Global Veterinaria 21 (4): 165-171, 2019
ISSN 1992-6197

DOI: 10.5829/idosi.gv.2019.165.171

# Inhibition of Protease Enzyme Activity of *Daphnia magna* from the *Cyanobacterium microcystis* Sp. Strain BM 25 Extracts

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**Abstract:** Cyanobacterial mass developments in eutrophic ponds and lakes are a major concern for lake management, as many cyanobacteria produce a huge variety of toxic secondary metabolites, e.g. microcystins. The aim of this research was to culture a strain of the *Cyanobacterium microcystis* sp strain BM25, to observe its biomass production and to isolate and purify protease inhibitors from this cyanobacterial biomass. Different secondary metabolites were isolated following a standard bioassay-guideline. Isolation was performed, with an enzymatic protease assay as bioassay. High performance liquid chromatography was used to identify different fractions of secondary metabolite from the strain BM25. Moreover, protease homogenates were isolated from *Daphnia magna* in order to test the inhibitors against naturally occurring major digestive proteases trypsin and chymotrypsin. It was measured that 60% MeOH and the 80% MeOH C18-SPE fraction inhibits chymotrypsin activity 98% (6 nmol pNA $min^{-1}mg^{-1}$) and 99 % (4 nmol pNA $min^{-1}mg^{-1}$), respectively. In contrast, trypsin activity was not inhibited by methanolic extracts of this cyanobacterium strain.

**Key words:** Cyanobacteria · Protease Inhibitors · Digestive Enzyme · Daphnia · HPLC · UV/Vis

## INTRODUCTION

In aquatic systems, the transfer of carbon and energy to the consumer level is to a large extent due to the interaction between the herbivore Daphnia and phytoplankton (e.g. Algae, Cyanobacteria) [1]. Cyanobacterial mass developments, so-called 'blooms', have become widespread in last few decades. Daphnia might also play a role in the control of cyanobacterial blooms because they are unselective filter feeders. Due to have this character, they cannot distinguish between good and bad food quality. However, cyanobacteria are low quality food source for this crustacean because cyanobacteria are deficient of fatty acids and sterols which are necessary for the growth of Daphnia [2]. Another reason is the production of secondary metabolites by cyanobacteria which are not necessary for their growth or reproduction but they have detrimental effects on Daphnia [3].

Some species of cyanobacteria produce secondary metabolites called cyanotoxins, which are harmful for terrestrial as well as aquatic organisms. Cyanobacterial toxins are classified on the basis of their toxic action and accordingly they are termed hepatotoxin, neurotoxin, cytotoxin, dermatotoxin and irritant toxins [4]. Toxic effects of various cyanotoxins have been investigated in mammals and aquatic organisms. Microcystis is a well-known cyanobacterial genus frequently producing hepatotoxins called microcystins [5]. Microcytins are cyclic hepta peptides consisting of short

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chain of amino acids linked by peptide bonds similar in structure to that of proteins. The cyclic nature of the microcystins makes them difficult to break down and to be metabolized by the animals that have ingested microcystins. Microcystins block protein phosphatases 1 and 2a with an irreversible covalent bond, which are important molecular switches in all eukaryotic cells [5]. In vertebrates, microcytins accumulate in the liver, where it damages liver tissue and promotes tumor growth in the liver [5]. The cyanobacterium *Microcystis* sp. produces a variety of bioactive metabolites which are cyclic and noncyclic peptides as well as depsipeptides with unusual amino acid compositions. The most known cyclic peptides are classified as microcystins, nodularins and hepatotoxins. Microcystins are produced by many cyanobacterial genus (eg. *Microcystis*). There are different variants of microcystins known. Among them chief Microcystin poisonings is Microcystin-LR which is found in freshwater environment [5]. There are also some non-toxic compounds produced by *Microcystis* sp. such as cyanopeptolins or micropeptolins, which are one of the class of cyanobacterial peptides.

Trypsin and chymotrypsin are the major protease enzymes found in the gut of Daphnia [6]. These enzymes are also called serine proteases which represent the most important digestive enzymes in the gut of *D. magna* [7]. On the other hand, protease inhibitors are another group of cyanobacterial secondary metabolites that interfere with growth and reproduction of zooplankton. In another study, it was reported that these inhibitors decreases protease activity and reducing somatic growth [8-11]. An experiment was done by Schwarzenberger *et al*. [12], it was found that the inhibitor content of *M. aeruginosa* BM25 is increased under p-limited condition [12] According to this study *M. aeruginosa* BM25 was grew in phosphorus depleted or nitrogen depleted medium and the content of three micropeptins, also chymotrypsin inhibitors, were measured. They found that the micropeptin content was different depending on the available nutrients. Under phosphorus deplete condition higher growth of *Daphnia magna* compared to Daphnia that were fed with p-depleted cyanobacteria.

