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Original Research Paper

Action of C-Phycocyanin Pigment and Cell Extracts of *Tolypothrix* sps. on the Biochemical Activity of Eri Silkworm and Their Antifungal Activity

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ABSTRACT

Soil samples were collected from different agro-climatic regions of north Telangana region of Andhra Pradesh. Sterilized nitrogen free BG-11 medium was used for isolation of nitrogen fixing cyanobacteria. Antifungal activity of four strains of *Tolypothrix* were studied. Methanolic extracts from biomass of selected Cyanobacteria were isolated and screened against four strains of fungi (*Candida albicans*, *Candida guilliermondii*, *Aspergillus niger* and *Aspergillus fumigatus*). The growth of all fungal strains tested were inhibited by the culture extracts and C-phycocyanin. Bioassay studies of Eri silkworm expressed no symptom of ill-health after feeding the C-phycocyanin treated castor leaves (*Ricinus cummunis*), and body weight and silk gland weights were also increased compared to control sample. The present study is also envisaged on the impact of phycocyanin of *Tolypothrix* species on the carbohydrate content and amylase activity in Eri silkworm (*Samia cynthia ricini*). According to the obtained results it is concluded that extracts from *Tolypothrix* sp. could be used traditionally in the treatment of microbial infections and also for the increase of carbohydrate and amylase activity in the silkworm under investigation.

INTRODUCTION

Cyanobacteria are a very old group of organisms and represent relics of the oldest photoautotrophic vegetation in the world that occur in freshwater, marine and terrestrial habitats (Mundt & Teuscher 1988). Cyanobacteria have drawn much attention as prospective and rich sources of biologically active constituents and have been identified as one of the most promising groups of organisms to be capable of producing bioactive compounds (Schlegel et al. 1999). Recent investigations on biologically active secondary metabolites from cyanobacteria led to the identification of wide range of compounds possessing antimicrobial, antiviral, antineoplastic and toxic properties (Falch et al. 1995, Moore 1996, Namikoshi & Rinehart 1996). The marine cyanobacterial group is well documented for its bioactive compounds possessing antimicrobial properties, while there are only a few reports available with freshwater cyanobacteria. In the present work, we report the C-phycocyanin, antifungal activity and the toxicity evaluation of the culture filtrate of four strains of *Tolypothrix* sp. and their impact on the carbohydrate content and amylase activity of Eri silkworm, *Samia cynthia ricini.*

MATERIALS AND METHODS

Soil samples were collected from different agro-climatic regions of paddy fields in North Telangana region of Andhra Pradesh. Soil samples in laboratory were cultured in G 11 media with or without nitrogen source. The cyanobacteria observed were transferred to the same medium. Unialgal cultures were prepared using sub-culturing methods. Each isolated cyanobacteria was cultured in a 500 mL flask containing 150mL of BG 11 medium without shaking for 30 days. The incubation temperature was 28°C ± 2 and illumination at 3000 lux with a white continuous fluorescent light.

Preparation of supernatant and cell extracts: The cultures were harvested after 30 days by centrifugation at 5000 rpm for 15 minutes. The aqueous supernatant was collected and the algal pellet was extracted with 15 mL of methanol, with shaking for 20 minutes. The culture supernatants and solvent extracts were dried under reduced pressure at 40°C and were stored at -10°C for further studies.

The most commonly used method for the extraction of phycobiliprotein C-phycocyanin was isolated following the method of Boussiba & Richmond (1979) and estimation as described by Bennett & Bogorad (1973). Ten mL of cyanobacterial culture was homogenized and centrifuged at 5000 rpm for 5 minutes. The pellet was washed and suspended in 2.0 mL of 0.05 M phosphate buffer (pH 6.8). The aqueous phase containing cyanobacterial cells was subjected to freezing and thawing. Then the content was centrifuged at 5000 rpm for 5 minutes and the supernatant was collected and stored in the refrigerator. Again the pellet was subjected to freezing and thawing until a colourless supernatant was obtained. The supernatant containing pigment was pooled

and final volume was observed. The pigment absorption was measured at 615 and 652 µm in a Beckman DU-64 spectrometer against 0.05 M phosphate buffer as blank. The concentration of C-phycocyanin was calculated using the following formula. The phycobilin pigments C-phycocyanin content in the cyanobacterial cultures were expressed as µg per mL of the cyanobacterial cells under investigation.

$$\text{C-Phycocyanin (PC)} = \frac{\text{E -615-0.474(E 652)}}{5.34}$$

Where, E 615 and and E 652 are the absorbance at 615, 652 µm respectively.

