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Toxic Effect of *Microcystis* Extract on Haemolymph Protein Profile In Cockroach *Periplaneta Americana*

Neelgagan Singh[1]*, Aditi Dubey[1], and Uma Shankar Gupta[2]
1.Department of Zoology, A. N. D. College, University Of Delhi, India
2.Department of Zoology, Dr H. S. Gour Vishwavidyalya Sagar (M.P.), India

ABSTRACT

The present study has been made to study the effects of microcystin on the physiology of cockroach. Toxic effects of microcystin have been reported in rat, fish etc. this toxin effects the liver thus the metabolism of the organism is much affected. In cockroach, intracoelomic treatment of *Microcystis* extract showed some fluctuations in concentration of protein fractions especially in fractions 3, 4, 5 and 6. A common decrease in protein concentration is observed in 4, 5 and 6 but fraction 3 showed an increase in concentration. Fractions 7 and 8 failed to separate both in male and female cockroach. Reduced protein concentration for fraction 4 and 6 observed in male cockroach. The concentration of protein in the haemolymph generally decreases due to the microcystin treatment. Similar observation was recorded for the insects in spectrophotometric analysis of protein concentration.

Keyword: Microcystin, cockroach, haemolymph, protein, toxin

*Corresponding Author Email: neelgagan.singh@gmail.com
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INTRODUCTION

Cyanobacterial blooms are an outcome of increasingly evident eutrophication in waterbodies around the world[1]. Most of these cyanobacteria are harmful to animals and humans as they produce toxins known as cyanotoxins. Cyanotoxins are classified into neurotoxins, hepatotoxins and skin irritants. The hepatotoxic cyanotoxins are produced by various genera such as *Microcystis, Anabena, Oscillatoria, Nodularia, Nostoc, Cylindrospermopsis*. Most hepatotoxins are generally referred to as microcystins (MCs), as they were first isolated from *Microcystis aeruginosa*[2]. Among the different variants of MC's, MCLR was found to be the most toxic followed by MCYR and MCRR. Comparative toxicity evaluation of these three toxins in mice have shown that although all three cause similar changes in serum levels of hepatic enzymes, LDH levels, DNA fragmentation and similar histopathological conditions, the intensity of toxicity was highest for MCLR[3].

The effect of Microcystin on the physiology in fish and mammals has been studied by many workers[4,5]. The toxins are generally bound to the cell membrane and are released as cells age and die, under stress. They could also passively leak out of cells, could be released from growing young cells, or released by lytic bacteria like *Pseudomonas* spp.[6]. Exposure to MCs was reported worldwide in animals and in humans for over a century[7]. Exposure occurs orally, but can also occur through inhalation or through dermal exposure.

Toxic effects of microcystin have been reported in rat, fish etc. this toxin effects the liver thus the metabolism of the organism is much affected. Very less work has been carried out on the toxic effects of microcystin in cockroaches. The present study has been made to study the effects of microcystin on the physiology of cockroach. It has been observed that the diversity of insects has affected in the Sagar Lake, which may due to the toxic effects of microcystin.

MATERIALS AND METHOD

Extraction of Microcystin Toxin

In Sagar lake *Microcystis aeruginosa* (cyanobacteria) is found throughout the year. However a thick layer or scum usually found between March to June. The water sample was collected and filtered on filter paper. Cells were dried at room temperature and stored at 8°C. The process was carried out as described by Harada *et al.*[8] with slight modification. 10 gm. of dried cells was extracted with 100 ml of methanol and water (70:30). The mixture was sonicated 3 times for 5 min. Total extract was frozen overnight and it was then centrifuged. The supernatant was separated and filtered through Whatmann No. 1 filter paper. The filtrate was concentrated on

Octadecil - Salane cartilage (C18), which was washed with 20 ml of distilled water, followed by 20 ml of 20% methanol and eluted with 20 ml of 100% methanol. The extract was completely dried by evaporation and solutions of different concentration of extracted microcystin solution were made in saline water.

