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Research Article

STUDY ON ISOLATION OF CYANOBACTERIA FROM JABALPUR REGION OF CENTRAL INDIA AND SCREENING THEIR PROTEASE INHIBITOR(S) ACTIVITY

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ABSTRACT

Central part of India is known for different environment throughout the year, in this way this part of the world has shown greater diversity among freshwater cyanobacteria. The present study showcases a screening program for the hunt of potent protease inhibitors compounds from cyanobacteria collected from Central India. A total of 176 cyanobacterial samples collected, from different habitats of the Jabalpur area of Central India, which finally yielded 25 uni-algal cultures belonging to eight genera and all these genera belong to different species of cyanobacteria. These strains were grown in the laboratory for many generations and screened for their inhibitory activity against three major proteases trypsin, chymotrypsin and papain using an endpoint and kinetic assay. Extracts from Microcystis sp. DL09, Gloeocapsa sp. DL17 and Microcystis sp. DL22 were shown the most potential protease inhibitory activity against all three enzymes. During kinetic studies the IC50 values for all the three enzymes did not vary much from those observed during endpoint assays. The proposed research showed that these Cyanobacteria have showed promising protease inhibitor activities. In the context of the possible use of such protease inhibitors in pharmaceutics and other areas will be great potential for the future.

Keywords: Cyanobacteria, Protease inhibitor, Trypsin, Chymotrypsin, Papain.

1. INTRODUCTION

Cyanobacteria are a large and widespread group of microorganisms found in a wide range of habitats, including aquatic, terrestrial, benthic and extreme environments over the world. Cyanobacteria are also important in many terrestrial environments and they live inside soils, on rocks and form symbiotic associations [1].Cyanobacteria can develop in large masses in eutrophic fresh and brackish water all over the world, including Australia, South-Africa, Brazil, Argentina, and the Northern European countries. Cyanobacteria sample was collected from different water bodies. In this way some species of cyanobacteria, i.e., Lyngbya sp., Lyngbya semiplena [2], Oscillatoria sp. [3], were collected from marine environment [4]. Some cyanobacteria can develop large masses in fresh water reported by another scientist.

Central part of India is also known for different environment throughout the year, in this way this part of the world has shown greater diversity among freshwater cyanobacteria. Large number of water bodies in this area harbour heavier planktonic as well as benthic cyanobacterial masses [5]. Similarly terrestrial and epiphytic cyanobacterial genera

also reported. They are most abundant in aquatic habitats as part of the plankton, some can be found tightly or loosely attached to surfaces of plants, rocks and sediments [6], and some can be found in fresh water. Recently Chaturvedi et al. [7] collected blooms from central India. The immense diversity within this group of microorganisms, apart from the variability of the morphology and range of habitats, is also reflected in the extent of their synthesis of natural products. Cyanobacteria have evolved to produce a diverse array of secondary metabolites that have aided species survival in these varied and highly competitive ecological niches [8]. These compounds have shown the evidence of vital biological activities important for our survival, which clearly indicates that cyanobacteria have a valuable potential for providing novel and diverse bioactive substances for drug discovery and can be considered a prime source for leads for drugs.

Cyanobacteria are known to produce metabolites with diverse biological activities, such as antibacterial, antifungal and antiviral, herbicides, anticancer and antiplasmodial activity [9]. Besides this they are also known to produce protease inhibitors [10]. Most of the protease secreted by bacteria and fungi has a tendency to react with protein and initiate the catabolism of protein by hydrolysis of the peptide bonds. Such enzymes have a wide presence in the living world and are present in almost all organisms. Proteases cause degradation of protein, but there are some special compounds which have the ability to counter the effect of proteases and stop the hydrolysis of another protein. Such compounds are known as protease inhibitors. These are among the most widely spread secondary metabolites, as they have been found in nearly every cyanobacterial bloom. The diverse physiologies of cyanobacteria serve as an excellent base for production of enzyme inhibitors. The most pronounce enzyme inhibitors are the inhibitors of proteases. Previously discussed most of the peptide and depsipeptides produced by cyanobacteria have the ability to act as protease inhibitors. Most of the peptide produced by the cyanobacteria has the ability to act as protease inhibitors.