Identification of cyanobacteria: Identification of the cyanobacteria was done by using morphological variation studies and taxonomical approaches mentioned in the published literature of Desikachary (1959), Anand (1989) and Santra (1993).

Antifungal bioassay: Antimicrobial activities of the C-phycocyanin and extraction solvent of *Tolypothrix* sp. were determined by the paper disk diffusion method. The following fungi were used as test organisms: *Candida albicans, Candida guilliermondii, Aspergillus fumigatus* and *Aspergillus niger*. Filter paper discs (6mm) were saturated with 50 µL of the test solution, dried under laminar air flow and placed on the Saubouraud's dextrose agar plate, which had been inoculated with a lawn of the test microorganisms. Plates were incubated for fungi at 25°C for a period of 24-48 hours. Discs treated with 50 µL methanol was used as negative control and Gentamycin discs were used (10 µg) as positive controls. The extracts and supernatants containing antifungal components produced distinct and clear circular zones of inhibition around the discs. The diameter of clear zones was determined in millimetres and used as an indication of antifungal activity. All tests were made in triplicate under sterile conditions.

The following formula was used for comparison of the antifungal produced activity of the sample with that of the standard.

$$\text{Antimicrobial index} = \frac{\text{Inhibition zone of sample}}{\text{Inhibition zone of the standard}} \times 100$$

Silkworm toxicity assay: The present study also included observations of the phycocyanin toxicity. Fifty number of second instar larvae of Eri silkworm (*Samia cynthia ricini*) were taken in a clean plastic tray of 20 cm diameter and 8 cm height. A quantity of 50 g of clean tender and chopped leaves of caster (*Ricinus communis*) was taken and one gramme of the C-phycocyanin was dissolved in minimum quantity of water and thoroughly mixed. The leaves were allowed to air dry. After starvation for 5 hr, the larvae were allowed to feed the treated leaves. Untreated leaves were also fed to another set of larvae which served as a control. Observations were made after 24 hr for the worm movement, death rate, feeding rate and symptoms of ill health. The larval body weight and gland weight of the silkworm were observed when the larvae passed on to the late fifth instar from early fifth instar.

Estimation of carbohydrate was done (Mokrash 1954) by collecting fifth instar larval haemolymph of both control and treated worms and expressed in terms of mg/mL. Haemolymph was collected from fifth instar silkworms by cutting the last abdominal leg and collecting in precooled tubes containing a few crystals of phenylthiourea. Digestive fluid was collected from the 4th day of fifth larval stadium. Larvae were starved for 4 h and digestive juice was collected from mid-guts after dissection of larvae. Digestive fluid was collected into precooled tubes. Five individual larvae were used for sample collection. Since the sex was not discernible during the larval stage, it is probable that the samples were derived from both sexes. The digestive fluid was centrifuged at 10,000 rpm for 10 min to remove undigested leaf particles and stored at -20°C until use. Amylase activity was measured with the standard procedure using soluble starch as substrate (Bernfeld 1955, Baker 1991). Maltose was used as a standard and the enzyme activity was expressed as mg of maltose released/mL/min at 37°C. Samples were diluted prior to assay to maintain linearity. Enzyme assays were carried out for five individual larval samples calibrated against controls for two trials and means of all the five values and trials were taken as the final values (Abraham et al. 1992).