Insects and Experimental Procedure

The cockroaches were reared at the temperature 26 ^{0}C with constant supply of food and water. Microcystin is a relatively hydrophilic molecule hence it would not be expected to pass readily through the waxy layer of insect epicuticle. Therefore Microcystin is not a contact acting toxin but must be ingested through the gut or injected into the body coelom to exert a toxic effect[9]. For experiments sublethal dose of 20µl (0.2% Microcystin toxin) were injected intracoelomically in first two groups of Cockroach respectively. For the third group or control insects were feed on control diet.

Collection of Haemolymph Samples

Samples of haemolymph were obtained by cutting antennae to obtain haemolymph in cockroach. 1 to 2µl haemolymph so exuded was soaked in Whatmann No. 1 filter paper. This soaked paper was directly applied to gel tubes for electrophoresis. For spectrophotometric analysis 2µl haemolymph was obtained from insect and it was diluted to 1ml.

Electrophoretic Technique

The insect haemolymph was examined by Polyacrylamide gel disc electrophoresis (PAGE). The treated haemolymph samples of insect were placed at one end of the tube containing the gel. The voltage was adjusted to 80. The separating gel was run at 100-150 volts, for 3-4 hours.

The Relative mobility (Rm) value of the separated protein fractions were calculated as described by Kulkarni and Mehrotra[10].

$$\text{Rm} = \frac{\text{Distance travelled by the protein band from origin}}{\text{Distance travelled by tracing dye from origin}} \times 100$$

Chemicals were used for the preparation of polyacrylamide gel as suggested by Laemmli's system[11].

Spectrophotometry

Spectrophotometry (by Lowry method) is used to determine the insect's hemolymph protein concentration in different groups. The principle behind the Lowry method of determining protein concentrations lies in the reactivity of the peptide nitrogen[s] with the copper [II] ions under alkaline conditions and the subsequent reduction of the folin-ciocalteu phosphomolybdicphosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed

oxidation of aromatic acids[12].

Statistical analysis

The result for the effect of microcystin on total protein concentration of insect's haemolymph were expressed as Mean ± SEM. The mean value of the results from the standard group were compared to the mean value of groups treated with extract using one way analysis of variance (ANOVA) followed by the Tukey's test for multiple comparison using. These results were considered as significant at $p<0.001$.

RESULTS AND DISCUSSION

The present study was conducted to observe the effect of microcystin extract on haemolymph protein in cockroach. As far as the author's knowledge is concerned few worker have done work on the effects of microcystins in insect, but no report is available on the haemolymph protein concentration in insect treated with microsytins. Innate immunity of insect to potential pathogens and parasites involves an array of reactions that include proteolytic cascades that regulate coagulation and melanization of haemolymph, production of reactive intermediates of oxygen and nitrogen, and secreted antimicrobial peptides[13]. In most of the insect studied fluctuations in protein concentration of the haemolymph are associated with oocyte maturation. Qualitative and quantitative changes in the haemolymph protein have been reported to occur in several insect species, during the course of egg maturation of *S. gregaria*[14], *T. molitor*[15], *Leptinotarsa decemlineata*[16], *Polytela glorarsa*[17]. Gupta and Rathore[18] demonstrated that Neem seed oil affects the haemolymph protein levels during the vitellogenic phase of *Poekilocerus pictus*.

Protein concentration in haemolymph of female (Table-1, Figure 1 and 2)

3rd day– In normal female the haemolymph protein concentration was observed 58.63±0.21 mg/ml. In insects treated with microcystin through intracoelomic injection it was decreased up to 55.25±0.21 mg/ml. **5th day**– In normal female the protein concentration was of 60.32±0.21 mg/ml. When insects treated with microcystin through intracoelomic injection it was decreased to 57.24±0.21 mg/ml. **7th day**– In normal female the protein concentration was observed of 55.13±0.21 mg/ml. In insects treated with microcystin through intracoelomic injection it was decreased to 47.83±0.21 mg/ml. **10th day**– The protein concentration detected in normal female was 54.01±0.21 mg/ml. Protein concentration was decreased in insects treated with intracoelomic injection of microcystin to 47.18±0.21 mg/ml. **15th day**- The protein concentration in normal female was of 61.16±0.21 mg/ml. The reduction in protein concentration occurred when female treated with intracoelomic injection of microcystin, 44.03±0.21 mg/ml. **20th day**-