A large number of reports starting from the report of Agrawal et al [11] for the presence of protease inhibitor from the water blooms of cyanobacteria from Central India have now been published. A large number of protease inhibitors belonging mainly to the classes of peptides and depsipeptides have been reported so far from the water blooms as well as from the laboratory cultures of cyanobacteria. The present study is an attempted to isolate local cyanobacterial genera with the ability to produce protease inhibitors. In the context of the possible use of such protease inhibitors in pharmaceutics and other areas, the proposed work is the first attempt in this direction. For the study, these cyanobacteria were collected from the Central India with the ability to produce protease inhibitors. Under axenic conditions, these strains were maintained under laboratory environmental and nutritional conditions for many generations and the protease inhibitory properties of all strains were compared against major proteases.

2. MATERIALS AND METHODS

2.1 Collection, Isolation and Identification of the Cyanobacteria

Cyanobacteria samples were collected from different region of Jabalpur areas as shown in (Fig. 1) during the study. All samples were collected from various ponds, ditches and lakes. These samples belong to terrestrial, benthic, epiphytic and planktonic habitats. The cyanobacteria natural sample collected from the field was taken to the laboratory. These natural samples were mixed with soil, dust and adherent particles. These samples were washed with distilled water 2-3 times to remove adherence soil and other impurities [12]. The samples examinations were done under microscope (Labomed, India) for the presence of cyanobacteria in the natural samples [13]. The natural samples having more than 40 % of green algae cell per observation field were discarded. The samples having cyanobacterial abundance were kept for further use. Natural samples of cyanobacteria were dried according to [14]. Dried powder was kept in an airtight bottle in -20°C until further use [15].

Cyanobacteria cells in these samples were cultured under laboratory condition using liquid media. The selection of media was based on the microscopic observation. Cyanobacteria showing the presence of heterocysts were shifted to BG-11 medium without nitrate (BG 11-NO3) [16]. Colonies of unicellular cyanobacteria were shifted to cyano medium [17]. Other cyanobacteria were shifted to BG-11 medium with nitrate, (BG 11+NO3). To obtain uni-algal cultures of various cyanobacteria streak plate method [18],

