the wavelength of 238 nm. Standard microcystin-LR was used to identify the microcystin-LR in both cyanobacterial extract and culture media. Sample injection volume was 20 μL.

The microcystin-LR fraction was collected from the semi-preparative Luna C_{18} 5 μm, 10 x 250 mm column, pooled and stored at -20°C for a confirmed identification of microcystin-LR, using HPLC-MS-MS.

8. Identification of microcystin-LR by HPLC-MS-MS

Microcystin-LR was identified by LC-200 series system coupled to API 300 Mass Spectrometer and TurboIonspray Interface. Chromatography was performed on Altima C18 column with the mobile phase of methanol and 2 mM ammonium acetate at the ratio of 1 to 1 volume. Injection volume of the sample was 8 μL. The ion source temperature was set at 400°C. A mass range of m/z 900-1100 was covered within 1.2 seconds and the data were collected in the positive ion mode.

9. Preparation of primary rat hepatocytes culture and MTT assay

Female Wistar rats (200-250 g) were used for rat hepatocyte preparation. Two-step collagenase perfusion of rat hepatocytes and MTT assay were slightly modified from the method of Chong et al., (2002). Primary cultured rat hepatocytes were plated in 96-well plates (8,000 cells/well), in 50 μL medium, and incubated before toxin treatment at 37°C for 24 h. After 24 h, hepatocytes were incubated with purified microcystin-LR from Huay Yuak cyanobacterial extract at the concentrations of 0, 0.98, 1.96, 3.93, 7.86, 15.61, 31.25, 62.50, 125, 250 and 500 ng/mL which were added in the medium and incubated for 24 h. After removal of 100 μL medium, 200 μL of MTT stock solution (5 μg/mL in PBS) was added into each well, the plate was incubated for 4 h at 37°C in 5% CO_2 / 95% air humidified atmosphere. Then, 100

μL of stop solution (dimethylsulfoxide, DMSO) was added into each well to lyze the cells and solubilize the formed formazan crystals. The formation of formazan was measured with an ELISA reader at 570 nm with a reference wavelength of 655 nm. Survival cells percentage was calculated as the percentage absorbance of sample relative to control

10. Data analysis

The absorbance in each concentration of microcystin-LR testing with the MTT assay was measured three times, then calculated to percentage of survival cells. The correlation between microcystin-LR concentrations in the *M. aeruginosa* cells and the culture media was evaluated for statistical significance by linear regression analysis.

RESULTS

1. Identification of cyanobacteria samples

The species of cyanobacteria was identified and it was revealed in April 2003 that the surface blooms in Huay Yuak reservoir, Chiang Mai were dominated by *Microcystis aeruginosa* Kütz. (over 95%).

2 Separation of microcystins in cyanobacterial extract

Cyanobacterial extract from the surface bloom samples and the laboratory cultures were rapidly separated by reversed-phase HPLC in 12 min. The chromatogram resulting after injection of a standard mixture containing microcystin-RR, -YR and -LR (3.33 μg/mL for each toxin, 20 μL injection volume) and the retention time at 4.9, 9.7 and 11.8 min, respectively, is shown in Fig. 1a. The main component of the surface bloom extract was microcystin-LR, and its peak showed a retention time of 11.8 min, whilst microcystin-RR was also found in small amounts at the retention time of 4.9 min (Fig. 1b). The quantity of microcystin-LR found in the surface bloom sample was 140 μg/g of dried cells. The purified microcystin-LR isolated from the surface cyanobacterial extract had almost 100% purity (Fig. 1c), and was pooled for the cytotoxicity test after a confirmed identification by LC-MS-MS.

3. Microcystin-LR concentrations in the algae cells and the culture media

The *M. aeruginosa* culture was harvested and quantitated every 2 days for microcystin-LR concentrations in the algae cells and culture media. The high concentration of microcystin-LR in the algae cells (day 2) was found at the initial sampling extract (258.62 μg/g of wet cells) and decreased to 173.15 μg/g of wet cells at the last sampling extract (day 30). In contrast, microcystin-LR in the culture media had a very low concentration (0.81 μg/mL) at day 2, but increased to 10.46 μg/mL at day 30. Therefore, the intracellular microcystin-LR concentrations in the algae cells correlated negatively with the extracellular microcystin-LR concentrations in the culture media as shown in Fig. 2.