From previous information it is clear that different species of cyanobacteria produce different compounds, few of these compound interfere digestive proteases of planktonic crustaceans. The aim of this study was to isolate specific compounds produced by *Microcystis* sp. strain BM 25 which inhibit proteases activity of *Daphnia magna*. If such an inhibitor would be produced by *Microcystis* sp. strain BM 25 then it would be identified by the subsequent fractionation of the extract using high- performance liquid chromatography (HPLC) and by the inhibition of digestive proteases of *Daphnia magna*, which was measured using spectrophotometer.

## MATERIALS AND METHODS

**Preparation and Fractionation of *Microcystis sp.* Strain BM25 Extracts:** Ten (10) Litre of a dense culture of *Microcystis* sp strain BM25 were centrifuged (17700g, Temperature 10°C) for 10 minutes and the supernatant was discarded. The pallet was dried using freeze-drier (Christ LOC-1m freeze dryer, ALPHA 1-4, Merrington, Shrewsbury, Shropshire, UK). Freeze-drying was stopped when the weight of the biomass was stable ($4^{th}$ measurement day-4). Freeze dried *Microcystis* sp. strain BM25 was transferred into a mortar by spatula and grinded using a pestle. The final weight of the powdered *Microcystis* sp strain BM25 was 5.7g. One gram (1g) of dried *Microcystis* sp strain BM25 was transferred into an Erlenmeyer flask and 100ml 60% MeOH were added and centrifuge (30,000g) for 3 minutes. Then, transfered *Microcystis* sp strain BM25 supernatant to a 1000 mL biker. Ultrapure $H_20$ (500mL) was added to dilute the methanol to a final concentration of 10%. Next step to prepare sample was solid-phase extraction (SPE). For this experiment C18 (BM25 ODS) cartridge was used as stationary phase and 10% 600ml prepared sample was used as mobile phase. By using the behavior of solute and different concentration of MeOH 20%, 40%, 60%, 80%, 100% (Concentrated MeOH) solution was prepared. The portion that passes through the stationary phase was discarded. The different fractions obtained from elution of the C18 SPE with 20%, 40%, 60%, 80% and 100% MeOH were evaporated to dryness using a rotatory evaporator and 40°C. The residue was re-dissolved in 10ml MeOH and transferred into a test tube. After that, the different fractions were evaporated to dryness with the speed vac evaporator (Temperature 14°C). The residue was re-dissolved in 1ml MeOH and transferred into a cap. Final volume of each fraction was 1ml.

**Analysis of protease inhibitors using HPLC-DAD:** HPLC-DAD is a form of liquid chromatography used to separate compounds that are dissolved in solution. HPLC instruments basically consist of a reservoir of mobile phase, a pump; an injector, a separation column and a detector. After injection, the different components in the sample pass through the column at different rates due to differences in their partition behavior

between the mobile phase and the stationary phase. The DAD detector was coupled to the HPLC in our experiment. All chromatographic analyses were performed on a binary high pressure gradient HPLC system of Shimadzu. The system was composed of SIL-20 AC Auto sampler, LC-20 AB Liquid Chromatograph, DGU-20 AB Degasser, CTO-10 AC Column Oven. Fractionation and sample purification 50µL Microcystis sp BM25 SPE was injected through the HPLC machine. In 40-80% gradient condition fractions were collected in different retention time. In gradient condition with 6 min: 55 % MeOH to 25 min: 55 % MeOH condition, 30µL 80% *Microcystis* sp. strain BM25 SPE of sample (10µL injected each run) injected through the auto sampler and collected fractions in different retention time.