The protein concentration in normal female was observed of 60.13±0.21 mg/ml. The reduction in protein concentration occurred in female treated with intracoelomic injection of microcystin, 42.19±0.21 mg/ml. **25th day**– In normal female the protein concentration was observed of 57.85±0.22 mg/ml. In insects treated with microcystin through intracoelomic injection it was decreased to 43.55±0.22 mg/ml.

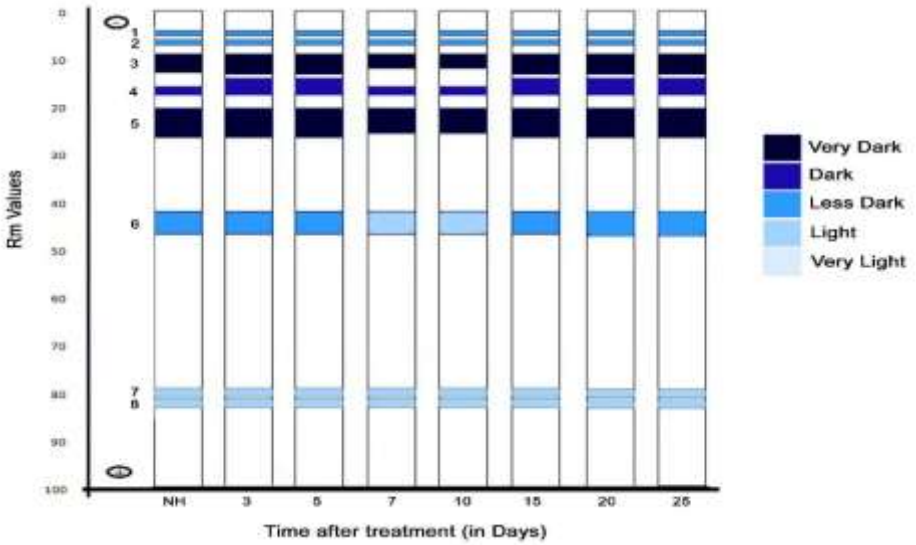

Figure 1: Haemolymph protein pattern of normal adult female cockroach

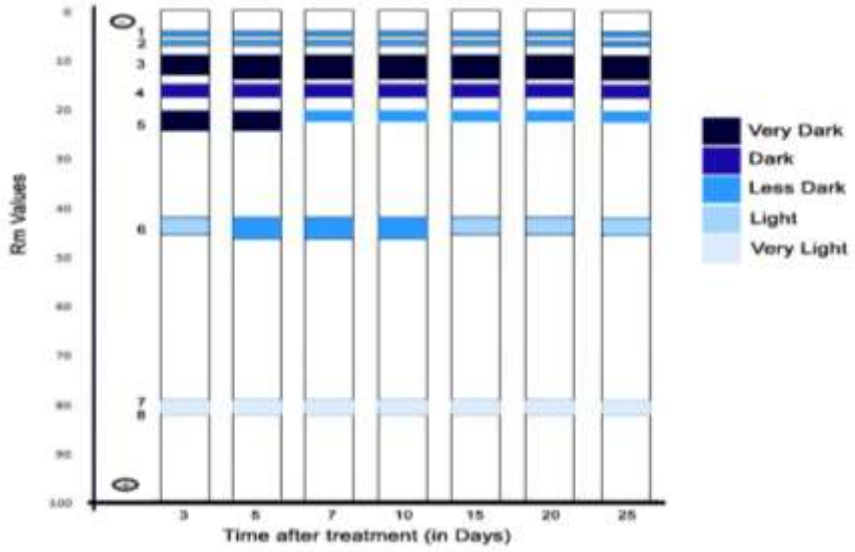

Figure 2: Haemolymph protein pattern of adult female cockroach treating by injecting microcystis toxin