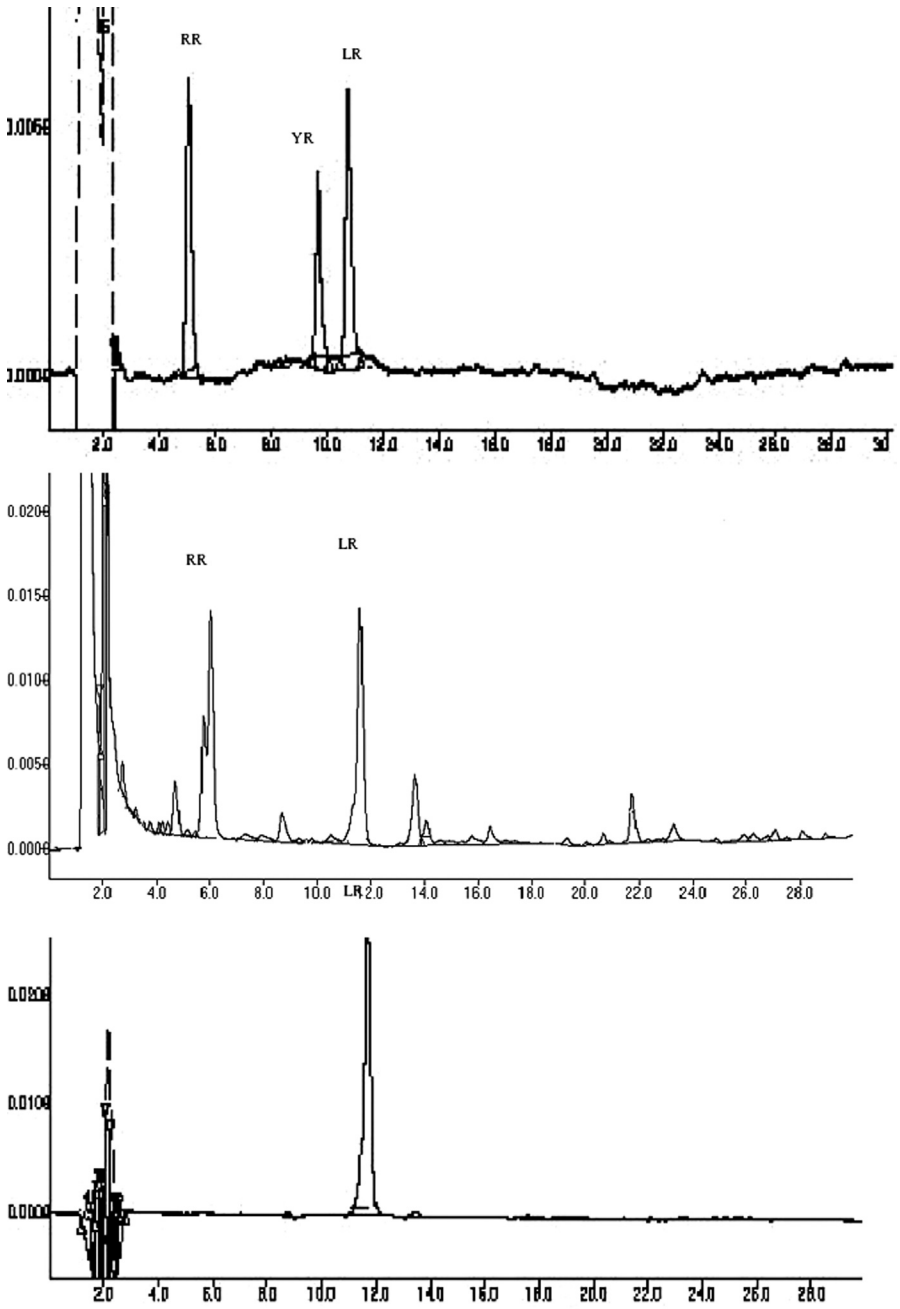

Figure 1: HPLC separation profile of microcystin-RR and -LR: (a) standard mixture containing microcystin-RR, -YR, -LR (3.33 µg/mL for each toxin); (b) the surface cyanobacterial bloom sample; (c) the purified microcystin-LR collected from the HPLC. The retention time of microcystin-RR, -YR and -LR was at 4.9, 9.7 and 11.8 min, respectively.

