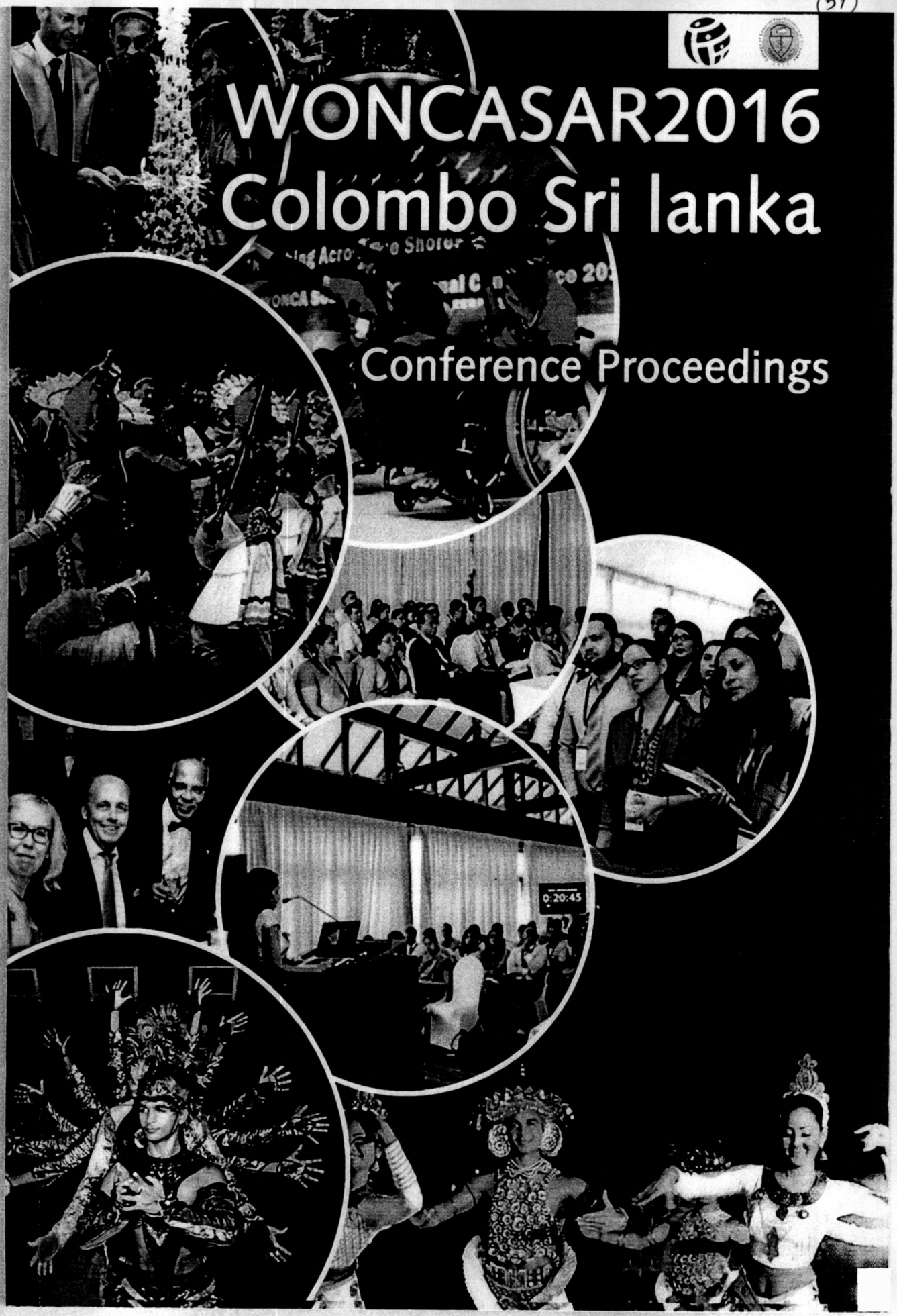

WONCASAR2016
Colombo Sri lanka

Conference Proceedings

DO MICROCYSTINS HAVE IMPACT ON HUMAN RENAL CELLS?

