Bull. Environ. Contam. Toxicol. (2004) 72:680–685

Bulletin of Environmental Contamination and Toxicology

Toxicity Assessment of Indian Marine Cyanobacterial Strains

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Received: 17 October 2002/Accepted: 9 January 2004

Cyanobacteria can undergo rapid growth under certain environmental conditions to form blooms in marine, brackish and freshwater bodies (Carmichael 1992; Hitzfeld et al. 2000, Chorus et al. 2000). The toxins produced by freshwater cyanobacteria can be largely divided into three classes according to their effects: hepatotoxins, neurotoxins and lipopolysaccharides many of which have been implicated in gastrointestinal problems and skin irritations (Carmichael 1992; Bell and Codd 1994). Exposure to cyanobacterial toxins can occur by different routes including dermal, inhalation, oral and intravenous. The bioaccumulation of cyanotoxins by aquatic animals including fish, molluscs and zooplankton has been reported (Amorin and Vasconcelos, 1999; Magalhaes et al. 2001). These cyanotoxins can induce serious illnesses in animals and humans, including hepatic, kidney, gastrointestinal, and allergy reactions and primary liver tumor promotion (Hitzfeld et al. 2000). Most of the toxins and bioactive compounds were reported from freshwater species. Reports on occurrence, isolation and toxicity of marine cyanobacteria are limited. Benthic and planktonic blooms of species of Oscillatoriales resulted in mortalities of farmed prawns, *Penaeus monodon* (Smith 1996). Fish kills through a disturbance of gill functioning due to inhibition of Na^+/K^+-ATPase by cyanobacterial blooms has been reported (Bury et al. 1998). Marine algal toxins are also responsible for human illnesses associated with consumption of sea-foods and in some cases, respiratory exposure to aerosolized toxins (Van Dolah, 2000).

In the present study, we have screened six Indian marine cyanobacterial strains by brine shrimp assay. The strains which were toxic in brine shrimp assay were further evaluated by mammalian cytotoxicity assay. Inhibition of Na^+/K^+- ATPase as a possible cause for the brine shrimp mortality was investigated by studying its inhibition *in vitro*. The *in vivo* lethality of the two strains which were found to be toxic by brine shrimp and cytotoxicity assays were confirmed by mouse bioassay.

MATERIALS AND METHODS

The axenic cultures of cyanobacterial strains used in the present study viz., *Oscillatoria formosa* BDU 40261 and BDU 91041, *Pseudanabaena schemedei* BDU 20761 and BDU 30313, and *Lyngbya sp.* BDU 90901 and BDU 90181, were obtained from the National facility for marine cyanobacteria, Bharathidasan University, Tiruchy, India. Cultures were maintained as static batch cultures in ASN III medium (Rippka et al.1979) under controlled room temperature of 25 ± 2^0C, 1000 lux light intensity and 16 hour photo-period. Cells were grown for 6 weeks, lyophilized and stored at -20^0C until

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used. Cell free extracts containing toxin (referred hereafter as toxin extracts) were prepared in either seawater, phosphate-buffered saline (PBS) or homogenization buffer (0.25 *M* sucrose and 6 m*M* EDTA) (Nidhi et al. 2002). A known amount of lyophilized cells was ultrasonicated and the resultant cell suspension was centrifuged at 30, 000 x *g* for 15 minutes. The supernatant was membrane filtered (0.22 μm) and stored at –20 ° C until required for testing. The African green monkey kidney (Vero) cells were obtained from the National Cell Science Centre, Pune, India. Monolayers of Vero cells grown in 24-well plates were treated with different concentrations of toxin extracts for 24 hr. Viability of the cells was determined by crystal violet dye exclusion assay (Wang et al. 1996). LDH activity in the culture media was measured spectrophotometrically as an index of plasma membrane damage and loss of membrane integrity (Rao et al. 2002). Enzyme activity was expressed as the percentage of extracellular LDH activity of the total LDH activity of the cells. Mitochondrial integrity was measured by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) by the mitochondrial enzyme succinate dehydrogenase (Mosman 1993).