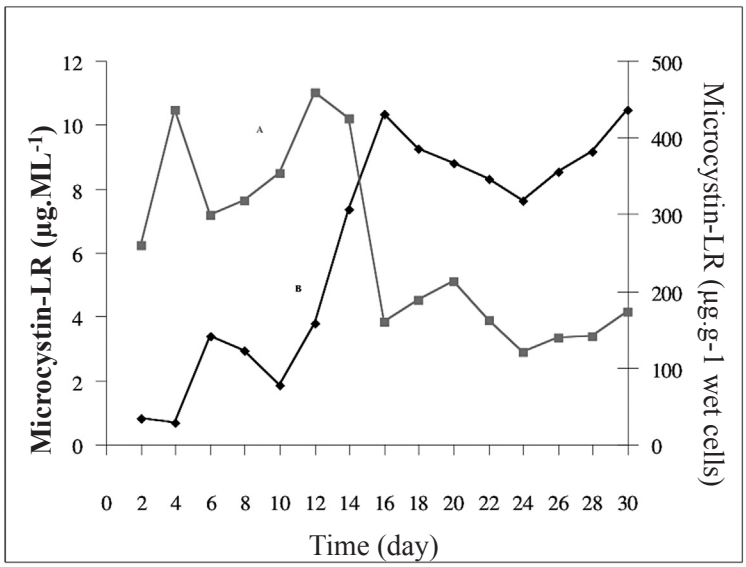

Figure 2: Relationship of microcystin-LR concentrations between in the algae cells (A) and culture media (B) collected from laboratory *M. aeruginosa* cultures at every two days for one month.

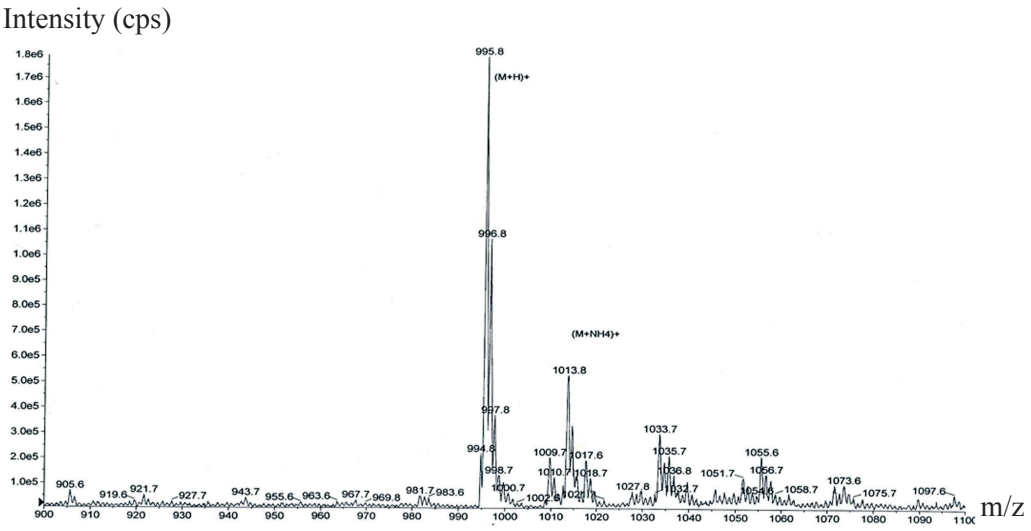

Figure 3: Mass spectrum of the purified microcystin-LR isolated from Huay Yuak *M. aeruginosa* cells extract identified by LC-MS-MS.

4. LC-MS-MS Identification of microcystin-LR

The mass spectrum of microcystin-LR analyzed by the LC-MS-MS in positive scan mode for a mass range between m/z = 900-1100 revealed a molecular mass of 995 (Fig. 3), which was fully compatible with the standard microcystin-LR MS-MS spectrum (Fig. 4). The typical microcystin-LR fragment ions were present at m/z 135 and 163 (Fig. 5), which corresponded to the Adda group of microcystin-LR.

