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# Biotoxic Cyanobacterial Metabolites Exhibiting Pesticidal and Mosquito Larvicidal Activities

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**Abstract** A freshwater bloom-forming cyanobacterium, *Microcystis aeruginosa*, and local soil isolate *Scytonema* sp. strain BT 23 were demonstrated to contain biotoxic secondary metabolites with pesticidal and mosquito larvicidal activities. A purified toxic constituent from *M. aeruginosa* showed an absorption maximum at 230 nm and its toxicity symptoms, Rf value on TLC, and retention time observed in an HPLC analysis were similar to those of the hepatotoxic heptapeptide microcystin-LR. The bioactive constituent of the *Scytonema* sp. was less polar in nature and exhibited two peaks at 240 and 285 nm. When applied to two cruciferous pests, *Pieris brassicae* and *Plutella xylostella,* the crude extracts and toxic principles from the two cyanobacteria showed significant antifeedant activity in a no-choice bioassay, and at higher concentrations exhibited contact toxicity to the insect larvae. The purified toxin from *M. aeruginosa* was found to be more effective and produced 97.5 and 92.8% larval mortality in the two pests, following 2 h of toxin treatment at a concentration of 25 μg per leaf disc (2.5 cm dia.). Meanwhile, similar treatment with the purified toxin from *Scytonema* sp. strain BT 23 only produced 73 and 78% mortality in the two pests. The cyanobacterial constituents also showed significant activity against *Culex* and *Anopheles* larvae. The *M. aeruginosa* toxin (20 μg $ml^{-1}$) caused 98.2 and 88.1% mortality in the *Culex* and *Anopheles* larvae, respectively, while the purified toxin from the *Scytonema* sp. was less toxic and only produced a 96.3 and 91.2% mortality, respectively, at a much higher concentration (40 μg $ml^{-1}$). Accordingly, the current results point to certain hitherto unknown biological properties of cyanobacterial biotoxins.

**Key words:** Cyanotoxins, microcystin, cyanobacteria, *Microcystis aeruginosa*, *Scytonema*, biopesticide

Cyanobacteria (blue-green algae) are known to produce a variety of secondary metabolites, including antialgal, antifungal, antiviral, and cytotoxic compounds [10, 16, 19, 20, 21]. Of particular interest are the biotoxic secondary metabolites produced by many bloom-forming species [5, 18, 28, 32]. Many cyanobacterial secondary metabolites with biotoxic and cytotoxic properties have already been isolated, purified, and characterized, and their activities against various organisms have been established [4, 6, 32]. Besides being toxic to animals, cyanotoxins also pose a threat to human health. The bioaccumulation of microcystins in aquatic vertebrates and invertebrates, and adherence of *M. aeruginosa* cells and microcystins to lettuce (*Lactuca sativa*) leaves upon irrigation with water containing cyanobacteria, increase the risk of human exposure to these deadly toxins [3, 7, 8].

Among the biotoxins recorded from cyanobacteria, hepatotoxic microcystins are the most ubiquitous compounds produced by *Microcystis aeruginosa*. Other species of *Microcystis* and strains of *Anabaena*, *Oscillatoria*, and *Nostoc* [4, 12, 24] also produce microcystins. Microcystin-LR is the most dominant and lethal hepatotoxin, although more than 60 chemical variants of microcystins have been reported [6], showing minor structural variations and varying degrees of hepatotoxicity [12, 23]. Several aspects of cyanobacterial toxins, such as their chemical nature, geographical distribution, lethal effects, and mode of action have already been investigated [4, 6]. However, there is relatively little information regarding the pesticidal or mosquito larvicidal effects of these secondary metabolites, even though Carmichael [5] has suggested that due to their novel chemical structures, cyanotoxins could, in theory, form the basis for the development of new pesticides.

Some of the early reports have indicated the absence of mosquito larvae in water bodies containing cyanobacterial blooms [27], yet the reason behind this is not very clear. Kiviranta *et al.* [17] speculated that most of cyanobacteria containing endotoxins may be poisonous to mosquito larvae. Aquatic cyanobacteria are also known to produce compounds that deter feeding by zooplankton [9, 11]. The survival and feeding of copepods and certain species of

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*Daphnia* have also been found to be adversely affected by hepatotoxins [3, 9].