Cyanobacteria are common inhabitants of surface waters which are found in every water supply, exposed to sunlight. The cyanobacteria are frequently found in marine, brackish and fresh waters, including freshwater surface drinking sources, such as lakes, ponds, rivers and reservoirs. Most importantly these blue green algal blooms can produce significant quantities of natural toxins when cell lysis takes place (Bownik 2010). When cyanobacteria produce such highly active natural biotoxins, the blue green algal blooms are known as a "harmful algal bloom (HAB)" (Carmichael 2001). It was documented that approximately 75% of water sources are containing cyanobacteria that consists of cyanobacterial secondary metabolites which can cause toxic effects on organisms and human (Ernst et al. 2005). Cyanobacteria produce a supreme array of bioactive secondary metabolites, including alkaloids, polyketides and non-ribosomal peptides considered as cyanotoxins (Nogueira et al. 2004). The toxic secondary metabolic compounds produced by cyanobacteria can affect organisms by causing health hazards to livestock and wildlife and even human intoxications have been documented (Feurstein et al. 2009a). In addition, absorption of cyanotoxins via root system of crops has also been documented. Thus, bioaccumulation may cause serious problems in human health (Ernst et al. 2005). To date at least 46 different species of cyanobacteria have been recorded as toxin producing organisms. Public awareness regarding the cyanotoxins arose after number of intoxication scenarios reported in different parts of the world (Chorus 2002) suggesting that these toxicants should be treated with great public consideration and proper monitoring should be maintained frequently. Due to lack of information that would confirm the presence of cyanotoxins in human food or water supplies and shortage of proper analytical methods, most of the cases were not documented officially (Pereira et al. 2012). Furthermore, advance water treatment facilities even in the developed countries endangered their consumers in severe cyanotoxin poisonings by oral route (Wolf and Frank 2002). Thus, the toxic cyanobacterial blooms in freshwater reservoirs have been considered as threats to human health (Zhang et al. 2011) causing diarrhoea, vomiting, nausea, etc (Botha et al. 2004). Microcystins (MCs) are potent cyclic peptide hepatotoxins produced by cyanobacteria, which pose a serious threat to human health through the consumption of contaminated waters or food (Brzuzan et al. 2013).

MICROCYSTINS ON HUMAN HEALTH

There is a wide spectrum of blue green algal toxins, predominantly affecting the nervous, hepatic and dermatologic systems (Neurotoxic, hepatotoxic and dermatotoxic). Among the different varieties of identified cyanotoxins, MCs is a major cyanobacterial toxin class with a family of hepatotoxic heptapeptides (Fischer and Dietrich 2000). Among more than 100 structurally different isoforms of MCs, Microcystin-LR (MC-LR) is the most commonly encountered variant of the family as it is the most potent toxic member of the group (Feurstein et al. 2011; Lankoff et al. 2004). The hepatotoxins are cyclic peptides, predominantly MCs, nodularins (NOD), and cylindrospermopsin (CYL). These are particularly toxic to the liver in part due to selective transport mechanisms that concentrate from the gut and blood into the liver cells and damage the liver