Table 1: Haemolymph protein concentration of Control and Treated Female Cockroach

T	Protein Concentration (mg/ml)							
	3 day	**5day**	**7day**	**10 day**	**15 day**	**20 day**	**25day**	**30day**
C	58.63±0.21	60.32±0.21	55.13±0.21	54.01±0.21	61.16±0.21	60.13±0.21	57.85±0.22	58.63±0.21
MI	55.25±0.21	57.24±0.21	47.83±0.21	47.18±0.21	44.03±0.21	42.19±0.21	43.55±0.22	55.25±0.21

T = Treatment, C = Control, MI = Microcystin through intracoelomic injection, Each value represent the Mean ± SEM (n=6) P<0.001.

Protein concentration in haemolymph of male (Table-2, Figure 3 and 4)

3rd day– In normal male the protein concentration was observed of 55.83±0.21 mg/ml. In insects treated with microcystin through intracoelomic injection it was decreased to 52.06±0.21 mg/ml. **5th day**– In normal male the protein concentration was of 57.71±0.21 mg/ml. In insects treated with microcystin through intracoelomic injection it was decreased to 53.23±0.21 mg/m. **7th day**- The protein concentration in male was observed of 58.77±0.21 mg/ml. The reduction in protein concentration was occurred in male treated with intracoelomic injection of microcystin i.e. 54.29±0.21 mg/ml. **10th day** – In normal male the protein concentration was observed of 60.56±0.21 mg/ml. when insects treated with microcystin through intracoelomic injection it was decreased to 58.53±0.21 mg/ml. **15th day** – In normal male the protein concentration was of 60.79±0.21 mg/ml. when insects treated with microcystin through intracoelomic injection it was decreased up to 57.78±0.21 mg/ml. **20th day**- The protein concentration in male was observed of 62.51±0.21 mg/ml. Increase in protein concentration occurred in male treated with intracoelomic injection of microcystin i.e. 64.84±0.21 mg/ml. **25th day**- The protein concentration in male was of 63.57±0.21 mg/ml. The increase in protein concentration occurred in male treated with intracoelomic injection of microcystin i.e. 65.33±0.21 mg/ml.

Table 2: Haemolymph protein concentration of Control and Treated Male Cockroach

T	Protein Concentration (mg/ml)							
	3day	5day	7day	10day	15day	20day	25day	30day
C	55.83±0.21	57.71±0.21	58.77±0.21	60.56±0.21	60.79±0.21	62.51±0.21	63.57±0.21	55.83±0.21
MI	52.06±0.21	53.23±0.21	54.29±0.21	58.53±0.21	57.78±0.21	64.84±0.21	65.33±0.21	52.06±0.21

T = Treatment, C = Control, MI = Microcystin through intracoelomic injection, Each value represent the Mean ± SEM (n=6) P<0.001.