The high degree of solubility of cyanobacterial toxins in water due to their polar molecules points to the biodegradable nature of these natural products [5]. However, the limited information available on the insecticidal/larvicidal effects of cyanotoxins [17, 22, 26] prompted the current authors to screen toxic cyanobacterial strains for their pesticidal properties. Accordingly, the present paper reports on the antifeedant and pesticidal effects of cyanotoxins from the bloom-forming cyanobacterium *M. aeruginosa* and a local rice field isolate, *Scytonema* sp. strain BT 23, against two serious pests, the cabbage white butterfly *Pieris brassicae* (L.) and diamondback moth *Plutella xylostella*, related to cruciferous vegetable crops. In addition, the activity of the cyanotoxins against *Culex* and *Anopheles* larvae is also reported.

## MATERIALS AND METHODS

### Organisms and Growth Conditions

The freshwater bloom-forming cyanobacterium *M. aeruginosa* was collected from the Lakshmikund pond situated in the central part of Varanasi city (India). Its axenic unialgal culture was maintained and routinely grown in a modified Jaworski medium [31]. The *Scytonema* sp. strain BT 23 was isolated from a soil sample from the Agricultural Farm at Banaras Hindu University and grown in a BG-11 nitrogen-free medium [25]. Cultures of both species were grown in a culture room at 25±1°C illuminated with cool white fluorescent tubes from a distance of 50 cm to achieve a 14.4 W $m^{-2}$ light intensity.

### Preparation of Crude Extract

The crude aqueous extract was prepared by sonicating 2 g of lyophilized cells of exponentially growing *M. aeruginosa* or *Scytonema* sp. strain BT 23 in double distilled water in a Branson Sonifier 450 (Branson Ultrasonics Corp., Danburry, CT, U.S.A.) for 5 min at the maximum output and duty cycles. After centrifugation of the extract at 10,000 ×g for 5 min, the resulting pellet was re-extracted if required. The supernatants were then mixed and evaporated to dryness in vacuum. The dried material was dissolved in double distilled water to obtain the desired concentration of the crude aqueous extract (toxin).

### Isolation and Purification of Toxin

The extraction and purification of the toxic principles from *M. aeruginosa* were performed according to the method of Harada *et al.* [13]. Lyophilized cells (4 g) were extracted three times in 5% acetic acid. After centrifugation (10,000 ×g for 10 min), the supernatant was evaporated to dryness in vacuo. The dried material was then loaded on a glass column (20×1.0 cm diameter) packed with silica gel (100–200 mesh size, E. Merck, Germany) and eluted with chloroform:methanol:water (65:35:10, v/v). The various fractions obtained were analyzed for their spectroscopic characteristics and in a mice bioassay test, and those fractions with the identical results were mixed together. For further purification, the cleaned-up toxic fraction was subjected to thin layer chromatography (20×20 cm TLC plates, coated with silica-gel 60 G) using chloroform:methanol: water (65:35:10; v/v; lower layer) as the mobile phase. The spots were visualized under short-wavelength UV radiation, then individual spots were scraped from the TLC plates and tested for toxicity based on a mice bioassay. A routine preparative TLC was employed to enrich the putative cyanotoxin. The absorption spectrum of the purified toxin was recorded on an ATI Unicam UV/Vis spectrophotometer (Unicam Ltd., Cambridge, U.K.). The purity and identity of the toxic constituent was checked by co-chromatography using TLC and HPLC based on standard microcystin-LR (obtained as a gift from Prof. W. W. Carmichael, Wright State Univ., U.S.A.). The active fractions exhibiting toxicity to mice were solubilized in 1% methanolic water, and subjected to bioassays against pests and mosquito larvae.

To isolate the toxin from *Scytonema* sp. strain BT 23, lyophilized cells (3.5 g) were extracted three times in methanol:water (1:1, v/v) by sonicating in an ice-bath for 15 min and then centrifuging the suspension (10,000 ×g for 10 min). The supernatant was concentrated under a vacuum to a half of its total volume. Thereafter, the concentrated suspension was partitioned with an equal volume of chloroform to obtain an aqueous methanol fraction and chloroform fraction. Both fractions were then tested for their toxicity against mice. Further purification and enrichment of the toxin present in the chloroform fraction was performed by TLC using hexane:acetone (9:1; v/v) as the mobile phase. The active toxic constituent was finally dissolved in 1% methanolic water using Tween 20 (0.01%) and subjected to an insect bioassay.