**Pre-Cultivation and Homogenate Preparation of *Daphnia magna*:** *Daphnia magna* clone B was used for this experiment which had been isolated. Twenty *D. magna* were taken in 6 glass vessels which were kept in low light condition and constant room temperature (20°C). Aged tap water was used as culture medium. The animals were fed daily with *Chlamydomonas klinobasis* (strain 56) that had been filtered first with a 30um net filter. Every second day animals were transferred in new glass vessels with fresh water and new food. After one week eggs were visible in their brood chamber. The green alga *C. klinobasis* strain 56 used as food for *D. magna*. This algal strain had been obtained from the Limnological Institute, University of Konstanz, Konstanz, Germany. It was cultivated in cyanophycean medium at 20°C at 130µE$m^{-2}$ $s^{-1}$. After pre-cultivation, 72 *D. magna* with eggs were collected in Eppendorf tube (1.5mL) and 360µL of water (5 µL ultrapure $H_20$ per each *D. magna*) were added. Homogenizer was washed with Ethanol before *D. magna* homogenate preparation. Homozenized *D. magna* was centrifuged (14,000g) for 3 minutes and transferred supernatant in new Eppendorf tube (1.5mL). Activity of the supernatant was assayed by using spectrophotometer (UV/Vis).

**Inhibition of Digestive Proteases of *Daphnia magna* Using Spectrophotometer:** The protein concentration of the supernatant of *Daphnia magna* homogenate was analyzed using a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA). Concentration of *D. magna* protein was measured (by using $C_1V_1=C_2V_2$ formula). After using spectrophotomer, activity was measured in optical density per minute (OD/min). Then specific proteolytic activity was determined as nmol pNA $min^{-1}$ $mg^{-1}$. Proteolytic activity of *D. magna* trypsin activity was assayed using the substrate N-benzoyl-DL-arginine p-nitroanilide (BApNA) at a final concentration of 1.4 mM in 7.5% (v/v) dimethylsulfoxide (DMSO). The reaction was carried out in 0.1 M phosphate buffer, pH 7.5. The solution was thermoequilibrated at 20°C before the addition of 5µl of *D. magna* homogenate to start the reaction. The hydrolysis of substrate was monitored continuously at 390 nm for 10 min and checked for linearity. Chymotrypsin activity was assayed as above, except that N-succinyl-alanine-alanine-proline- phenylalanine p-nitroanilide [S(Ala)2ProPhepNA] was used as a substrate at a final concentration of 0.95 mM in 7.5% (v/v) DMSO. Crude extract of *Microcystis* sp. strain BM25 SPE was prepared to observe inhibition of digestive proteases of *D. magna*. All the assays were carried out in triplicate and the mean data of the treatments are presented in results section.

**Statistical Analysis:** Statistical analyses were conducted with the program Sigmaplot 11.0. The data were analyzed using one way ANOVA and a post hoc analysis [Tukey's honestly significant difference (HSD)]. Graph were made with Microsoft office Excel 2007.

## RESULTS

An extract was produced from *Microcystis* sp. strain BM25 from freeze-dried biomass. Then, this extract was loaded onto a C18 solid-phase extraction and successively eluted with 20%MeOH, 40% MeOH, 60% MeOH and 100% MeOH. These SPE fractions were tested for the inhibition of trypsin and chymotrypsin in *Daphnia magna* homogenate. Inhibition of chymotrypsin activity was measured by preparing different fractions i.e. 20%, 40%, 60%, 80% and 100% of *Microcystis* sp. strain BM25. Without addition of any SPE-fraction, the Daphnia homogenate had a chymotrypsin activity. Inhibition of trypsin was measured different volume of this cyanobacterial strain and no significant inhibition observed (Appendix: Figure A1).

Addition of aliquots of the 20% MeOH and the 100% MeOH SPE-fractions had almost no effect on the chymotrypsin activity. For 20% MeOH SPE-fractions, chymotrypsin activity was 287nmol pNA$min^{-1}mg^{-1}$and for 100% MeOH SPE-fractions, chymotrypsin activity was 289 nmol pNA $min^{-1}mg^{-1}$. However, addition of aliquots of 60% MeOH and 80% MeOH SPE-fractions resulted in chymotrypsin activities of 6 nmol pNA $min^{-1}$ $mg^{-1}$ and 4 nmol pNA $min^{-1}$ $mg^{-1}$ (Figure 2). This indicates that the