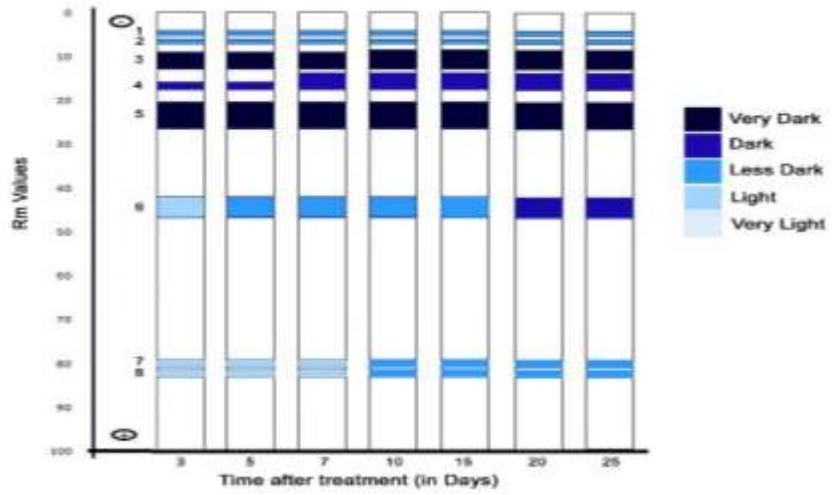

Figure 3: Haemolymph protein pattern of normal adult male cockroach

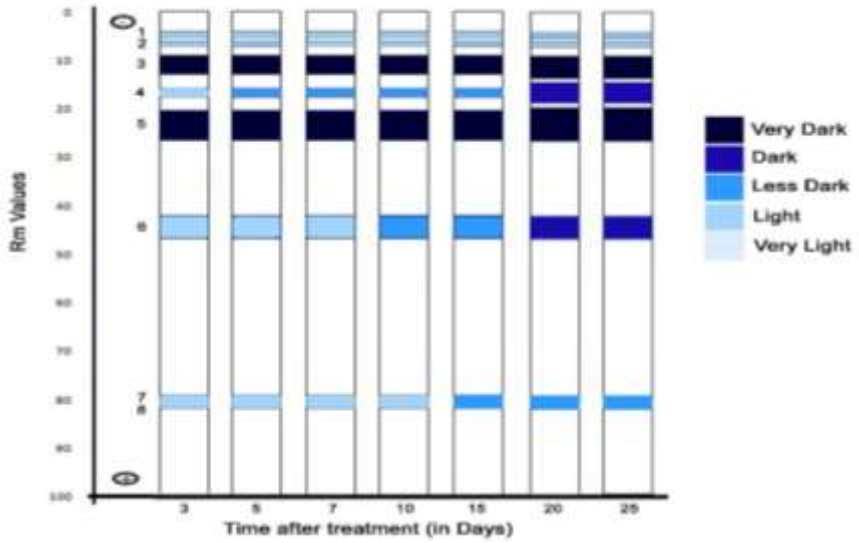

Figure 4: Haemolymph protein pattern of adult male cockroach treating by injecting microcystis toxin

Protein concentration analysis of control adult cockroach

In the haemolymph of cockroach a total of eight protein fractions were observed by Polyacryamide gel disc electrophoresis. Out of eight protein fractions, four protein fractions i.e., fractions 3, 4, 5 and 6 showed high protein concentration and major changes during the ovarian maturation. Since the early publication of Telfer[19] on the *Cecropia* silkworm, the female-specific blood protein, vitellogenin, has been shown in many species of insects[20] including *Blattella germanica*[21], and ample evidence has been accumulated on its fluctuation in blood, and sequestration by vitellogenic oocytes[20,22]. The site of synthesis of vitellogenin has been shown to be the fat body[23,24], and the localization of vitellogenin in this organ was clearly demonstrated immunohistochemically[25]. Protein fractions 3, 4, 5 and 6 showed fluctuations in their protein

concentration with the vitellogenesis in cockroach. Protein fractions 3, 4, 5 and 6 showed a marked decline in protein concentration from 7 days to 10 days, after that protein concentration increased again. Spectrophotometric analysis of protein concentration of female cockroach showed a high protein concentration in newly emerged adult female, it's declined after 5 days to 10 days and increased again after 10 days. Tanaka[26] studied immune histochemical of vitellogenin during embryogenesis in the cockroach *Blattela germanica*. Observations showed that some presence of yolk in oocytes of adult female cockroach and loses evenness of yolk after 7 days on the other hand vitellophage started from 7 days. This kind of major transformation in vitellogenesis has been showed by protein fractions 3, 4, 5 and 6 during 7 to 10 days, so it can be assumed that these protein fractions are vitellogenic protein fractions. The proteins which are taken up by the ovary are called vitellogenic proteins[16]. Gupta and Rathore[18] observed similar results in *P. pictus.*