by deranging the cytoskeletal architecture of the hepatocytes (Fischer and Dietrich 2000). All members of MCs are severe hepatotoxins and their effects have been confirmed both from acute human and animal poisoning incidents reported in different parts of the world (Chorus et al. 2000; Duy et al. 2000; Manage et al. 2009b) and from in vivo and in vitro experimental studies (Gupta et al. 2003; Z˚egura et al. 2003). Microcystin is also believed to cause damage to cells DNA by the activation of endonucleases (Jochimsen 1998). Cylindrospermopsin is a protein synthesis inhibitor, resulting in wide spread necrosis of the tissues of many organs such as liver, kidney and pancreas. Experimentally, acute high dose administration of MC can lead to death from hepatoencepholopathy within hours, and chronic administration to mice of sublethal amounts of Microcystis extracts in drinking water results in increased mortality with chronic active liver disease, even at fairly low doses and in relatively short time periods (Heinze 1996). In laboratory experimental animals, teratogenic activity has been demonstrated with oral administration of Microcystis extracts; approximately 10% of otherwise normal neonatal mice had small brains with extensive hippocampal neuronal damage (Astrachan 1980; Carmichael 1992). There are relatively few case reports and fewer epidemiologic studies on the human health effects of the blue green algal toxins which have been documented (Carmichael 1992; Chorus 2002; Falconer 1992; Jalaludin and Smith 1992). Humans can be exposed to the cyanobacteria and their toxins through direct skin contact or by drinking contaminated waters; other possible routes of exposure include inhalation of aerosols, consumption of contaminated food, and even through dialysis (Codd 1999; Chorus 2002). Occupational exposures for fishermen, watermen, and scientists, as well as recreational exposures for the general public, are both possible (Codd 1999). Chorus 2002; Junshi 1990; Yu et al. (1989; 1995) have studied the possible relationship between the consumption of blue green algal contaminated surface drinking water (pond, ditch, river and well water or deep well) and an increased risk for primary hepatic cancer and chronic gastrointestinal diseases in China. China has an extremely high rate of primary liver cancer, previously associated with Hepatitis B and Aflatoxin exposures (Yu 1995). However, reportedly large epidemiologic studies in 1973 and in 1983 were performed in Haimen, Quidong and Nanhui Counties (Guangxi province, China) to evaluate drinking water source, exposure and risk of primary hepatic cancers. These studies found that not only a significantly increased risk (SIR) of primary liver cancer in areas of high surface drinking water consumption (SIR=2.6) compared with areas of non-surface drinking water consumption (SIR=0.34), but also a strong dose response relationship.

GUIDELINES FOR MICROCYSTINS

Consequently, the effective removal of MCs from water sources is a major goal for all water utilities and the protection of aquatic ecosystem. MCs are heat and chemical stable structures and cannot be eliminated by heating even at 100°C. Furthermore, MCs are known to be chemically stable compounds (Lahti et al. 1995) and the most conventional drinking water treatments have limited efficacy in removing dissolved MCs (Svrcek and Smith 2004), and sometimes produce carcinogenic substances and other mutagens as well (Ishii et al. 2004). Conventional water treatment procedures (such as coagulation, flocculation, clarification and sand filtration) are normally efficient methods of removing cyanobacterial cells, but are not effective in removing or destroying dissolved cyanobacterial toxins. As a solution to protect humans and other animals from cyanotoxin exposure and intoxication, the World Health Organization (WHO) defined a drinking water guideline value of 1.0 µg/L for MC-LR, revealing

the importance of these toxins as a potential public health hazard (WHO 1998). Furthermore, Health Canada calculated a Tolerable Daily Intake (TDI) of 0.013 µg of MC-LR per kg of body weight per day (defined as a 60-kg adult consuming 1.5 L of water per day, with an MC-LR content of 0.5 ngmL-1 water; Fischer et al. 2001). Different countries have adopted different concentrations of MC-LR and CYL for their drinking water quality standards. In Australia, MC-LR 1.3 µgL-1; Canada, MC-LR 1.5 µgL-1; Japan, MC-LR 1.0 µgL-1; New Zealand, MC-LR 1.3 µgL-1and CYL 3 µgL-1. Based on the data in the present study and the other cyanotoxin and cyanobacteria research conducted by the University of Sri Jayewardenepura in collaboration with NWSDB Sri Lanka and JICA, in 2014 MC-LR 1 µgL-1 and CYL 2 µgL-1 was included into SLS 714 (2013) drinking water quality standards (SLS 614:2013 UDC 663.6).

In Sri Lanka, there were some records about sudden fish and cattle deaths attributed to the presence of toxic cyanobacterial blooms (Jayatissa et al. 2006; Piyasiri and Pathmalal 1999; Sethunga and Pathmalal 2010) and recently some researchers have highlighted but not confirmed the effect of cyanotoxins on chronic renal disease in the north central province in Sri Lanka. Manage et al. (2009) recorded the hepatotoxic effect of the M. aeruginosa on Wister rats in vitro and another research revealed that the contamination level of MC-LR in some drinking, irrigation and aesthetic water bodies in Sri Lanka ranged between 10- 20 µgL-1 (Sethunge and Manage 2010). These values were much higher than the WHO recommended values of 1.0 µgL-1 for MC-LR in drinking and recreational water quality standards (WHO 1998).