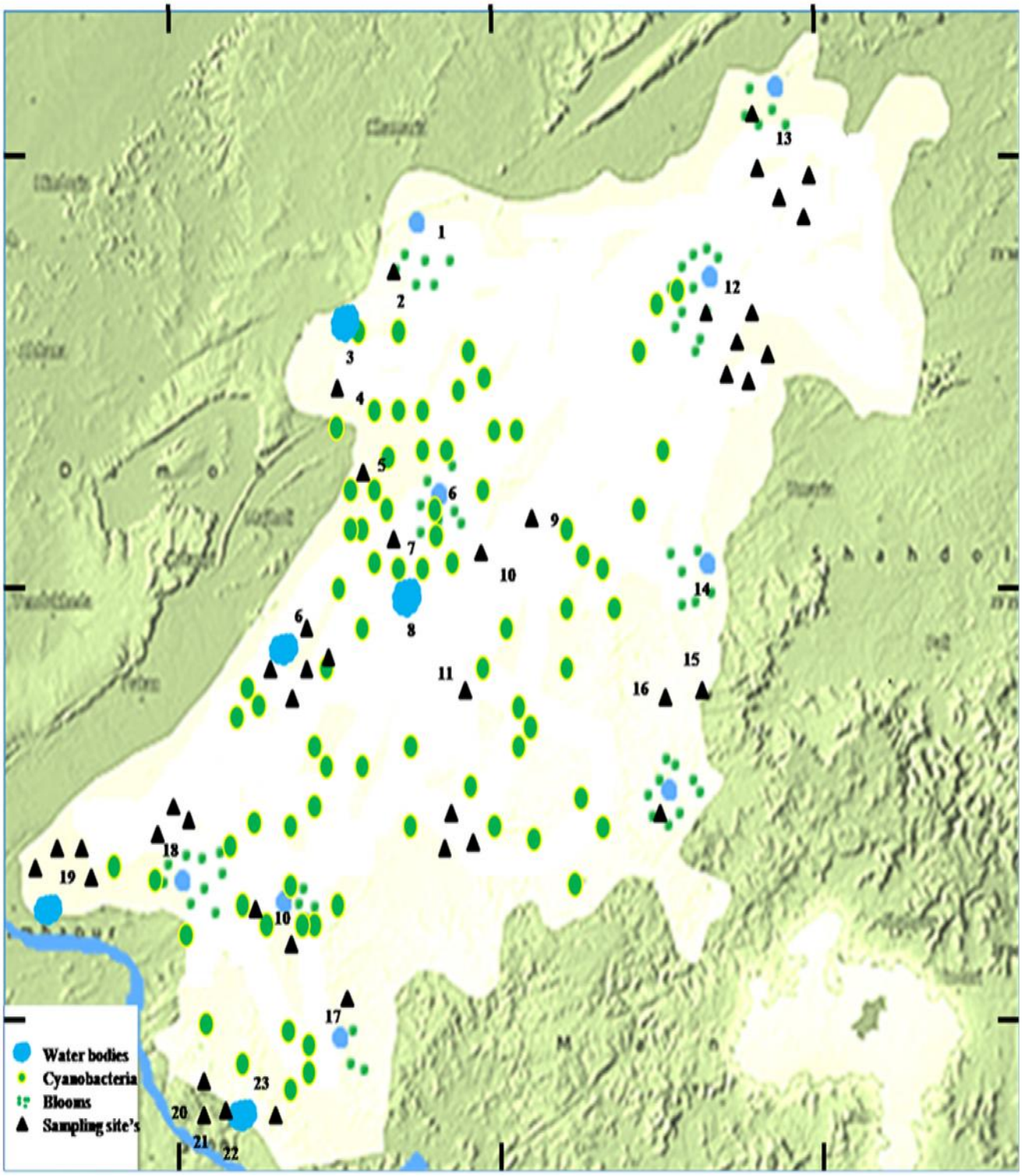

Figure1: Map of sampling site of cyanobacteria in Jabalpur region.

1. Vijay Nagar 2. Ukhari road 3. Gulaua chowk 4. Garha 5. Yadav colony 6. Ranital 7. Madan Mahal 8. Local pond 9. Wright town 10. Napier town 11. Srinath ki talaiya 12. Hnuman tal 13. Adhartal lake 14. Kahambh tal 15. Sanjivini Nagar 16. Cantonment area 17. Gwarighat 18. Ganga Sagar 19. Supatal 20. Tewar 21. Panchwati ghat 22. Saraswati ghat 23. Bhedaghat

light and dark plate method [19] and dilution methods [17] were used during the study. The cyanobacteria were identified using literature and identification keys [20], [1]. The isolated cultures were maintained in different medium under white fluorescent lights (1,500 lux) with a light–dark cycle of 16:8 h at 28 ± 2 °C. To make bacteria-free culture of cyanobacteria, cultures were constantly given treatment with antibiotics. The cultures were grown in media supplemented with cycloheximide (20 µg ml^{-1}) [21], ampicillin and penicillin G (100 µg ml^{-1}) [22]. All the cultures were grow for several generations to make bacteria free culture and the sterility of the cultures was verified by inoculating culture broth to nutrient broth. For bulk culturing, the cultures were allowed to grow exponentially for 30 days and the filaments and colonies were harvested by centrifugation and freeze dried. Dried powder was stored at -20°C until use.

2.2 Preparation of cyanobacterial extracts-

For extraction, 100 mg dried powder of each Cyanobacterium species were extracted thrice with 100 ml of 75 % methanol. All the three extracts were pooled, dried under vacuum and redissolved in 1 ml of 50 % methanol. This extract was used as a source of protease inhibitors from cyanobacteria [23].