Primary screening of toxicity was carried out by *Artemia salina* (Brine shrimp) bioassay using toxin extracts prepared in sea water (Nidhi et al. 2002). Whole larval homogenate was used as enzyme source and Na^+/K^+ -ATPase activity was determined by the modified method of Holliday (1985). Briefly, larvae were separated form seawater and rinsed in cold homogenizing buffer (0.25 *M* sucrose and 6 m*M* EDTA) and homogenized in a hand operated glass homogenizer for 30 strokes in ice. The homogenate was passed through a sieve of 100 mesh (Sigmaware™, USA) and used immediately as crude enzyme. Enzyme extracts were treated with various concentrations of toxins prepared in homogenizing buffer and incubated on ice for 60 min before the assay. Na^+/K^+ -ATPase activity was assayed as phosphate liberated from adenosine triphosphate (ATP) by crude homogenate. Enzyme activity was measured as the difference between phosphate liberated in an assay medium with potassium present at optimal concentration and a medium without potassium and with 1 m*M* ouabain, a specific inhibitor of the enzyme. For each assay 67 μl aliquots of fresh crude homogenate was added to 200 μl of assay medium containing either 167 m*M* NaCl, 50 m*M* KCl, 33 m*M* imidazole-HCl at pH 7.2 or 217 m*M* NaCl, 1.67 m*M* ouabain, 33 m*M* imidazole-HCl at pH 7.2. After preincubation for 10 min in a water bath at 30^0C, the assay was started by adding 67 μl of a solution containing 25 m*M* ATP and 50 m*M* $MgCl_2$ (pH 7.2, adjusted with crystalline imidazole-HCl). The assay was terminated after 15 min by addition of 1.5 ml ice-cold Bonting's reagent containing 560 m*M* H_2SO_4, 8.1 m*M* ammonium molybdate and 176 m*M* $FeSO_4$. Phosphate concentrations in the reaction mixtures were measured spectrophotometrically as the reduced phosphomolybdate complex. Controls received only homogenizing buffer. Protein concentration was determined by Lowry's method (Lowry et al. 1951). Toxin extracts prepared in PBS were used for determining LD_{50} in male mice by intraperitoneal route (Dixon 1965). All assays were conducted in triplicate and results are mean±SE of three experiments. For LC_{50} value calculation, percent brine shrimp larval mortalities were converted to probits and plotted against log dose of concentration. The data were analyzed by Student's *t*-test and the level of significance was set at $p \leq 0.05$.

RESULTS AND DISCUSSION

In the present study we evaluated the laboratory cultures of six marine cyanobacteria isolated from Indian marine water bodies. As a primary screening test for toxicity,

Artemia salina bioassay was used. Out of six strains only two *O. formosa* strains BDU 40261 and 91041 induced larval mortality (Fig. 1A). The LC_{50} concentrations of BDU 40261 and 91041were 114.2 and 144.6 μg/ml respectively. In comparison, none of the other species showed any toxicity even at 4 mg/ml. Concentrations beyond 4 mg/ml lead to turbidity after 12 hr and high mortality rates were observed in control wells. Such high mortality may be due to oxygen insufficiency and not due to toxicity of the strains *per se*. Larval mortality was 100% at 500 μg/ml in both the toxins. In order to ascertain the cause of larval mortality, Na^+/K^+-ATPase activity of whole body homogenate was determined (Fig. 1B, C). The toxins at higher concentration (10, 25, 50 μg) significantly reduced the enzyme activity in BDU 40261and 91041. The death of larvae can be attributed to disturbance in gill ion concentration, which is very essential to maintain homeostasis.

Based on brine shrimp assay *O. formosa* BDU 40261 and 91041 were further evaluated for in vitro cytotoxicity in African green monkey kidney (Vero) cells. Kidney cells derived from monkeys have been used to study the toxic effects of natural toxins (Annalaura and Zucco 1995). Viability of the toxin-treated monolayer was determined by the ability of the cells to take up vital dye crystal violet. Toxin extracts induced concentration dependent cytotoxicity and was characterized by loss of viability (Fig. 1D), decrease in MTT reduction (Fig. 1E) and increased LDH leakage (Fig. 1F). In contrast to the crystal violet assay and MTT test, LDH leakage was more in BDU 91041 treated cells than with BDU 40261. Light microscopy of control cells showed regular, round and oval shaped cells with compact monolayer with very little intercellular spaces (Fig. 2A). Each cell had a prominent central nucleus but few occasional cells were binucleated. Most of the cells maintained normal morphology up to 6-8 hours even at highest concentration tested. At 12-16 hr there was an increase in number of detached cells in both BDU 40261 and 91041 treated wells at all the concentrations (Fig. 2 B, C). At 24 hr post-treatment areas of cell loss were evident in monolayer (Fig. 2D). Cells adjacent to areas of cell loss became spindle shaped with fine plasma membrane extensions. At higher toxin extract concentrations there was increase in number of detached cells and wide gaps in monolayer.