RESULTS

Certain cyanobacteria have drawn much attention as prospective and rich sources of biologically active constituents and have been identified as one of the most promising groups of organisms capable of producing bioactive compounds (Fish & Codd 1994, Schlegel et al. 1999). Although the potential of cyanobacteria as biofertilisers is well known, attention has recently been focused on the biotechnological potentials of cyanobacteria for obtaining pharmacolog-ically active secondary metabolites (Carmichael 1992). The marine cyanobacteria are well documented for their bioactive compounds possessing antimicrobial properties, while there are only few reports available with freshwater cyanobacteria.

Antifungal activity of culture filtrate and C-phycocyanin: The results of solvent extracts and C-phycocyanin of the selected strains of cyanobacteria *Tolypothrix ceylonica*, *T. fragilis*, *T. nodosa* and *T. tenuis* were against the pathogenic fungi (*Candida albicans*, *C. guilliermondii*, *Aspergillus niger* and *A. fumigatus*) have been given in Table 1. In general, the inhibition of culture extracts was more followed by

the C-phycocyanin pigment. In the study of antifungal activity *T. fragilis* culture extracts have shown higher inhibition (11.7 mm) followed by C-phycocyanin from *T. tenuis* (9.4 mm) on the pathogenic fungi of *Aspergillus fumigatus*. The culture extract from *Tolypothrix tenuis* (7.7 mm) and C-phycocyanin from *T. fragilis* (6.6 mm) showed higher inhibition of *Aspergillus niger* among the different treatments. The culture extract of *T. fragilis* (11.6 mm) and C-phycocyanin of *T. tenuis* (9.4 mm) expressed higher inhibition on the pathogen of *Candida albicans* under study. The *Candida guilliermondii* appears to be more resistant by expressing the minimum inhibition zones for culture extracts (1.3-4.8 mm) and C-phycocyanin (2.7-4.2 mm) when compared with the control (Gentamycin).

In the present study the effects of C-phycocyanin pigments of various strains of *Tolypothrix* (*T. fragilis*, *T. nodosa* and *T. tenuis*) on carbohydrate content in the haemolymph of silkworms were found to be as 43.3 ± 1.4, 44.91 ± 1.2, 42.8 ± 2.8 and 49.5 ± 0.7 respectively and control was observed as 40.8 ± 3.63 mg/mL. The percent increase in carbohydrate content in the haemolymph of silkworm treated with the four strains of *Tolypothrix* was more (6.12, 10.04, 5.04 and 21.32) as compared to control under study (Table 2).

The digestive amylase activity values after treatment with *Tolypothrix ceylonica* have expressed as 0.027 ± 0.001, followed by *Tolypothrix fragilis* (0.033 ± 0), *Tolypothrix nodosa* (0.026 ± 0.001) and *Tolypothrix tenuis* (0.03 ± 0.001 mg/mL) compare to control (0.026 ± 0.002 mg/mL/min). The percentage increase of phycocyanin treated samples (*T. ceylonica, T. fragilis, T. tenuis*) of digestive amylase activity of silkworm have shown 3.84%, 26.92%,0% and 15.38 respectively (Table 2) .

Silkworm toxicity test: Certain cyanobacteria have drawn much attention as prospective and rich sources of biologically active constituents, and have been identified as one of the most promising groups of organisms capable of producing bioactive compounds (Fish & Codd 1994, Schlegel et al. 1999). Cyanobacteria are known to produce metabolites with diverse biological activity such as antibacterial, antifungal, antiviral, anticancer, antiplasmodial, algicide, antiplatelet aggregation and immuno-suppressive activities (Borowitzka 1995, Jaki et al. 2000, Kajiyama et al. 1998). Patterson & Carmeli 1992, Gerwick et al. 1994, Luesch et al. 2000, Papendorf et al. 1998, Papke et al. 1997, Rho et al. 1996, Koehn et al. 1992, Ghasemi et al. 2003). Few members of Cyanobacteria are very toxic to human beings and also to animals (Tyagi et al. 1999). The present study also deals with observations of the toxicity cyanobacterial pigment C-phycocyanin obtained from *Tolypothrix* sps. Toxicity of phycocyanin pigments was tested in a sensitive insect silkworm and the results were presented in Table 3. Phycocyanin pigment from the four cyanobacterial cultures was mixed individually with the mulberry leaves fed to the silkworm larvae once in a day caused no toxicity. The movement of the worms was normal. No symptom of ill health and abnormal secretion was detected. In all the treated worms 100 percent survival was observed. The body weight of the silkworm and silk gland weight were increased when compared to control worms ranging from 14.82-20.01% and 54.81-73.32% respectively.