2.3 Protease inhibitors assays

2.3.1 Endpoint assays-

The endpoint assays were performed using Hammerstein's casein as a substrate. Casein was dissolved in 100 mM Tris/HCl buffer, pH 8.5, at a concentration of 1 % (w/v). The enzymes solution for three enzymes, trypsin, chymotrypsin and papain were prepared in 1 mg ml-1 concentration in 100 mM Tris/HCl buffer, pH 8.5 (trypsin), 100 mM Tris/HCl buffer, pH 8.8 (chymotrypsin) and 100 mM phosphate buffer, pH 6.5 (papain) with 2 mM DTT for all these enzymes. For enzyme inhibition assay, 50 µl of each enzyme was pre-incubated with 50 µl of each cyanobacterial extract for 30 min at 37 °C. Controls for each enzyme were run that received 50 % methanol in lieu of any cyanobacterial extract. Appropriate blank (cyanobacteria extract) was run with all sets that received no enzyme. The reactions was started immediately by the addition of 100 µl substrates (1 % casein) and stop the reaction with 1.2 ml of 10 % tricholoro acetic acid exactly after 20 min incubation. Exactly after 10 min the reaction mixture was centrifuged and the absorbance was measured at spectrophotometer, 280 nm. Tyrosine was released in the end of the reaction measured using standard curve of tyrosine in 0.1 N HCl (R2 = 0.992) at 280 nm. The enzyme inhibition activity of all cyanobacteria extract during endpoint assays is presented as nM of tyrosine min-1 (mg protein)-1. All these assays were carried out in triplicate sets and the data were analyzed by using one-way ANOVA and post hoc comparisons (Tukey) according to Agrawal [24].

2.3.2 Kinetic assays-

Enzyme inhibition activity with kinetic assays were performed with specific chromogenic substrates i.e. BApNA and [S(Ala)2ProPhepNA]. BApNA used as a substrate for trypsin activity at a final concentration of 1.4 mM in 7.5 % (v/v) dimethylsulfoxide (DMSO). The reaction was carried out using 0.1 M Tris/HCl buffer, pH 8.5. The enzyme and inhibitor solution was pre-incubated for 30 min at 37 °C before the addition of substrate to start the reaction. After 10 min the hydrolysis of substrate monitored at 390 nm. P-nitroanilide (pNA) released at the end of the reaction and was measured using standard curve of pNA in DMSO (R2 = 1) at 390 nm. The same procedure as followed for papain and chymotrypsin with different substrate and buffer solution as well. 100 mM phosphate buffer, pH 6.5 supplemented with 2 mM DTT for papain assays. [S(Ala)2ProPhepNA] used for chymotrypsin assays at a final concentration of 0.95 mM in 7.5 % (v/v) DMSO. Tyrosine was released in the end of the reaction measured using standard curve of tyrosine in 0.1 N HCl (R2 = 0.992) at 280 nm. The enzyme inhibition activity during kinetic assays is presented as nM pNA min-1 (mg protein)-1. All these assays were carried out in triplicate sets and the data were analyzed by using one-way ANOVA and post hoc comparisons (Tukey) according to Patel et al [25]. 50 % inhibitory activity in both endpoint and kinetic assays were performed with all cyanobacterial extract. Different concentration of cyanobacterial extract was used calculation of 50 % inhibitory activity. For this 0, 1, 10, 100, 1,000 and 10,000 µg dry weights used these entire dry weight equivalent to cyanobacterial extracts. Sigma Graph Pad Prism® version 6.0 used for the IC50 calculation by fitting a sigmoidal dose–response curve.

3. RESULTS

In this study, all cyanobacteria samples were isolated from different sampling sites of the Jabalpur area of Central India (Fig. 1). In total, 176 samples of different cyanobacteria from different habitat were collected, out of them 25 uni-algal cultures were isolated. These uni-algal cultures belong to eight genera, identified based on their morphological features according to Desikachary [20] and Whitton and Potts [1]. These genera include Oscillatoria sp., Lyngbya sp., Nostoc sp., Anabaena sp., Fischerella sp., Microcystis sp., Gloeocapsa sp. and Calothrix sp. Out of them, some species of cyanobacteria belong to the same genera so avoiding confusion each cyanobacteria species gave a unique lab id and number and maintained in the laboratory during the study. The morphological features of the uni-algal cultures are shown in (Fig. 2). The isolated cyanobacteria were cultured and maintained in an axenic state in the laboratory.

3.1 Protease inhibition during endpoint assays

When the cultured biomasses of all cyanobacteria strains were extracted with 75 % aqueous methanol, the degree of inhibition by these extracts of different strains of cyanobacteria differed very much against all tested enzymes in both the assays: endpoint and kinetic (Table 1).

3.1.1 Endpoint assays-

Protease inhibitory activity of three enzymes during endpoint assays performed with or without to five mg dry weight equivalent cyanobacterial extracts in the reaction mixture. Endpoint assays were performed with Hammarsten's casein, used as substrate for trypsin, chymotrypsin and papain. Enzyme activity was represented as an amount of end product tyrosine released per min per mg protein. Absorbance of the reaction mixture calculated with standard curve of tyrosine. The data presented are mean ± SD (n = 3). The activities were present in term of [µM tyrosine min-1 (mg protein)-1].