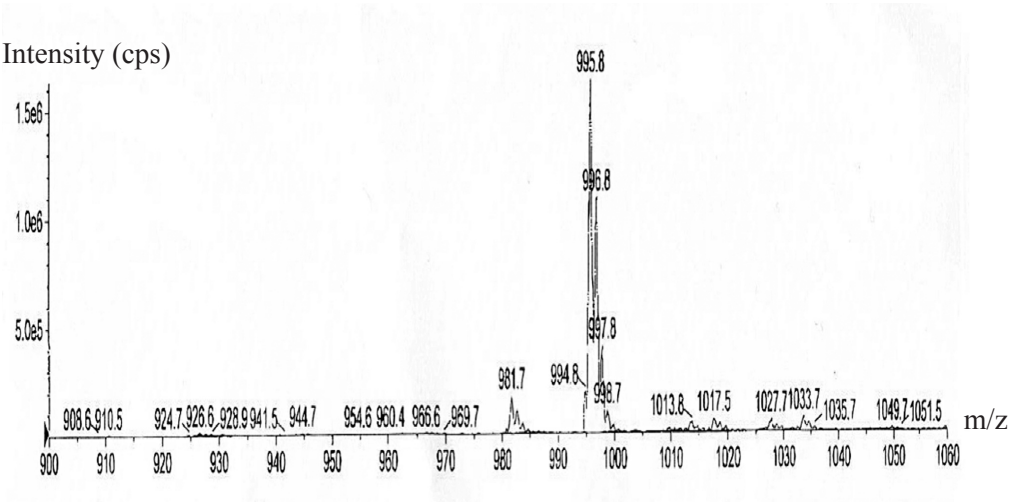

Figure 4: Mass spectrum of the standard microcysin-LR indentified by LC-MS-MS showing the molecular ion at m/z 995.8.

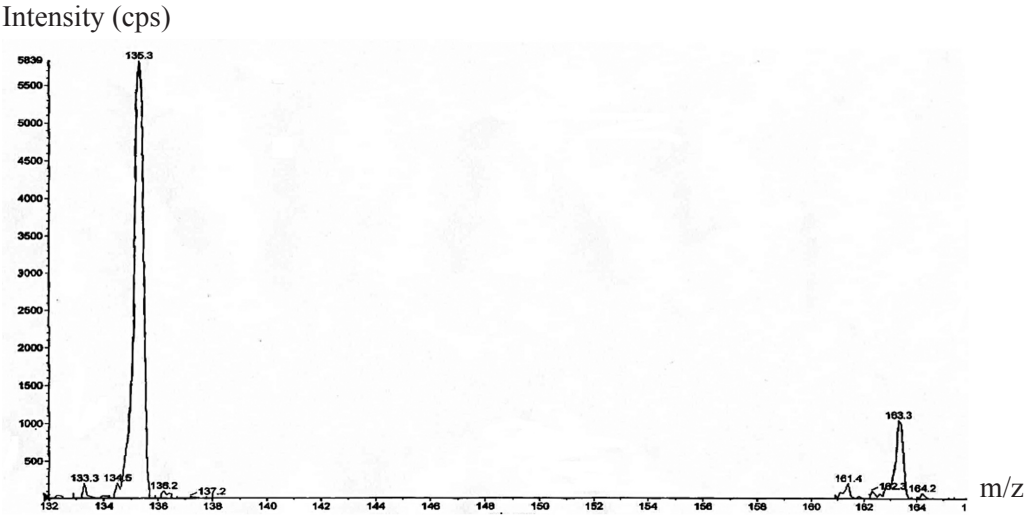

Figure 5: Mass spectrum obtained from the fragmentation of microcystin-LR showing the molecular ion at m/z 135 and 163.