### Toxicity Test

Known concentrations of the cell-free cyanobacterial extracts or purified toxic constituents dissolved in normal saline (0.9% NaCl) or 1% methanolic water were injected intraperitoneally (i.p.) into male mice (Park's Albino strain weighing 20±2 g) to evaluate the toxicity. The control mice were only injected with normal saline. Four different dose levels were tested using 3 mice per dose level. The mice injected with the toxins were closely observed over a period of 7 h. The $LD_{50}$ value was taken as the midway concentration between the doses at which 100% of the test mice survived and 100% died.

### Insect Bioassay

Late-second or early-third instar larvae of *Pieris brassicae* and *Plutella xylostella* were used in the bioassays. The

larvae were starved for 24 h prior to the toxin treatment to equalize their physiological conditions. To evaluate the contact toxicity, fresh cabbage (*Brassica oleracea*) leaf discs (2.5 cm dia.) previously coated with different concentrations of the crude extract and purified constituents in 1% aqueous methanol were kept in petri dishes of 2.5 cm in diameter. After the complete evaporation of the solvent, 10 larvae were placed on each petri dish containing leaf discs. The control leaf discs were only treated with the corresponding solvent. After 2 h, the larvae were transferred to another set of petri dishes containing fresh untreated leaves. The mortality was recorded after 48 h.

The feeding inhibition bioassay was performed using the no-choice leaf-disc method, as described by Alkofahi *et al.* [2], and calculated according to Hassanali and Lwande [14]. The mosquito larvicidal activity was tested against second instar larvae of *Culex* and *Anopheles* sp., as described by Slimestad *et al.* [29]. Corrections for mortality due to natural causes were made by subtracting the percentage of dead larvae in the untreated control, maintained separately for each treatment, from the corresponding value in the treated series. The mean corrected mortality was then calculated by Abbott's formula [1]. Each experiment was performed in triplicate and subjected to statistical analysis.

## RESULTS

The toxicity assessment of the crude extracts of *M. aeruginosa* and *Scytonema* sp. strain BT 23 based on a mice bioassay revealed a toxic nature for both organisms. Crude extracts of the two organisms killed the test mice within one hour of toxin administration (i.p.) with $LD_{50}$ value of 60 and 56 mg per kg body weight. The separation of the cleaned-up fraction of *M. aeruginosa* obtained from column chromatography on a TLC plate resulted in three spots (MT-1 to MT-3), all exhibiting fluorescence under UV radiation (Table 1). Among the spots, only MT-1 (R*f* 0.54) was shown to be toxic. Upon rechromatography of the pooled fraction of MT-1, a single spot appeared on the TLC plate. Co-chromatography on the TLC plate using standard microcystin-LR revealed a close mobility between the toxic

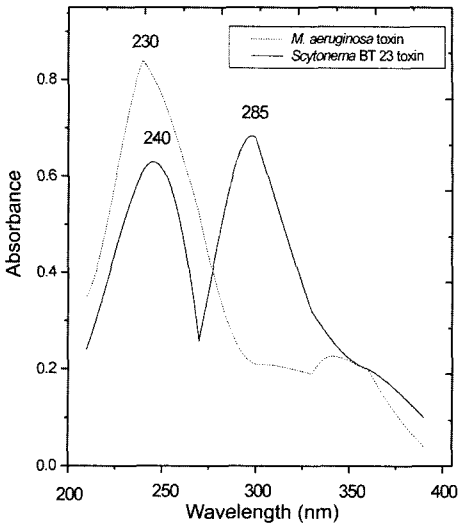


**Fig. 1.** Absorption spectra of purified toxins from *Microcystis aeruginosa* and *Scytonema* sp. strain BT 23.

constituent of spot MT-1 (λ max 230 nm, methanol) (Fig. 1) and standard microcystin-LR. The identity of the toxic constituent of spot MT-1 was further confirmed by an HPLC analysis, since the retention times (tr) for the standard microcystin-LR and toxic constituent of spot MT-1 (R*f* 0.54) were both identical (11.4 min) under the HPLC analyses.

Among the six UV fluorescent spots (ST-1 to ST-6) obtained after TLC separation of the chloroform fraction of *Scytonema* sp. strain BT 23, only spot ST-6 (R*f* 0.84) exhibited toxicity to mice (Table 1). The spot was enriched through preparative TLC followed by rechromatography, which yielded a single spot at R*f* 0.84. The compound showed two absorption peaks at 240 and 285 nm in methanol (Fig. 1), and it was soluble in less polar solvents (e.g. diethylether, ethyl acetate, and ethanol) but insoluble in water, indicating that the compound might be weakly polar. However, due to the unavailability of an authentic standard, the identity of the toxic constituents present in the *Scytonema* cells could not be confirmed. Purification of the extract by preparative TLC resulted in an appreciable recovery of 0.14 and 0.2% (dry wt. basis of the purified constituents) from *M. aeruginosa* and *Scytonema* sp. strain BT 23, respectively.