DISCUSSION

The cyanobacteria such as *Fischerella ambigua* (Flach et al. 1995), *Fischerella musciola* (Hagmann et al. 1996), *Nostoc commune* (Jaki et al. 2000), *Scytonema hofmanni* (Pignatello et al. 1983), *Hapalosiphon fontinalis* (Moore et al. 1987), *Anabaena* sp. (Frankmolle et al. 1992), *Nostoc spongiaeforme* (Hirata et al. 1996), *Microcystis aeruginosa* (Ishida et al. 1997), *Phormidium* sp. (Fish & Codd 1994) have been reported as important cyanobacterial members to produce antimicrobial substances. These reports are in agreement with the present study, since the extracts from *Tolypothrix* sps. had similar effects on the pathogenic microbes used in the present investigation.

Screening efforts aimed to identify antimicrobial agents in cyanobacteria have revealed several promising compounds. Some of these substances identified are Nostocyclyne A (Plotono & Carmeli 2000), Nostofungicidine (Kajiyama et al. 1998), Kawaguchipeptin B (Ishida et al. 1997), Nostocin A (Hirata et al. 1996), Ambigol A and B (Falch et al. 1995), Hapalindoles (Moore et al. 1987), and Scytophycins (Ishibashi et al. 1986).

A few studies have been made to screen cyanobacteria from paddy-fields for the production of antimicrobial substances. Possibly the synthesis of highly active toxin is a defence option of cyanobacteria in these environmental conditions against other organisms like bacteria, fungi, viruses and eukaryotic microalgae (Mundt et al. 2001). Antimicrobial activity of *Tolypothrix* sps. not yet been studied and the screening programme is among the first studies done for assessment of its antibacterial and antifungal activity. The toxicity tests are important for the algal products because, various members of cyanobacteria produce toxins like cytotoxins, hepatoxins and neurotoxins. In the present investigation C-phycocyanin pigment from the four strains of *Tolypothrix, T. ceylonica, T. fragilis, T. nodosa, T. tenuis* have not expressed toxicity in the silkworm toxicity assay. The results on silkworm clearly showed that the phycocyanin pigments are safe and also increase body weight and silk gland weight under the study.

Table 1: Antifungal activity of cyanobacterial culture extract and C-phycocyanin.

Name of cyanobacteria	Treatments	Inhibition annules (mm ± SEM)* *Aspergillus fumigatus*	*Aspergillus niger*	*Candida albicans*	*Candida guilliermondii*
Tolypothrix ceylonica	Culture extract	11.4 ± 0.1	7.4 ± 0.1	8.1 ± 1.4	4.8 ± 0.2
	phycocyanin	7.8 ± 0.4	5.2 ± 0.3	6.7 ± 0.5	4.2 ± 0.1
Tolypothrix fragilis	Culture extract	11.7 ± 0.0	6.7 ± 0.2	11.6 ± 0.2	3.1 ± 0.0
	phycocyanin	8.8 ± 0.3	6.6 ± 0.4	8.9 ± 0.4	2.7 ± 0.1
Tolypothrix nodosa	Culture extract	6.7 ± 0.2	6.8 ± 0.5	11.4 ± 0.0	4.3 ± 0.2
	phycocyanin	9.4 ± 0.0	4.9 ± 0.3	8.1 ± 0.2	3.0 ± 0.3
Tolypothrix tenuis	Culture extract	8.8 ± 0.4	7.7 ± 0.5	8.0 ± 0.1	1.3 ± 0.5
	phycocyanin	6.5 ± 0.3	5.5 ± 0.2	9.4 ± 0.4	2.8 ± 0.4
Gentamycin		13 ± 0.0	12 ± 0.0	14 ± 0.0	12 ± 0.0

*Data are presented as mean of three readings ± SEM.