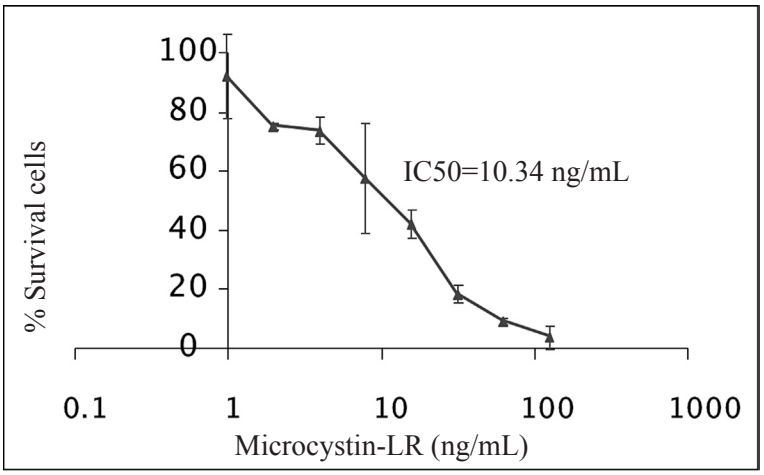

Figure 6: Inhibition curve of purified microcystin-LR (0-125 ng/mL) on primary cultured rat hepatocytes incubated for 24 h. Values are the mean of triplicate measurements.

5. Cytotoxicity of microcystin-LR on primary cultured rat hepatocytes

The purified microcystin-LR was collected from HPLC, pooled, lyophilized and kept for the cytotoxicity test. Figure 6 shows the survival curve of primary cultured rat hepatocytes incubated with microcystin-LR concentrations that ranged between 0-125 ng/mL for 24 h. It revealed the prominent dose-dependent curve of the microcystin-LR and the primary cultured rat hepatotoxicity. The IC50 calculated from these curves was 10.34 ng/mL.

DISCUSSION AND CONCLUSION

The concentration of microcystin-LR found in Huay Yuak cyanobacterial blooms in April, 2003 (140 µg/g of dried cells) was lower than the previous reports. Ruangyuttikarn et al., (2004) found microcystin-LR at 1.86 mg/g of dried cells in the cyanobacteria collected from the same reservoir in the year 2001. Mahakhant et al., (1998) found at 0.70-0.80 mg/g of dried sample in cyanobacterial blooms collected from two reservoirs and a pond in Thailand. In addition, it was also different from reports of other cyanobacterial blooms in many countries (Kaya and Watanabe, 1990; Falconer, 1994; Oudra et al., 2001; Shen et al., 2003). These differences in microcystin concentrations may be due to several factors such as temperature, light intensity, hydrologic stability and nutrients at the sampling time and site that affect toxin production and cell toxicity (Watanabe and Oishi, 1985). Additionally, the genetic differences among various strains may result in different quantitative toxin production.

The MS-MS spectrum of the surface bloom samples showed a $[M+H]^+$ ion at m/z 995 (Fig. 3), corresponding to the expected molecular weight of microcystin-LR (994 Da) (Botes et al., 1985; Watanabe et al., 1988). The typical fragmentation ion at m/z 135 was also observed, which was formed by α-cleavage of a methoxy group of Adda group of the microcystins (Namikoshi et al., 1992). Monitoring at m/z 135 was useful for differentiation of microcystins from other types of compounds and for identifying microcystins in environmental samples which contain a large number of interfering compounds.

The relationship between microcystin-LR in the algae cells and the culture media was useful to predict the possibility of microcystin release into the water. Tsuji et al., (1996) reported that the range of measured concentrations for dissolved microcystin was 0.1-10 μg/L. However, the concentration of dissolved toxins was much higher in aging or declining blooms as shown in Fig. 2.

The result from this study agreed with earlier studies which showed the functional and morphological mitochondrial alterations caused by microcystins (Berg et al., 1988; Khan et al., 1995; Brattacharya et al., 1996). Ding et al., (1998) found that mitochondrial damage was closely associated with *Microcystis* cyanobacterial extract-induced cell injury in cultured rat hepatocytes. McDermott et al., (1998) found the nuclear and mitochondrial changes preceded apoptosis in rat hepatocytes following treatment with microcystin. Recently, it has been reported that mitochondria were one of the target organelles of hepatotoxicity caused by microcystins (Majsterek et al., 2004).

Cytotoxicity of microcystin-LR using the MTT assay which can assess the mitochondrial function and the amount of the formazan (product from the reduction of MTT dye by mitochondrial dehydrogenase enzymes) generation was directly proportional to the number of viable cells. Our result of IC50 of 10.34 ng/mL was significantly lower than the previous report by Bouaïcha and Maatouk (2004) which produced a 24 h IC50 of 48 ng/mL. However, these data could not provide enough evidence of differential toxicity due to the different procedure of rat hepatocytes preparation, rat hepatocytes culture condition and MTT procedure. Another reason for this difference might be due to the presence of some impurities in the samples.