On the basis of the results on in vitro toxicity, acute (24 hr) LD_{50} of *O. formosa* BDU 40261 and 91041 were determined in male mice. The LD_{50} values of BDU 40261 and 91041 were 180.1 and 174.9 mg/kg body weight (fiducial limits 151.7-213.5 and 150.4-203.6 respectively). The animals were lethargic, and quiet without any visible discomfort and did not show typical symptoms of two common cyanobacterial toxins viz., microcystin or anatoxin-a poisoning. No lethality or sub-lethal symptoms were observed in other strains even up to 2 g/kg body weight concentration corroborating the results of brine shrimp and in vitro cytotoxicity assays.

In vivo toxicity by mouse bioassay was carried out to corroborate the in vitro results. The LD_{50} of the two strains varied from 175-180 mg/kg body weight. The interesting observation in the study was lack of dose dependent increase in toxicity. Even at 2 to 3 LD_{50} concentration, there was no decrease in time to death or enhanced toxicity symptoms in animals. In conclusion, results of our study indicate the occurrence of toxic strains of cyanobacteria in Indian marine water bodies and the reports on bioaccumulation of cyanotoxins by aquatic organisms and zooplankton and possible subsequent human toxicity through food chain underlines the need for identifying potentially toxic strains and their distribution. The results of our study also indicate the