Table 2: Effect of phycocyanin pigment on carbohydrate content in the haemolymph and digestive amylase activity.

Source of pigment	Carbohydrate content in haemolymph (mg/mL)	Percentage increase (%)	Digestive amylase activity mg/mL/min	Percentage increase (%)
Tolypothrix ceylonica	43.3 ± 1.4	6.12	0.027 ± 0.001	3.84
Tolypothrix fragilis	44.91 ± 1.2	10.04	0.033 ± 0	26.92
Tolypothrix nodosa	42.8 ± 2.8	5.04	0.026 ± 0.001	-
Tolypotrix tenuis	49.5 ± 0.7	21.32	0.03 ± 0.001	15.38
Control	40.8 ± 3.63	-	0.026 ± 0.002	-

• The values are expressed in terms of standard error of the mean.

Table 3: Effect of phycocyanin pigment on the body weight and silk gland weight of Eri silkworm (S*amia cynthia ricini*).

Name of cyanobacteria	Body weight of silkworm (mg)	Percentage increase	Silk gland weight (mg)	Percentage increase
Tolypothrix ceylonica	7447 ± 0.23*	20.01	1093 ± 0.13*	54.8
Tolypothrix fragilis	7634 ± 0.41*	15.345	1224 ± 0.21*	73.32
Tolypothrix nodosa	7125 ± 0.34*	14.829	1124 ± 0.22*	59.24
Tolypothrix tenuis	7339 ± 0.54*	18.27	1094 ± 0.26*	54.91
Control	6205 ± 0.48	-	706 ± 0.10	-

Values are mean ± SEM for ten larvae. *Statistically significant difference in comparison with the control group with $p < 0.001$

Trehalose is the major and metabolically active, non-reducing disaccharide in the insect blood, which is synthesized in the fat body, and utilized during spinning, flight and starvation of insects (Saito 1960, Hori 1969). From the present studies the increase in carbohydrate content in the silkworms treated with phycocyanin extracted from various *Tolypothrix* sps. suggests that carbohydrates are determinant factor for the normal growth and development of the larva, which ultimately determines the quality of silk produced. The impact of biochemical parameters on yield revealed the importance of amylase activity for the survival of silkworm, *Bombyx mori* (Chatterjee et al. 1993). According to Hori (1969), certain food compounds can stimulate or inhibit digestive enzymes. Hirata & Yosuo (1974) found that silkworm strains, which have more amylase activity showed better cocoon weight, shell weight and shell percentage, and rate of synthesis of amylase is dependent upon the rate of feeding. Studies on quality of silk fibre through application of rhizobacteria suggests that rhizobacteria could be an effective tool for enhanced biomass production in some plants, which in turn has an impact on the growth of silkworms to produce more silk fibre of good quality (Unni et al. 2008).

CONCLUSION

Many secondary metabolites are potent toxins, causing health problems for animals and humans when the producer organisms occur in masses in water bodies. The toxins produced by cyanobacteria are grouped into three categories on the

basis of the bioassay methods used to screen them like cytotoxins, hepatotoxins and neurotoxins. Cytotoxins are studied with cultured cell lines, there are still no data on cytotoxins from natural sources that are lethal to animals. Recent investigation on biologically active secondary metabolites from cyanobacteria led to the identification of wide range compounds possessing antimicrobial, antiviral, antineoplastic and toxic properties. The ability to produce antimicrobial substances may be noticed not only as a defensive instrument for the strains but also as a good source of new bioactive compounds from pharmaceutical point-of-view. The present study, aimed at the preliminary investigation of antifungal activity and toxicity evaluation studies of *Tolypothrix* sp., indicated that this group of selected strains displays a potential that warrants further work.

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