**Table 1.** Separation of toxic constituents from *Microcystis aeruginosa* and *Scytonema* sp. strain BT 23.

| Parameters | Organisms | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *M. aeruginosa* | | | *Scytonema* sp. strain BT 23 | | | | | |
| Spots on TLC[a] | MT-1 | MT-2 | MT-3 | ST-1 | ST-2 | ST-3 | ST-4 | ST-5 | ST-6 |
| Rf[b] value | 0.54 | 0.66 | 0.93 | 0.22 | 0.31 | 0.52 | 0.63 | 0.74 | 0.84 |
| Absorption maxima (nm, methanol) | 230 | 204 | 220 | 210 | 210, 230 | 220 | 265 | 214 | 240, 285 |
| $LD_{50}$ to mice (μg kg body $wt^{-1}$) | 100 | -- | -- | -- | -- | -- | -- | -- | 100 |

[a]Mobile phase - chloroform:methanol:water (v/v, lower phase) for *M. aeruginosa*; haxane:acetone (v/v) for *Scytonema* sp. strain BT 23.
[b]Detection under low-wavelength UV radiation.

**Table 2.** Antifeedant activity of crude and purified toxins of *M. aeruginosa* and *Scytonema* sp. strain BT 23.

| Source of toxin | Mean feeding inhibition (%) after 48 h[a] | | | | | |
|---|---|---|---|---|---|---|
| | Concentration (µg per leaf disc) | Crude extract | | Concentration (µg per leaf disc) | Purified toxin | |
| | | *P. brassicae* | *P. xylostella* | | *P. brassicae* | *P. xylostella* |
| *M. aeruginosa* | | | | | | |
| | 100 | 31.3±3.4 | 26.8±2.1 | 2.5 | 19.6±1.7 | 17.3±2.4 |
| | 250 | 63.3±3.6 | 49.7±2.4 | 5.0 | 45.6±3.1 | 43.1±2.1 |
| | 500 | 83.0±6.2 | 64.0±3.1 | 10.0 | 84.0±4.2 | 64.6±2.3 |
| *Scytonema* sp. strain BT 23 | 100 | 34.3±3.2 | 27.0±2.4 | 2.5 | 33.3±3.1 | 29.3±2.3 |
| | 250 | 54.6±4.2 | 49.0±2.4 | 5.0 | 59.0±2.1 | 46.3±3.1 |
| | 500 | 93.0±5.2 | 74.0±2.1 | 10.0 | 94.3±3.6 | 78.6±2.1 |
| $SE_{(d)}$[b] | | 1.66 | 1.32 | | 1.96 | 1.67 |
| CD at 5%[c] | | 3.92 | 3.12 | | 4.63 | 3.94 |

[a]Mean feeding inhibition (%)=(1 –T/C)×100; where T and C represent feeding on toxin treated and untreated (control) leaf discs, respectively.
[b]$SE_{(d)}$=Standard error of difference of means.
[c]CD=critical difference.

The toxicity of the compounds purified in the form of TLC spots MT-1 and ST-6 was evident from the fact that they killed mice within 40 min of administration, with an $LD_{50}$ of 100 µg per kg body weight. The toxin-treated mice also showed clinical signs of hepatotoxicoses, suggesting the hepatotoxic nature of the compounds. The clinical symptoms observed in the mice treated with the *Scytonema* sp. strain BT 23 toxin were similar to those observed with microcystin-LR, even though its spectral and solubility characteristics differ markedly. The current results did not exhibit any similarity with microcystins nor scytonemins, nonetheless, the compounds were extremely hepatotoxic.