This study indicated that the mitochondrial function had been altered after exposure to microcystin. It also indicated that microcystin-LR found in cyanobacterial samples from Huay Yuak reservoir had high toxicity on rat hepatocytes. Therefore, exposure to microcystin-LR contamination in the water could result in cellular damage and negative health consequences for people who use this reservoir. To prevent the potential hazard of microcystins on human health in Chiang Mai's reservoirs, specific monitoring and control of cyanobacterial growth are necessary.

ACKNOWLEDGEMENTS

This study was supported by the Faculty of Medicine Research Fund, the Graduate School, Chiang Mai University, Chiang Mai, Thailand and the National Research Center for Environmental Toxicology, University of Queensland, Australia.

REFERENCES

Aguete, E.C., A. Gago-Martínez, J.M. Leão, J.A. Rodrigues-Vãzquez, C. Menàrd, and J.F. Lawrence. 2003. HPLC and HPCE analysis of microcystins RR, LR and YR present in cyanobacteria and water by using immunoaffinity extraction. Talanta 59: 697-705.

Bergs, K., J. Wyman, W.W. Carmichael, and A. Dabholkar. 1988. Isolated rat liver perfusion studies with cyclic heptapeptide toxins of *Microcystis* and *Oscillatoria* (Freshwater cyanobacteria). Toxicon 26: 827-837.

Botes, D.P., P.L. Wessels, H. Kruger, M.T.C. Runnegar, S. Santikarn, R.J. Smiths, J.C.J. Barna, and D.H. Williams. 1985. Structural studies on cyanoginosins-LR, -YR, -YA, and-YM, peptide toxins from *Microcystis aeruginosa*. J. Chem. Soc. Perkin. Trans. 1: 2747-2748.

Bouaïcha, N., and I. Maatouk. 2004. Microcystin-LR and nodularin induce intracellular glutathione alteration, reactive oxygen species production and lipid peroxidation in primary cultured rat hepatocytes. Toxicol. Lett. 148: 53-63.

Brattacharya, R., P.V. Rao, A.S. Bhaskar, S.C. Pant, and S.N. Dube. 1996. Liver slice culture for assessing the hepatotoxicity of freshwater cyanobacteria. Hum. Exp. Toxicol. 15: 105-110.

Carmichael, W.W. 1989. Freshwater cyanobacteria (blue-green algae) toxins. p. 3-16 In C.L. Ownby and G.V. Odell (eds) Natural toxins: Characterization, pharmacology and therapeutics. Oxford, Pergamon Press.

Carmichael, W.W. 1994. The toxins of cyanobacteria. Sci. Am. 270: 78-86.

Chong, M.W.K., B.S.F. Wong, P.K.S. Lam, G.R. Shaw, and A.A. Seawright. 2002. Toxicity and uptake mechanism of cylindrospermopsin and lophyrotomin in primary rat hepatocytes. Toxicon 40: 205-211.

Codd, G.A. 2000. Cyanobacterial toxins, the perception of water quality, and the prioritisation of eutrophication control. Ecol. Eng. 16: 51-60.

Dawson, R.M. 1998. The toxicology of microcystins. Toxicon 36: 953-962.

Ding, W.X., H.M. Shen, Y. Shen, H.G. Zhu, and C.N. Ong. 1998. Microcystic cyanobacteria cause mitochondrial membrane potential alteration and reactive oxygen species formation in primary cultured rat hepatocytes. Environ. Health. Perspect. 106: 409-413.

Eriksson, J.E., H. H?gerstrand, and B. Isomaa. 1987. Cell selective cytotoxicity of a peptide toxin from the cyanobacterium Microcystis aeruginosa. Biochim. Biophys. Acta. 930: 304-310.

Falconer, I.R. 1994. Health problems from exposure to cyanobacteria and proposed safety guidelines for drinking and recreational water. p. In G.A. Codd, T.M. Jefferies, C.W. Keevil, and E. Potter (eds.) Detection methods for cyanobacterial toxins. Cambridge, The Royal Society for Chemistry.