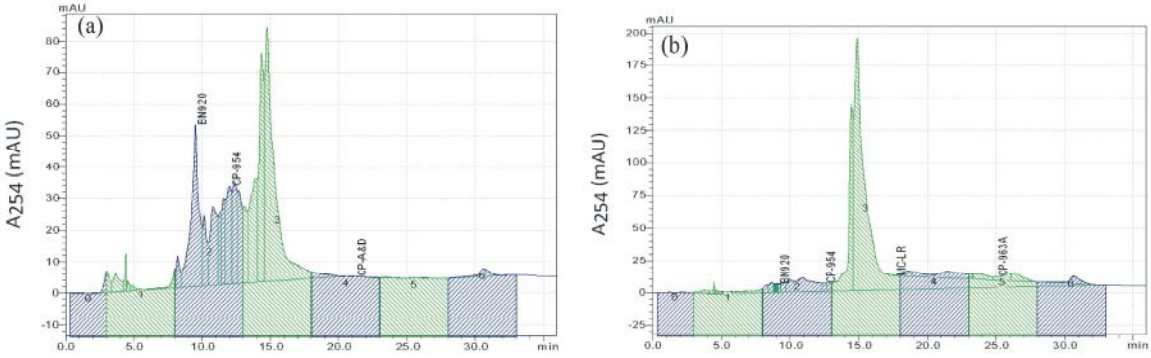


Fig. 1: High performance liquid chromatography and fractionation of 50µL of an extract that had been eluted from a C18-SPE cartridge with (a) 60 % MeOH and (b) 80%. of *Microcystis* sp strain BM25. Numbers and vertical lines indicate retention times for fractions number 0-6. A gradient of 6 min: Several peaks are seen in the fractions 1-3, which indicates the existence of compounds among these fractions.

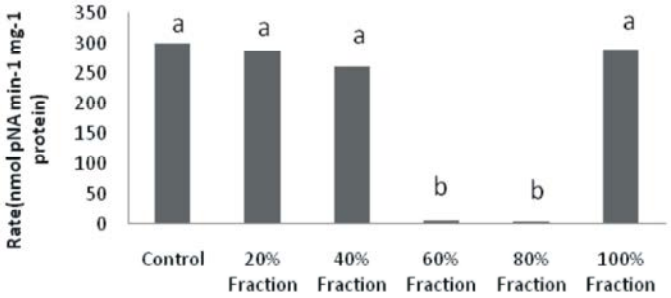


Fig. 2: Effects of an extract of *Microcystis* sp. strain BM25 on chymotrypsin activity from homogenate of *D.magna*. The crude extract of this cyanobacterial strain had been loaded onto a C18-solid phase extraction (SPE) cartridge and successively been eluted by 20% MeOH, 40 % MeOH, 60% MeOH, 80% MeOH, 100% MeOH and aliquots of each SPE fraction were added inhibition of chymotrypsin activity were determined. It was observed that among different percentage of fractions only the 60% and 80% fractions of *Microcystis* sp. strain BM25 completely inhibited chymotrypsin activity of *D. magna*. Shown are mean values ± SD (n=3). Different letters indicate a significant difference among the content of the respective inhibitor (Tukey's HSD after one-way ANOVA, P <0.05).

60% MeOH and 80% MeOH SPE fractions inhibited the Daphnia chymotrypsin by 98% and 99 %. Therefore, only the 60% MeOH and 80% MeOH SPE fractions used were for the subsequent HPLC analyses.

After that, 50 µL of 60% MeOH and 80% MeOH SPE fractions were injected to HPLC to separate chymotrypsin inhibitors from other compounds. The sample was chromatographed on a gradient with 6 min: 40% MeOH to 25 min: 80% MeOH and seven fractions were collected from each SPE-fractions thus that the entire HPLC run was collected in fractions. A few peaks were detected at different retention times (Figure 1a and Figure 1b), which indicates that different compounds were present among the fractions. After collecting the fractions were evaporated to dryness and re-dissolved in the 250 µL of methanol. Aliquots of this methanolic solution were added to *Daphnia magna* homogenate and then the chymotrypsin activity was determined with the aim to identify which specific fraction would inhibit *D. magna* chymotrypsin activity. In each assay 25 µL of the fractions was used (Appendix: Figure 1B and Figure 2A) without any additions (control) had a chymotrypsin activity of 367 nmol pNA min $min^{-1}mg^{-1}$ (Appendix: Figure 1B). Addition of aliquots of the HPLC fractions 0, 1, 2, 5 and 6 did not change this activity (Appendix: Figure 1B). However, addition of HPLC fraction 4 resulted in an activity of 292 nmol pNA $min^{-1}mg^{-1}$and addition of HPLC fraction 3 led to chymotrypsin activity of 14 nmol pNA $min^{-1}$ $mg^{-1}$. This means that HPLC fraction 4 inhibited the Daphnia chymotrypsin activity by 20% and fraction 3 inhibited 96 %.