CELLULAR UPTAKE OF MICROCYSTINS

As a result of the complex molecular structure and amino acid composition, MCs are somewhat hydrophilic and have large molecular weights (~1kDa; Carmichael et al. 1988). Hence, MCs are incapable in passing by passive diffusion through the cell membranes, and MCs are transported by active transport through specific transporters (Fischer et al. 2010). Thus, MC uptake is mediated by various organic anion transporting polypeptides responsible for the sodium-independent uptake of large amphipathic endogenous and exogenous organic anions into cells and across the blood-brain barrier (e.g: rodent Oatps / human OATPs). (Fischer et al. 2005; Menezes et al. 2013). Fischer et al. (2005) identified the organic anion transporting polypeptides OATP1b2, OATP1A2, OATP1B1, and OATP1B3 as MC-LR transporters in the rat and man. The tissue distribution of the identified MC-LR transporting OATPs explains the preferential organ toxicity of MCs in liver and brain. OATP mediated and MC congener dependent transport is responsible for MC induced neuronal toxicity (Feurstein et al. 2009). Although MCs are reputed as liver disruptors, damage may also occur in other vital organs such as the intestines, kidneys, thymus of quail and male reproductive organs (Towner et al. 2002; Zeller et al. 2011; Zhang et al. 2011). MCs are mainly excreted by the hepatocytes, but up to 9% can be eliminated by urine (Bischoff 2001) which makes the kidneys a potential target organ for MC-LR (Menezes et al. 2013). Furthermore, OATP-A, one of the known MC active transporters has been identified at mRNA level in the human kidney (Hagenbuch and Meier 2003). Thus the kidney might also be an important target organ for MCs (Dias et al. 2009).

MICROCYSTINS AND CHRONIC RENAL DISEASE IN SRI LANKA

Moreover, Chronic Kidney Disease of uncertain etiology (CKDu) became a burning health problem in some parts of the country, especially in North Central, North Western, Uva and Eastern provinces in Sri Lanka (WHO 2009). Predominantly vulnerable community for this condition is male farming community. Several hypotheses have been made to explain the causal associations between the high prevalence of the disease in the region and existing environmental factors (Chandrajith et al. 2010; Jayasumana et al. 2013; WHO 2009).

CKD is typically associated with diabetes, hypertension, infections and environmental nephrotoxins (Eckardt et al., 2009). Exact aetiology of the CKDY is still unknown though there were recognized causes of CKD worldwide including diabetes mellitus, hypertension, glomerulonephritis, obstructive uropathy and congenital diseases such as polycystic kidney disease (Wanigasuriya 2012). The report for CKD in Sri Lanka published by WHO suggested many hypothesis for the cause of the disease and cyanobacterial toxins is one of the hypothesis for CKD prevalence in Sri Lanka. Cases of CKD in Sri Lanka with unknown aetiology (CKD) have increased frighteningly over the recent decades leading to national concern and urgent need for action. This is highlighted by hospital admissions for CKDu in the Anuradhapura General Hospital, the main hospital in the North Central province which increased by 27% between 1992 and 2006 (Chandrajith et al. 2010). Currently more than 8000 patients are receiving treatment for CKDu. Progression of CKDu is generally symptomless until the advanced stages of disease where the kidneys are damaged irreversibly resulting in mortality unless intervened with dialysis and/ or transplantation. Since last decade there has been extensive research funded by major research bodies in Sri Lanka and the World Health Organization (WHO), to determine prevalence, risk factors, treatment strategies and socioeconomic impact of CKDu (Wanigasuriya 2012).

Given that, the risk factors responsible for the occurrence of CKDu in Sri Lanka have not been clearly identified, further investigations are a priority. Furthermore, in 2013 WHO report highlighted that microcystins contaminated water is among the major hypothesis for the CKDu in the affected areas (WHO 2013). Thus, the role of microcystins, some of the most potent natural toxins, in CKDu has been considered but not investigated properly. Thus, studies are essential if public health is to be safeguarded since providing safe drinking water is one of the most critical factors to guarantee long-term population health. This is more critical in developing countries like Sri Lanka as majority of water bodies which are used as source water for drinking is contaminated with cyanobacteria and microcystins. Thus, studies on molecular identification of toxin producing cyanobacteria to develop water treatment solutions has become an important timely research area to provide novel solutions to problems of current national importance.