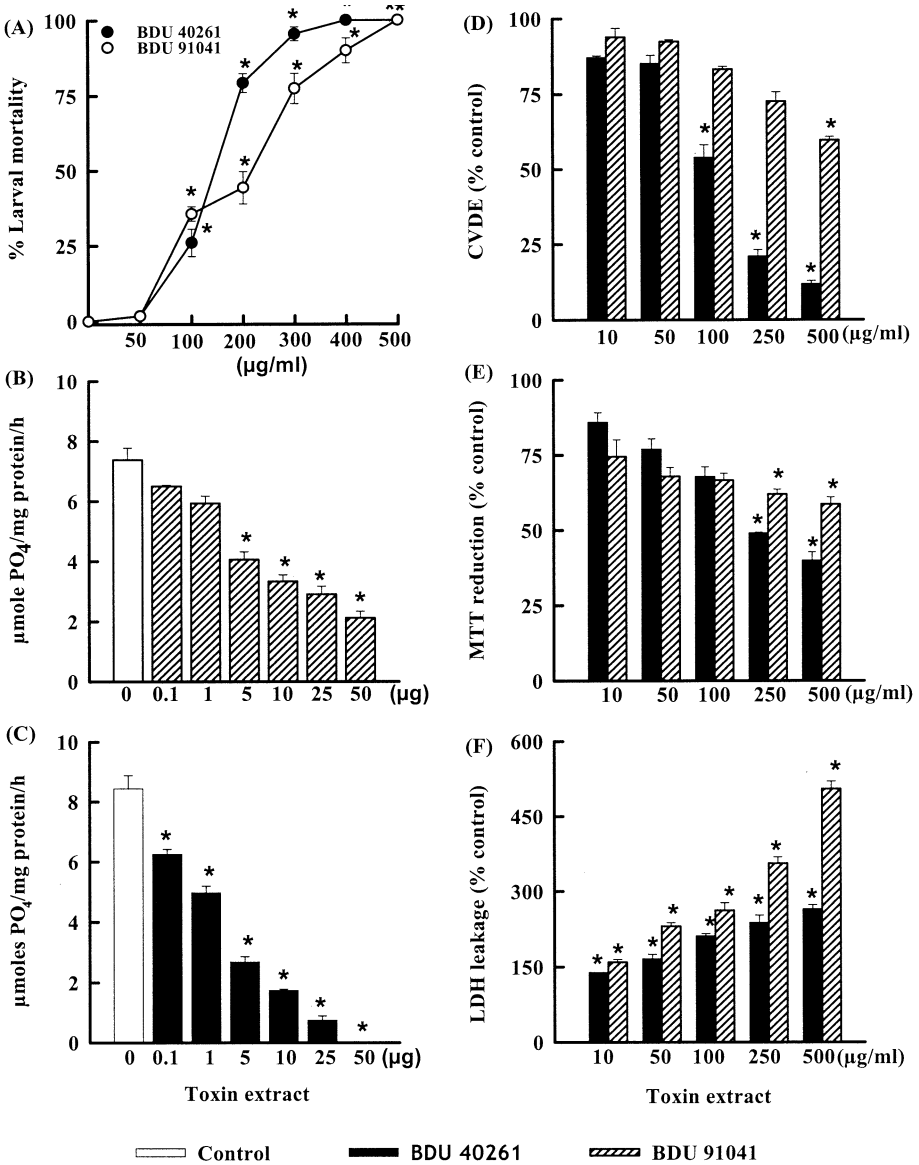

Figure 1. Effect of *Oscillatoria formosa* BDU 40261 and 91041 toxin extracts on *Artemia salina* larval mortality (A); Na^+/K^+ – ATPase activity of whole larval homogenates (B, C); viability of Vero cells by crystal violet dye exclusion assay (CVDE) (D); reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) by the mitochondrial enzyme succinate dehydrogenase (E); and lactate dehydrogenase (LDH) leakage (F). Each value represents mean ± SE of three replicates. * Significantly different from control at $p \leq 0.05$ by Student-*t* test.

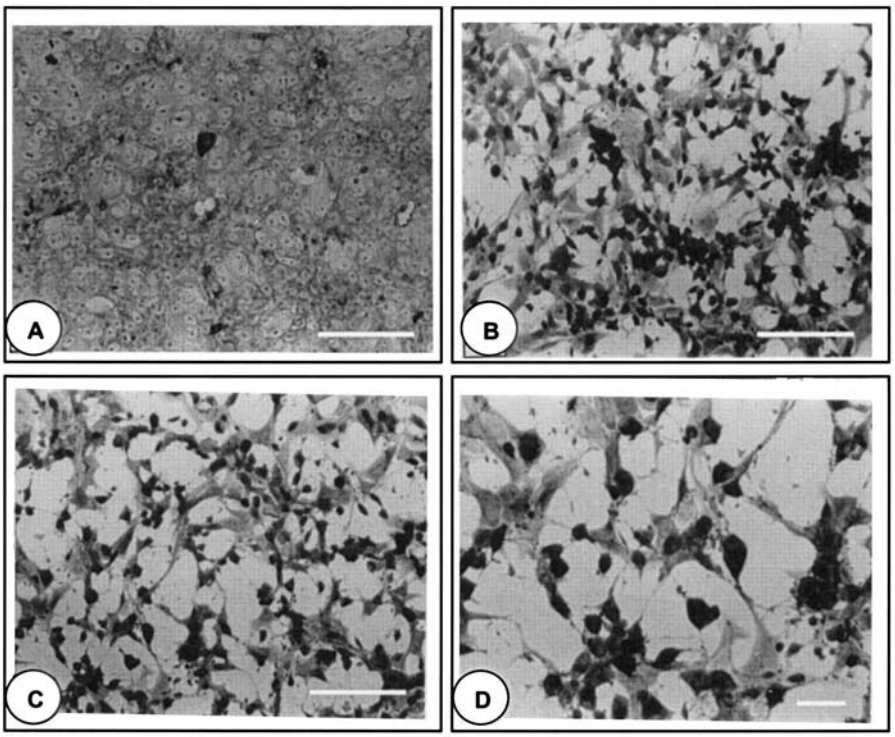

Figure 2. Cytotoxic effect of *Ocillatoria formosa* BDU 40261 and BDU 91041 toxin extracts in Vero cells. (A) Control cells with compact monolayer ; (B) Effect of 100 μg/ml BDU 40261 after 12 hr ; (C) Effect of 100 μg/ml BDU 91041 after 12 hr ; and (D) Effect of 100 μg/ml BDU 91041 after 24 hr. Note the wide gaps in monolayer at 24 hr post-treatment due to loss of cells. Bar equals 5 μm.

utility of brine shrimp assay together with mammalian cytotoxicity assay as a rapid and reliable primary toxicity screening system. Further studies are in progress to characterize the chemical nature of the toxin present in the strains and their mechanism of *in vivo* toxicity.

Acknowledgments.

We thank Mr. K. Sekhar, Director, DRDE and Dr. R. Vijayaraghavan, Head, Division of Pharmacology and Toxicology for support and encouragement. We are thankful to Dr. M.M. Parida, for providing Vero cells. Nidhi Gupta is thankful to Defence Research and Development Organization for the award of Senior Research Fellowship.

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