**Appendix A:**

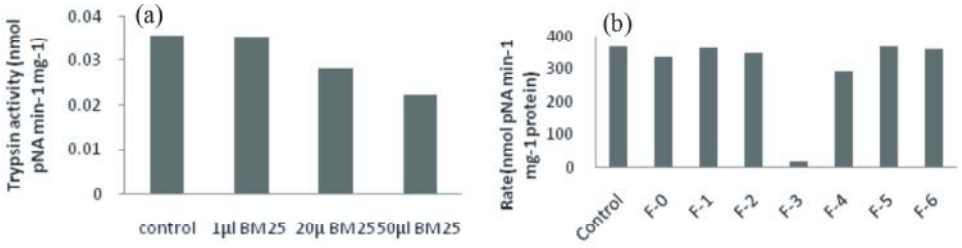


Fig. 1: (A) Effects of an extract of *Microcystis* sp strain BM25 on trypsin activity from homogenate of *D. magna* were determined by monitoring the release of p-nitroaniline (pNA).No significant inhibition of trypsin observed. (B) Chymotrypsin activity was measured by collecting seven fractions from HPLC. In HPLC 80% MeOH SPE- fraction was injected. And 25µl of the HPLC-fractions were used in each measurement. It was found that fraction 3 inhibits chymotrypsin activity. Shown are mean values ± SD (n=3).

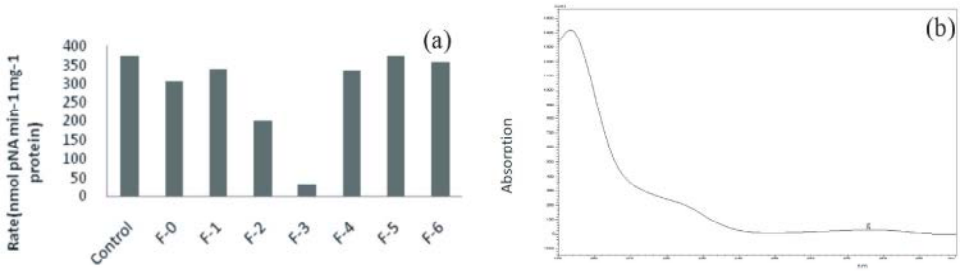


Fig. 2: (A) Chymotrypsin activity was measured by collecting seven fractions from HPLC. In HPLC 60% MeOH SPE-fraction was injected. And 25µl of the HPLC-fractions were used in each measurement. Fraction 3 inhibits chymotrypsin activity more than any other fractions. However, it was found that fraction 2 also inhibited chymotrypsin activity but to some lesser extent. Shown are mean values ± SD (n=3). Different letters indicate a significant difference among the content of the respective inhibitor (Tukey's HSD after one-way ANOVA. (B) Injected volume was 10 µl of 80% *Microcystis* sp BM25 at 55-55% isocratic condition. UV-spectrum of the peak with a retention time of 20.98 min and the spectrum shows maximum at 196 nm, 225 nm and 276 nm.

For 60% MeOH C18-SPE fraction (Figure 2A), *D.magna* homogenate had a chymotrypsin activity of 375 nmol pNA $min^{-1}mg^{-1}$ (Appendix: Figure 2A). Addition of aliquots of the HPLC fractions 0, 1, 4, 5 and 6 did not change this activity (Appendix: Figure 2A). However, addition of HPLC fraction 2 resulted in an activity of 200 nmol pNA $min^{-1}mg^{-1}$and addition of HPLC fraction 3 led to chymotrypsin activity of 32 nmol pNA $min^{-1}mg^{-1}$ (Appendix: Figure 2A). This means that HPLC fraction 2 inhibited the Daphnia chymotrypsin activity by 47% and fraction 3 inhibited 91 %. It indicates that both, the 60% MeOH and the 80% MeOH C18-SPE fraction contain the same chymotrypsin inhibitor.

10µl of 80% and 60%MeOH C18-SPE fraction was injected into HPLC using gradient with 6min 55% and 25min 55% condition and seven fractions were collected. After 20.98min retention time, a peak was observed at a wavelength of 254nm (Appendix: Figure 2B) for 80% MeOH C18-SPE fraction. Finally, inhibition of chymotrypsin activity was determined by bioassay and it was observed that fraction 4, 3 and 5 inhibits chymotrypsin activity than the other fractions.

## DISCUSSION

Crude extract of *Microcystis* sp. strain BM25 was assayed to compare inhibition of trypsin and chymotrypsin activity. There was no significant inhibition observed for trypsin activity but the 60% and 80% SPE fractions of this *Cyanobacterium* strain completely inhibited chymotrypsin activity of *D. magna*. This finding is in line with Schwarzenberger, Sadler and Von [13] and it is therefore clear that ingestion of this cyanobacterial strain by *D. magna* leads primarily to interference with the animal's chymotrypsin and not with its trypsin activity [13]. Therefore it was concluded that this strain BM25 of *Microcystis* contains considerably more chymotrypsin inhibitors than

trypsin inhibitors and thus all further work focused on the purification of chymotrypsin inhibitors from this *Cyanobacterium*.