Both cyanobacterial strains were bioassayed for their feeding inhibition and pesticidal activities against two pests, *P. brassicae* and *P. xylostella*. The cell extracts and purified toxins of both cyanobacteria significantly inhibited the feeding activity of the insect larvae. A negative correlation between increased doses of the toxins and the feeding activity of the larvae was observed (Table 2). The crude extract of *M. aeruginosa* at a concentration of 500 µg per leaf disc caused an 83 and 64% feeding inhibition in *P. brassicae* and *P. xylostella*, respectively, whereas the purified toxin elicited similar feeding inhibition values at a fairly low concentration (10 µg per leaf disc) in the two pests. Interestingly, with the *Scytonema* sp. strain BT 23 extract, the feeding inhibition was higher, reaching to 93 and 74% at 500 µg per leaf disc, however, the purified toxin (10 µg per leaf disc) showed 94.3 and 78.6% feeding inhibition, respectively, against the two pests (Table 2).

A 2 h exposure to higher concentrations of the toxins proved lethal to the larvae. The *M. aeruginosa* extract (2.5 mg per leaf disc) caused 92–96% mortality of both pests after 48 h (Fig. 2), while the purified toxin from *M. aeruginosa* produced 97.5 and 92.8% mortality in the *P. brassicae* and *P. xylostella* larvae, respectively, at a concentration as low

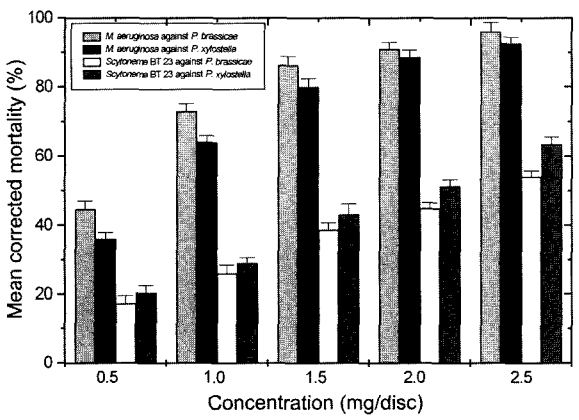


**Fig. 2.** Effect of crude extracts of *Microcystis aeruginosa* and *Scytonema* sp. strain BT 23 on *P. brassicae* and *P. xylostella*.

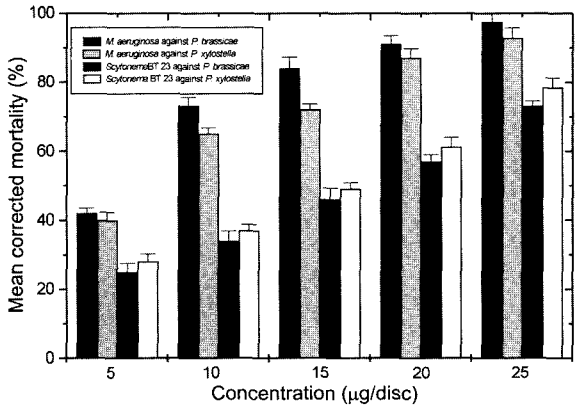


**Fig. 3.** Effect of purified toxic constituents of *Microcystis aeruginosa* and *Scytonema* sp. strain BT 23 on *P. brassicae* and *P. xylostella*.

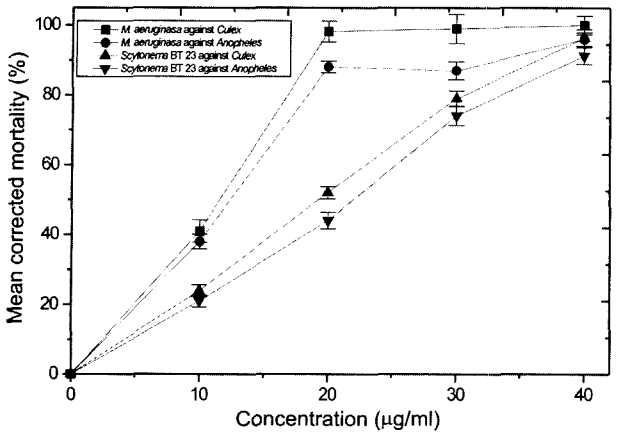


**Fig. 4.** Mosquito larvicidal effect of purified toxic constituents of *Microcystis aeruginosa* and *Scytonema* sp. strain BT 23 on *Culex* and *Anopheles*. Each point represents mean±SD.

as 25 µg per leaf disc (Fig. 3). In contrast, the *Scytonema* sp. strain BT 23 extract only produced 54 and 63% larval mortality, respectively, at 2.5 mg per leaf disc (Fig. 2), while the purified toxin of the strain resulted in a higher mortality than the crude extract at 73 and 78%, respectively, at 25 µg per leaf disc (Fig. 3).