Guzman, R.E., P.F. Solter, and M.T. Runnegar. 2003. Inhibition of nuclear protein phosphatase activity in mouse hepatocytes by the cyanobacterial toxin microcystin-LR. Toxicon 41: 778-781.

Henning, K., J. Cremer, and H. Meyer. 1992. Cytotoxicity of cyanobacterium *Microcystis aeruginosa.* J. Vet. Med. B 39: 307-310.

Honkanen, R.E., J. Zwiller, R.E. Moore, S.L. Daily, B.S. Khatra, M. Dukelow, and A.L. Boynton. 1990. Characterization of microcystin-LR, a potent inhibitor of type 1 and type 2A protein phosphatases. J. Biol. Chem. 265: 19401-19404.

Ito, E., F. Kondo, K. Terao, and K.I. Harada. 1997. Neoplastic nodular formation in mouse liver induced by repeated intraperitoneal injections of microcystin-LR. Toxicon 35: 1453-1457.

Kaya, K., and M.M. Watanabe. 1990. Microcystin composition of an axenic clonal strain of *Microcystis viridis* and *Microcystis viridis*-containing water blooms in Japanese freshwaters. J. Appl. Phycol. 2: 173-178.

Khan, S.A., S. Ghosh, M. Wickstrom, L.A. Miller, R. Hess, W.M. Haschek, and V.R. Beasley. 1995. Comparative pathology of microcystin-LR in cultured hepatocytes, fibroblasts, and renal epithilial cells. Nat. Toxins 3: 119-128.

Kiatpradub, S. 2003. Eutrophication and water quality in Huay Yuak reservoir, Chiang Mai Province in the year 2002. Master's degree independent study. Chiang Mai University, Chiang Mai.

Komárek, J., and K. Anagnostidis. 1999. Cyanoprokaryota 1. Teil: Chlorococcales. Stuttgart, Gustav Fischer Verlag Jena.

Lawton, L.A., C. Edwards, and G.A. Codd. 1994. Extraction and high-performance liquid chromatographic method for the determination of microcystins in raw and treated waters. Analyst 119: 1525-1530.

MacKintosh, C., K.A. Beattie, S. Klumpp, P. Cohen, and G.A. Codd. 1990. Cyanobacterial microcystin LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. FEBS Lett. 264: 187-192.

Mahakhant, A., T. Sano, P. Ratanachot, T. Tong-a-ram, V.C. Srivastava, M.M. Watanabe, and K. Kaya. 1998. Detection of microcystins from cyanobacterial water blooms in Thailand freshwater. Phycol. Res. 46: 25-29.

Majsterek, M., P. Sicinska, M. Tarczynska, M. Zalewski, and Z. Walter. 2004. Toxicity of microcystin from cyanobacteria growing in a source of drinking water. Comparative Biochim. Physiol. part C 139: 175-179.

McDermott, C.M., C.W. Nho, W. Howard, and B. Holton. 1998. The cyanobacterial toxin, microcystin-LR, can induce apoptosis in a variety of cell types. Toxicon 36: 1981-1996.

Namikoshi, M., K.L. Rinehart, R. Sakai, R.R. Stotts, A.M. Dahlem, V.R. Beasley, W.W. Carmichael, and W.R. Evans. 1992. Identification of 12 hepatotoxins

from a Homer Lake bloom of the cyanobacteria *Microcystis aeruginosa*, *Microcystis viridis*, and *Microcystis wesenbergii*: nine new microcystins. J. Org. Chem. 57: 866-872.

Nishiwaki-Matsushima, R., T. Ohta, S. Nishiwaki, M. Suganuma, K. Kohyama, T. Ishikawa, W.W. Carmichael, and H. Fujiki. 1992. Liver tumor promotion by the cyanobacterial cyclic peptide toxin microcystin-LR. J. Cancer Res. Clin. Oncol. 118: 420-424.

Otsuka, S., S. Suda, R. Li, M. Watanabe, H. Oyaizu, S. Matsumoto, and M.W. Watanabe. 1999. Phylogenetic relationships between toxic and non-toxic strains of the genus *Microcystis* based on 16S to 23S internal transcribed spacer sequence. FEMS Microbiol. 172: 15-21.