Recent study, HEK-293 and ACHN cells were exposed for 24 h to pure MC-LR (1.0–200 μM) and the cytotoxic effects were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Sulphorhodamine B (SRB) cell viability assays. MC-LR induced apoptotic effects were evaluated by assessing morphological changers using phase contrast and fluorescence microscopy, estimating expression of selected apoptotic related genes (P53, Bax, and Survivin) by real time PCR analysis and estimating level of caspase 3 and caspase

9 expressions. MC-LR containing cyanotoxin extract was prepared from bloom sample collected from Beira Lake by 100% methanol and dried under reduced pressure. HPLC-DAD analysis was performed to quantify the MC-LR. HEK-293 and ACHN cells were exposed for 24 h to different concentrations of cyanobacterial crude extract (1.0 – 200 µg/mL) and the cytotoxic effects were evaluated by (MTT) and (SRB) cell viability assays. Morphological changes induced by MC-LR containing cyanotoxin extract was evaluated by phase contrast microscopic observations. Cytotoxicity study was carried out to evaluate cytotoxic effects of some selected MCs (MC-LR, MC-RR, MC-LF and MC-LW) and Nodularin (NOD) on HEK-293 and ACHN cells. HEK-293 and ACHN cells were treated with different concentrations of MC-LR, MC-RR, MC-LF, MC-LW and Nodularin (1.0–200 µM) for 24 h and cytotoxicity was evaluated by Sulphorhodamine B (SRB) assay and the respective IC50 values were calculated using statistical tools.

Table1. IC50 values of different types of cyanotoxins for HEK-293 and ACHN cell lines

MCs	IC50 µM	
	HEK-293	ACHN
MC-LR	16.57±0.035	62.36±0.037

MCs	IC50 µM	
	HEK-293	ACHN
MC-RR	85.96±2.296	159.14±1.160
MC-LF	1158.16±9.025	1589.78±3.206
MC-LW	1068.09±6.148	1268.76±6.143
Nodularin	58.96±1.256	98.34±0.978

A significant cytotoxicity was induced in both types of cells by the toxins tested. All the toxins had a significantly higher cytotoxicity on normal kidney cells than on the kidney adenocarcinoma cells. Furthermore, MC-LR had the lowest IC50 values (16.57±0.035 for HEK-293 and 62.36±0.037 for ACHN cells) while MC-LW had the highest IC50 values (1158.16±9.025 for HEK-293 and 1589.78±3.206 for ACHN cells). Therefore, this study demonstrated that cyanotoxins could cause cytotoxic effects on kidney cells. MC-LR was the most toxic while MC-LW was least toxic cyanotoxin on both cell types tested. MC-RR, MC-LF and Nodularin had moderate cytotoxicity on human renal cells. According to the results, MC-LR was selected to carry out further cell culture studies due to its higher toxicity and it was the most common cyanotoxin reported in natural water bodies with toxin producing cyanobacteria.

CYTOTOXICITY ASSESSMENT OF MC-LR

The cytotoxic effect of MC-LR on HEK-293 and ACHN cells were assessed by SRB and MTT assays. Figures 1 (A) and (B) show the relative cell survival at each concentration of MC-LR compared to control treatment assessed by SRB and MTT assays respectively. Exposure to MC-LR caused a significant ($p<0.001$, One-Way ANOVA) dose dependent cytotoxicity in both cell lines. When IC50 values were compared using the results of the SRB assay, a comparatively lower IC50 value was observed in HEK-293 (16.32 µM [16.24 µg/mL]) than in ACHN cells (90.85 µM [90.41 µg/mL]; $p<0.01$, paired t test) MTT assay also showed a significantly ($P < 0.001$) lower IC50 in the HEK-293 cells than in the ACHN cells, but the value for HEK-293 cells was much higher than the value determined by the SRB assay. The IC50 values of MTT assay for HEK 293 cells and ACHN cells were 72.62 µM (72.27 µg/mL) and 97.09 µM (96.62 µg/mL) respectively.