Trypsin assay- During endpoint assays, the activity of trypsin was reduced by cyanobacteria extracts when used in five mg dry weight equivalent concentrations compared to control. The trypsin (control) activity was observed 44.19 ± 2.64 µM tyrosine min-1 (mg protein)-1. During the assays Microcystis sp. DL 22 shows maximum activity 3.16 ± 0.89 µM tyrosine min-1 (mg protein)-1 and 92.80% enzyme inhibition. Three cyanobacteria extract Oscillatoria sp. DL 08 shows 79.10% reduction; Microcystis sp. DL 09 shows 90.30 % and Gloeocapsa sp. DL 17 shows 82.20 % enzyme inhibition. Five species of cyanobacteria Calothrix sp. DL 01, Anabaena sp. DL 19, Nostoc sp. DL 20, Nostoc sp. DL 21 and Nostoc sp. DL 25 were unable to inhibit this enzyme. The enzyme inhibition observed less than 30 % of these species. Remaining extracts of sixteen cyanobacteria shows greater than 30 % enzyme inhibition compared as a control.

Chymotrypsin assays- Similarly chymotrypsin also reduced by the cyanobacteria extracts as compared to control. During the assays chymotrypsin (control) activity was observed 76.91 ± 0.39 µM tyrosine min-1 (mg protein)-1. The maximum activity shows by Microcystis sp. DL22 6.50 ± 0.15 µM tyrosine min-1 (mg protein)-1 and 91.54% inhibition. Microcystis sp. DL 09 shows 91% enzyme inhibition and Gloeocapsa sp. DL 17 shows 86.72% enzyme inhibition. Three extracts of cyanobacteria species Oscillatoria sp. DL 18, Nostoc sp. DL 20 and Nostoc sp. DL 21 were unable to inhibit this enzyme and shows less than 30 % enzyme inhibition. Out of them nineteen extract of cyanobacteria was shown average enzyme activity and shows greater than 30 % enzyme inhibition.

Papain assays - During endpoint assays with papain, a similar trend was observed when treated with five mg equivalent to all cyanobacterial extracts. The degree of inhibition observed different for all cyanobacteria extract. The papain (control) activity shows 43.20 ± 3.09 µM tyrosine min-1 (mg protein)-1. Gloeocapsa sp. DL 17 shows the maximum enzyme activity 10.60 ± 0.71 µM tyrosine min-1 (mg protein)-1 and 75.40% enzyme inhibition. Oscillatoria sp. DL 08 shows 74.20 % enzyme inhibition. Remaining extract of cyanobacteria species shows enzyme inhibition activity between 30-70%.

3.1.2 Kinetic assays-

Enzymatic activity of three enzymes during kinetic assays performed with or without to five mg dry weight equivalent cyanobacterial extracts in the reaction mixture. Substrate BApNA and [S(Ala)2 ProPhepNA] were used for the study. BApNA used as substrate for trypsin and papain and [S(Ala)2 ProPhepNA] for chymotrypsin. Enzyme activity was represented as an amount of end product pNA released per min per mg protein. Absorbance of the reaction mixture