Since the toxins of both cyanobacteria severely affected the mortality of the two pests, the existence of mosquito larvicidal activity was investigated. Interestingly, when tested against *Culex* and *Anopheles* larvae, the toxins from the two cyanobacteria manifested significant larvicidal activity after 24 h of treatment (Fig. 4). In fact, many of the larvae suffered loss of moulting or pupation in the presence of the crude extracts or purified toxins. The crude extract from *M. aeruginosa* produced 97 and 93% larval mortality in *Culex* and *Anopheles*, respectively, at a concentration of 300 µg $ml^{-1}$, whereas the *Scytonema* extract produced 90 and 87% larval mortality, respectively, at a similar concentration. Likewise, the purified toxin of *M. aeruginosa* elicited 98.2 and 88.1% mortality in the *Culex* and *Anopheles* larvae, respectively, at a concentration of 20 µg $ml^{-1}$. However the toxin of *Scytonema* sp. strain BT 23 was found to be less effective as it only induced 96.3 and 91.2% mortality, respectively, at a much higher concentration (40 µg $ml^{-1}$).

## DISCUSSION

The current findings indicated that both *M. aeruginosa* and *Scytonema* sp. strain BT 23 contain substances that were toxic to mice. The spectral and chromatographic characteristics of the *M. aeruginosa* toxin and clinical signs observed in the mice bioassay suggested that the toxin isolated from *M. aeruginosa* was a microcystin, meanwhile the toxin of *Scytonema* sp. strain BT 23, although chemically distinct, also exhibited clinical symptoms similar to those observed with microcystins. The exact chemical nature of the toxin isolated from *Scytonema* sp. may only be revealed after its detailed chemical characterization. Conceivably, it may be a new class of compound. It is pertinent to mention here that the presence of several variants of hepatotoxic microcystins and other secondary metabolites in a number of cyanobacteria has already been reported [4, 32].

The toxins of both cyanobacteria exhibited antifeedant and pesticidal effects on insect pests. At lower concentrations, both toxins inhibited the feeding of the insect larvae, while at higher concentrations caused larval mortality. The differences in the mortality and feeding deterrence activity between the cyanotoxins may have been due to the differences in their chemical nature. It would appear that the toxins acted as both biopesticides and antifeeding compounds. The current results are comparable to those of Sathiyamoorthy and Shanmugasundaram [26], who reported on the antifeedant and pesticidal activity of a glycine-rich peptide toxin of *Scytonema* MKU 106 against two cotton pests, *Heliothis armigera* and *Stylepta derogata.*

Until now, there have been relatively few reports on the larvicidal activity of cyanobacterial secondary metabolites, including toxins [17, 22]. The current findings indicated that the extracts and purified toxins of both *M. aeruginosa* and *Scytonema* sp. strain BT 23 were strongly larvicidal to the *Culex* and *Anopheles* sp. In addition, these toxins also probably affect the moulting process and subsequent developmental processes. Since such processes are controlled by the neurosecretory cells of the ventral nerve cord [15], it would appear that the toxic constituents may affect the neurosecretory cells. This was apparent from the observations that many of the mosquito larvae failed to moult and pupate with higher concentrations of the toxins. However, further study is needed to reveal the exact cause of the mosquito larvicidal activity.

Since the late 1970s, *Bacillus thuringiensis* (Bt) in the form of a crude spore crystal mixture has become the preferred agent for pest control in many countries [15]. However, the effectiveness of Bt as an agent for mosquito and pest control is limited due to its narrow specificity and costly production. As such, there is an urgent need for alternative microbiocides, especially in view of the hazardous effects of synthetic chemical pesticides. Accordingly, the current report on the pesticidal values of toxic constituents from cyanobacteria would appear to be promising; because a) they represent a new class of secondary metabolites, b) being natural products, they are biodegradable, and c) they are effective against a range of insect-pests and mosquitoes [5, 9, 17, 22, 26]. Although the relatively higher mammalian toxicity associated with cyanotoxins restricts their further exploitation in pest control, chemically modified versions of these novel compounds could be produced to minimize mammalian toxicity [5]. This is borne out by the existence

of over 60 different natural variants of microcystins, some with a lesser degree (or even none) of mammalian toxicity than microcystin-LR [4, 6]. Consequently, further experiments on the structure-bioactivity of cyanotoxins, their mechanism of action, effect on nontarget organisms, bioaccumulation, and biodegradation are critical to ascertain their usefulness as pest control agents.

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