After HPLC, several peaks indicated the existence of different compounds present in *Microcystis* sp. strain BM25 60% and 80% MeOH SPE fractions. After subsequent bioassays revealed that there were compounds present which inhibited *D. magna* chymotrypsin activity. Fraction 3 was inhibitory as it was for the 60% and 80% MeOH C18-SPE fraction. This indicates that both, the fraction contain the same chymotrypsin inhibitor. These results demonstrated that *Microcystis* sp. strain BM25 contains chymotrypsin inhibitors which interfere with the digestive proteases of *D. magna*. Recently the two major types of proteases in *D. magna* were categorized as trypsins and chymotrypsins [13, 14]. Numerous cyanobacteria produce protease inhibitors, which are mostly cyclic or linear peptides. A number of such peptides have been isolated from natural water blooms, which inactivate trypsin or chymotrypsin activity [13, 14]. Protease inhibitors are a group of cyanobacterial secondaryy metabolites that interfere with growth and reproduction of herbivorous zooplankton. These protease inhibitors are found in many cyanobacterial blooms which inhibit digestive serine proteases of *D. magna* [13]. These serine proteases represent the most important digestive enzymes in the gut of this small crustaceans Daphnia [14].When cyanobacterial cells with protease inhibitors are ingested, then the protease inhibitors negatively affect the fitness of *D. magna* by decreasing the protease activity in the gut and thus finally reducing somatic growth of Daphnia [15].

An experiment was done to identify protease inhibitors in cyanobacteria and it was found *Microcystis aeruginosa* produces few secondary metabolites which inhibits digestive protease from a major phytoplankton grazer in freshwater lakes i.e the genus Daphnia [15, 16]. Two major classes of digestive proteases, trypsin and chymotrypsin, in *D. magna* have been found to have multiple inhibitors with different specificities against them which were contained by *Microcystis aeruginosa* PCC 7806 [16, 17]. Those inhibitors possess a chemical nature which is still not known. Cyanopeptolins A-D and microcystins are known to be produced by *M. aeruginosa* PCC 7806 but, trypsin or chymotrypsin are not inhibited by microcystins [18, 19]. However, bovine trypsin has been inhibited by Cyanopeptolin A [20]. Whether cyanopeptolins belongs to the inhibitors of *D. magna* trypsins needs to remain tested because there are subsequent difference in sensitivity found between proteases of bovine origin and from *D. magna* to synthetic inhibitors and inhibitors from *Microcystis aeruginosa* [20-22].

In conclusion, it is clear that chymotrypsin activity of *D. magna* is inhibited by *Microcystis* sp. strain BM25. After HPLC and bioassay it is observed that peaks is correlated with the chymotrypsin inhibition of *D. magna*. However, as separation of these two peaks by HPLC has not yet been successful, different gradients, column, mobile phase, temperature was used to separate chymotrypsin inhibitors from other compounds. It remains to be seen, if it is one or two major inhibitors. And it remains to be tested by LC-MS, if these inhibitors are known chymotrypsin inhibitors or yet unknown compounds.

## CONCLUSIONS

Extracts of *Microcystis* sp. strain BM25 inhibit one of the major proteases of *Daphnia magna*, i.e. chymotrypsins. Chymotrypsin activity was inhibited but trypsin activity was not inhibited by methanolic extracts of this *Cyanobacterium* strain. It was observed that 60% MeOH and the 80% MeOH C18-SPE fraction inhibits chymotrypsin activity 98% and 99 % respectively. After HPLC, there were some fractions were collected from 60% and 80% MeOH C18-SPE fraction. HPL-fractions number 3 inhibited chymotrypsin activity completely. It was true for both *Microcystis* sp. strain BM25 60% and 80% MeOH C18-SPE-fractions inhibits chymotrypsin activity completely. So, it is assumed that there are common chymotrypsin inhibitors present in 60% and 80% MeOH C18-SPE-fractions. Separation of peaks is not been successful yet. Further research work is needed to identify chymotrypsin inhibitor by LC-MS. It is still unknown that which compound of this *Cyanobacterium* inhibits chymotrypsin activity of *D. magna*.

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