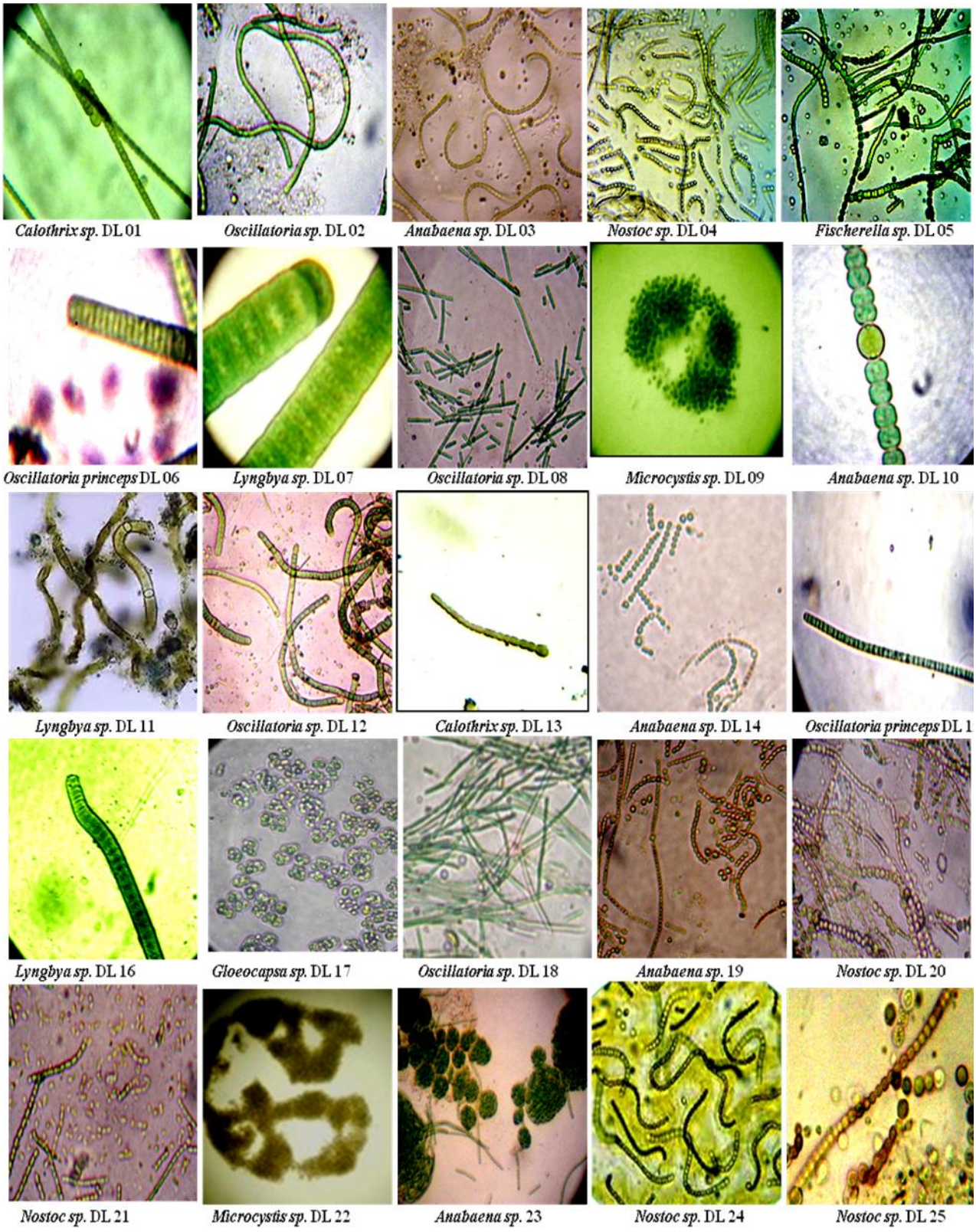

Figure 2: Cyanobacteria isolated from Central India.

Table 1: Enzymatic activities of trypsin, chymotrypsin and papain during endpoint assays as well as kinetic assays with or without to 5 mg dry weight equivalent cyanobacterial extracts in the reaction mixture.