In several insects e.g. *Hylophora cecropia*[19]; *Schistocerca gregaria*[27] and *Leucophaea*[28] female specific proteins have been reported, which are incorporated into the ovary and are named female sex specific vitellogenic proteins. In cockroach no sex specific proteins were observed, but the concentration of the protein fractions 4, 5 and 6 increases in relation to the ovarian maturation. In cockroach a low concentration of fraction 3, 4 and 6 was observed in the newly emerged adult male, but the concentration of the fraction 3 was high as in the case of the female. The protein concentration of fraction 4 was observed low after emergence, but it increases after 7 days. The concentration of fraction 6 increases in later stage. The protein fractions 3, 4 and 6 show fluctuation in various stages. Spectrophotometric analysis of protein concentration of male cockroach showed a low protein concentration in newly emerged adult male, it's increased after 5 days and remain almost same for 10 to 25 days. Lensky and Kalinsky[29] have reported the presence of three haemolymph proteins in the reproductive organs, seminal vesicles, mucous gland and penis of honey bee drones. De Loof and de Wilde[16] in *Leptinotarsa* have reported that due to castration accumulation of blood protein does not take place.

Protein concentration analysis of microcystin treated insect

The effects of *Microcystis* extract in insects through intra-coelomic injection on the haemolymph proteins of insects has not been reported as far as the author's knowledge is concerned. There have been several studies of microcystin toxicity in aquatic invertebrates, but only few workers had done work in this direction as little is known of microcystin toxicity in insects[30, 31,32]. In cockroach, intracoelomic treatment of *Microcystis* extract showed some fluctuations in concentration of protein fractions especially in fractions 3, 4, 5 and 6. A common decrease in protein concentration is observed in 4, 5 and 6 but fraction 3 showed an increase in

concentration. Fractions 7 and 8 failed to separate both in male and female cockroach. Reduced protein concentration for fraction 4 and 6 observed in male cockroach. The concentration of protein in the haemolymph generally decreases due to the microcystin treatment. Similar observation was recorded for the insects in spectrophotometric analysis of protein concentration. It has been reported that only two types of proteins seem to bind covalently to microcystin-LR, protein phosphatases 1 and 2A and the b subunit of ATP synthase[33]. Dittmann *et al.*[34] reported a generalized association of microcystin-LR to proteins, and recently, differential fractionation of *Microcystis* cells showed microcystins bound to a protein fraction primarily composed by phycobiliproteins[35].

Vela *et al.*[36] analyzed several proteins in presence of microcystin–LR and showed that no new protein isoforms observed, also presence of microcystin–LR per mol of protein altered the peptide profile, some peaks of HPLC disappeared while others increased in size. They published some results which indicated that the binding of microcystin to a wide range of proteins *in vitro* is a fact, even though in our opinion its physiological meaning is still unclear. These results are pointing to a non-specific binding which does not seem to cause a change in the isoelectric point of the proteins tested. They also showed that the unspecific binding of microcystin-LR to proteins does not reflect a physiologically relevant situation, the fact that the elution profile of lysozyme after tryptic digestion is affected by the presence of microcystin-LR, suggests that this interaction may strongly affect results when working with microcystin-LR in the presence of proteins. According to Juttner and Luthi[35] data concerning toxicity of the biological samples, the coupling of microcystin to proteins has a significant practical interest, since it may strongly affect the determination of the toxin and its toxicity. Few workers have reported the decrease in the total protein concentration in fish after treatment with microcystin. A highly significant decrease in the total protein concentration as compared to the normal was reported by Vezie *et al.*[37]. Reduction in total protein was also reported by Kopp and Hetesa[38] in their experiments.

CONCLUSION

In conclusion, it can be said that *Microcystis* produce a toxin microcystin, which has got a toxicological importance for the human and their livestock. The findings from this work clearly showed that the microcystin treatment majorly effects the haemolymph protein profile in cockroach. As Bt toxin used as microbial bio pesticide, microcystin can be used as natural pesticide for controlling various pests. Several studies have also explored the use of genetically

engineered cyanobacteria, specifically expressing the insecticidal proteins from *Bacillus thuringiensis* to control insects.

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