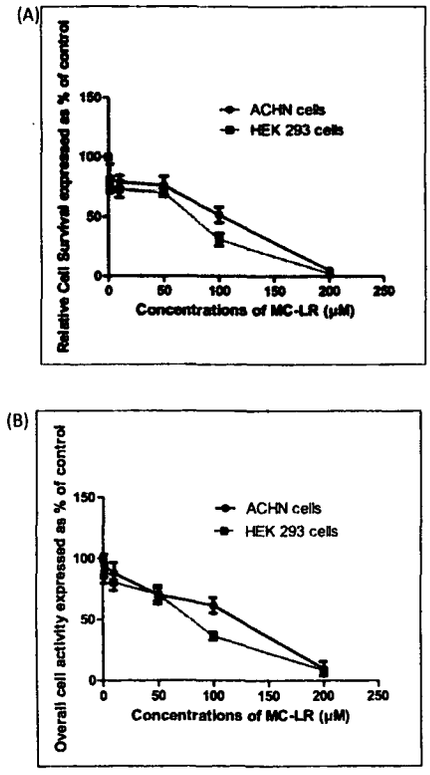

Figure 1. Comparative dose dependent cytotoxicity of MC-LR on HEK-293 (A) and ACHN (B) cell lines

CYTOTOXICITY ASSESSMENT OF CYANOBACTERIAL CRUDE EXTRACT

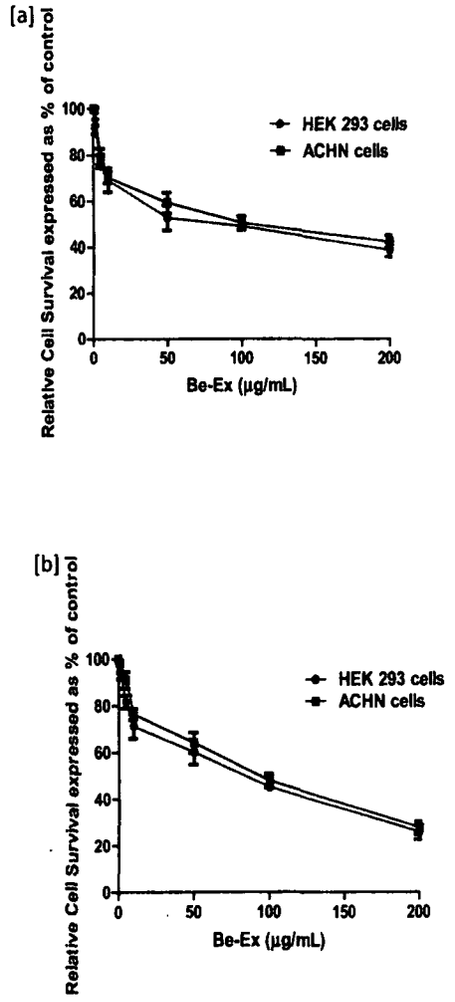

Figure 2 Viability of ACHN and HEK-293 cells exposed to different concentrations of cyanobacterial crude extract for 24 h assessed by SRB assay [a] and MTT assay [b]. Results are expressed as the mean percentage of three replicates relative to control ± standard deviation (*p< 0.001).

In conclusion, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and SRB (Sulphorhodamine B) assays for cell viability revealed a significant decrease in cell viability in both cell lines after treatment with MC-LR at 50 μM for 24 h ($p<0.001$). Moreover, MC-LR treated ACHN and HEK-293 cells exhibited a marked dose dependent loss of confluence as judged by phase contrast microscopy. Similarly, fluorescence microscopic observations following Acridine Orange-Ethidium Bromide (AO/EB) staining confirmed that both cell types were undergoing apoptosis after treatment with MC-LR for 24 h. Present study provides direct evidence that MC-LR exposure can induce apoptosis related morphological changes, up regulation of the expression of Bax and p53 genes modulate expression of Survivin gene and increase activity of caspase 3 and 9 in Human embryonic Kidney (HEK-293) and Human kidney adenocarcinoma (ACHN) cells. Hence, Bax, p53, Survivin, caspase 3 and 9 are most likely to be involved in MC-LR induced cellular damage. Moreover, this study contributes to elucidate the toxicological mechanism underlying the effects of MC-LR on Human kidneys.

Professor Pathmalal M Manage
Centre for Water Quality and Algae Research,
Department of Zoology,
University of Sri Jayewardenepura, Sri Lanka

Full papers published in any index journal