	Protease activity					
	Endpoint assays			Kinetic assays		
	Trypsin	Chymotrypsin	Papain	Trypsin	Chymotrypsin	Papain
	[μM tyrosine min^{-1}(mg protein)$^{-1}$]			[nM *p*NA min^{-1} (mg protein)$^{-1}$]		
Control	44.19 ± 2.64	76.91 ± 0.39	43.20 ± 3.09	242.66 ± 3.51	964.36 ± 16.26	381.23 ± 1.40
Calothrix sp. DL 01	32.22 ± 0.06 (27%)	47.98 ± 2.36* (37.6%)	27.5 ± 0.60* (36.3%)	168.73 ± 3.97*(30.4%)	460.77 ± 13.45*(52.5%)	239.09 ± 0.48*(37.28%)
Oscillatoria sp.DL02	22.49 ± 0.05*(49.1%)	45.05 ± 0.94*(41.4%)	17.82 ± 1.20*(58.75%)	28.2 ± 0.36*(88.3%)	239.6 ± 32.26*(75.1%)	81.73 ± 0.86*(78.5%)
Anabaena sp. DL03	30.31 ± 0.53(31.4%)	47.13 ± 1.67*(38.7%)	25.37 ± 0.96*(41.2%)	123.15 ± 4.88*(49.2%)	117.65 ± 1.71*(87.8%)	168.47 ± 2.35*(55.8%)
Nostoc sp. DL04	30 ± 0.17*(32.1%)	45.61 ± 1.91*(40.6%)	21.34 ± 1.30*(50.6%)	142.32 ± 1.20*(41.3%)	364.18 ± 4.00*(62.2%)	94.41 ± 1.66*(75.2%)
Fischerella sp. DL05	29.69 ± 0.57(32.8%)	46.35 ± 2.26*(39.7%)	25.92 ± 0.64*(40%)	138.04 ± 2.51*(43.1%)	373.72 ± 11.93*(61.2%)	177.16 ± 1.58*(53.5%)
Oscillatoria princeps DL06	24.93 ± 0.86*(43.52%)	35.36 ± 0.82*(54.02%)	24.64 ± 1.07*(42.96%)	103.5 ± 5.3*(57.34%)	319.13 ± 8.0*(66.9%)	156.89 ± 3.04*(58.8%)
Lyngbya sp. DL07	16.93 ± 3.14*(61.1%)	27.21 ± 1.46*(64.62%)	14.64 ± 1.97*(66.11%)	38.50 ± 0.42*(84.13%)	296.26 ± 0.46*(69.27%)	54.90 ± 0.11*(85.50%)
Oscillatoria sp.DL08	9.20 ± 0.74*(79.1%)	35.05 ± 0.39*(54.4%)	11.11 ± 1.48*(74.2%)	18.2 ± 0.36*(92.4%)	19.87 ± 3.89*(90.4%)	51.73 ± 0.86*(86.4%)
Microcystis sp. DL09	4.26 ± 1.44*(90.3%)	6.88 ± 0.68*(91%)	15.60 ± 0.71*(63.8%)	13.25 ± 0.67*(94.53%)	81.22 ± 1.23*(91.5%)	142.42 ± 2.51*(62.6%)
Anabaena sp. DL10	23.26 ± 0.28*(47.3%)	35.01 ± 0.92*(54.4%)	18.13 ± 1.58*(58%)	68.81 ± 0.97*(71.64%)	311.09 ± 8.77*(67.7%)	78.39 ± 2.05*(79.4%)
Lyngbya sp. DL11	26.01 ± 1.62*(41.14%)	43.61 ± 0.97*(43.2%)	26.56 ± 0.66*(38.51%)	74.52 ± 0.60*(69.2%)	353.08 ± 1.33*(63.3%)	192.23 ± 1*(49.5%)
Oscillatoria sp.DL12	20.33 ± 1.23*(53.9%)	34.55 ± 0.69*(55%)	20.98 ± 0.65*(51.4%)	85.47 ± 1.67*(64.68%)	287.83 ± 2.22*(70.1%)	73.17 ± 2.28*(80.8%)
Calothrix sp. DL 13	23.32 ± 0.28*(47.2%)	35.61 ± 0.76*(53.6%)	27.06 ± 0.59*(37.3%)	96.24 ± 3.20*(60.34%)	326.66 ± 6.67*(66.12%)	216.27 ± 4.93*(43.27%)
Anabaena sp. DL14	18.78 ± 0.75*(57.5%)	28.54 ± 0.91*(62.8%)	27.46 ± 0.41*(36.43%)	58.81 ± 0.97*(75.76%)	303.76 ± 4.17*(68.5%)	224.15 ± 7.63*(41.2%)
Oscillatoria princeps DL15	29.2 ± 1.51(33.9%)	46.62 ± 2.42*(39.3%)	20.60 ± 0.51*(52.3%)	134 ± 2.07*(44.6%)	379.21 ± 9*(60.6%)	89.09 ± 1.55*(76.6%)
Lyngbya sp. DL16	23.62 ± 0.78*(46.5%)	44.41 ± 0.53*(42.2%)	19.15 ± 0.62*(55.67%)	86.91 ± 5.17*(63.08%)	348.10 ± 2.30*(63.9%)	68.82 ± 2.11*(81.94%)
Gloeocapsa sp.DL17	7.85 ± 0.30*(82.2%)	10.21 ± 0.70*(86.72%)	10.60 ± 0.71*(75.4%)	10.53 ± 0.93*(95.67%)	71.24 ± 1.11*(92.61%)	37.01 ± 2.79*(90.29%)
Oscillatoria sp.DL18	25.89 ± 1.55*(41.14%)	65.31 ± 0.61*(15.32%)	21.22 ± 0.98*(32.3%)	170.75 ± 3.90*(29.6%)	861.98 ± 6.01(10.6%)	379.4 ± 1.16*(0.47)
Anabaena sp. DL19	30.79 ± 0.99 (30.2%)	48.41 ± 1.91*(37%)	21.04 ± 1.19*(51.2%)	164.46 ± 2.06*(32.2%)	451.53 ± 15.62*(53.1%)	151.92 ± 2.05*(60.1%)
Nostoc sp. DL20	32.1 ± 0.13(27.4%)	52.43 ± 2.57*(31.8%)	14.67 ± 1.98*(66%)	119.49 ± 1.10*(50.7%)	219 ± 3.89*(77.29%)	59.46 ± 0.80*(84.4%)
Nostoc sp. DL21	31.33 ± 0.74*(29.1%)	53.49 ± 1.07*(30.5%)	27.61 ± 0.62*(36%)	140.5 ± 0.10*(42.1%)	864.56 ± 6.06*(10.3%)	232.02 ± 2.02*(39.13%)
Microcystis sp. DL22	3.16 ± 0.89*(92.8%)	6.50 ± 0.15*(91.54%)	14.13 ± 2.45*(67.29%)	12.54 ± 0.93*(94.88%)	84.52 ± 4.03*(91.2%)	134.1 ± 2.07*(64.8%)