Oudra, B., M. Loudiki, B. Sbiyya, R. Martins, V. Vasconcelos, and N. Namikoshi. 2001. Isolation, characterization and quantification of microcystins (heptapeptides hepatotoxins) in *Microcystis aeruginosa* dominated bloom of Lalla Taker Koust lake-reservoir (Morocco). Toxicon 39: 1375-1381.

Peerapornpisal, Y., W. Sonthichai, M. Sukchotiratana, S. Lipigorngoson, W. Ruangyuttikarn, K. Ruangrit, J. Pekkoh, R. Prommana, N. Panuvanitchakorn, N. Ngernpat, S. Kiatpradab, and S. Promkutkaew. 2002. Survey and monitoring of toxic cyanobacteria in water resources for water supplies and fisheries in Thailand. Chiang Mai J. Sci. 29(2): 71-79.

Prescott, G.W. 1970. How to know the freshwater algae. Iowa: W.M.C. Brown Company Publishers Dubuque.

Ruangyuttikarn, W., I. Miksik, J. Pekkoh, Y. Peerapornpisal, and Z. Deyl. 2004. Reversed-phase liquid chromatographic-mass spectrometric determination of microcystin-LR in cyanobacterial blooms under alkaline conditions. J. Chromatogr. B 800: 315-319.

Runnegar, M.T.C., S. Kong, and N. Berndt. 1993. Protein phosphatase inhibition and in vivo hepatotoxicity of microcystins. Am. J. Physiol. 265: G224-230.

Sekijima, M., T. Tsutsumi, T. Yoshida, T. Harada, F. Tashiro, G. Chen, S.Z. Yu, and Y. Ueno. 1999. Enhancement of glutathione S-transferase placental form positive liver cell foci development by microcystin-LR in aflatoxinB1-initiated rats. Carcinogenesis 20: 161-165.

Shen, P., Q. Shi, Z.C. Hua, F.X. Kong, Z.G. Wang, S.X. Zhuang, and D.C. Chen. 2003. Analysis of microcystins in cyanobacteria blooms and surface water samples from Meiliang Bay, Taihu Lake, China. Environ. International. 29: 641-647.

Sivonen, K., and G. Jones. 1999. Cyanobacterial toxins. p.41-111. In I. Chorus and J. Bartram (eds.) Toxic cyanobacteria in water. A guide to their public health consequences, monitoring and management. E &FN Spon, London.

Skulberg, O.M., G.A. Codd, and W.W. Carmichael. 1984. Toxic blue-green algal blooms in Europe: a growing problem. Am Bio. 13: 244-247.

Toivola, D.M., J.E. Eriksson, and D.L. Brautigan. 1994. Identification of protein phosphatase 2A as the primary target for microcystin-LR in rat liver homogenates. FEBS Lett. 344: 175-180.

Tsuji, K., S. Setsuda, T. Watanuki, F. Kondo, H. Nakazawa, M. Suzuki, and K.I. Harada. 1996. Microcystin levels during 1992-5 for lakes Sagami and Tsukui Japan. Natural Toxin 4: 189-194.

Ueno, Y., S. Nagata, T. Tsutsumi, A. Hasegawa, M. Watanabe, H.D. Park, G.C. Chen, and S.Z. Yu. 1996. Detection of microcystins, a blue-green algal hepatotoxin, in drinking water sampled in Haimen and Fusui, endemic areas of primary liver cancer in China, by highly sensitive immunoassay. Carcinogenesis 17: 1317-1321.

Watanabe, M.F., S. Oishi, S, K.I. Harada, K. Matsuura, H. Kawai, and M. Suzuki. 1988. Toxins contained in *Microcystis* species of cyanobacteria (Blue-green algae). Toxicon 26: 1017-1025.

Watanabe, M.F., and S. Oishi. 1985. Effect of environmental factors on toxicity of a cyanobacterium (*Microcystis aeruginosa*) under culture conditions. Appl. Environ. Microbiol. 49: 1342-1344.

WHO. 1998. Guidelines for Drinking-water Quality. 2nd ed. Addendum to Volume 2, Health Criteria and Other Supporting Information, World Health Oraganization, Geneva.