Anabaena sp. DL23	28.09 ± 0.96*(36.4%)	47.02 ± 1.73*(38.86%)	26.09 ± 0.39*(39.6%)	129.5 ± 0.32*(46.63%)	418.57 ± 5.62*(56.5%)	183.57 ± 2.54*(51.8%)
Nostoc sp. DL24	31.09 ± 1.12*(29.6%)	50.75 ± 2.42*(34%)	26.82 ± 0.48*(37.91%)	174.42 ± 1.29*(28.1%)	456.07 ± 12.96*(52.7%)	204 ±7.30*(46.46%)
Nostoc sp. DL25	28.42 ± 2.17*(35.6%)	47.60 ± 1.97*(38.1%)	24.71 ± 1.04*(42.8%)	133.3 ± 2.31*(45.05%)	428.76 ± 1.16*(55.5%)	125.74 ± 2.03*(67%)

Endpoint assays were performed with casein as a substrate while kinetic assays were performed with BApNA for trypsin and papain and [S(Ala)2ProPhepNA] for chymotrypsin. Enzyme activity was represented as an amount of end product released per min per mg protein. The data presented are mean ± SD (n = 3). Figures in parentheses show percent reduction of enzyme activity as compared to control. * Denotes significant difference in activity against control (one-way ANOVA, Dunnett's multiple comparison test, $p < 0.05$).

calculated with standard curve of pNA. The data presented are mean ± SD (n = 3). The activities were present in term of [nM pNA min-1 (mg protein)-1].

Trypsin assays- The trypsin (control) activity was observed 242.6 ± 3.51 nM pNA min-1 (mg protein)-1. Gloeocapsa sp. DL 17 shows the maximum trypsin inhibition activity 10.53 ± 0.93 nM pNA min-1 (mg protein)-1 and 95.67% inhibition. Five cyanobacteria extract Oscillatoria sp. DL 02 shows 88.30% enzyme inhibition, Lyngbya sp. DL 07 shows 84.13% inhibition, Oscillatoria sp. DL 08 shows enzyme inhibition 92%, Microcystis sp. DL 09 shows 94.53% and Microcystis sp. DL 22 shows 94.88% reduction. Out of them sixteen extract of cyanobacteria species shows enzyme inhibition between 30-70% compared as a control. Three species of cyanobacteria Calothrix sp. DL 01, Oscillatoria sp. DL 18 and Nostoc sp. DL 24 were unable to inhibit this enzyme and enzyme inhibition activity found less than 30 %.

Chymotrypsin assays- During the assays chymotrypsin (control) activity was observed 964.36 ± 13.28 nM pNA min-1 (mg protein)-1. Gloeocapsa sp DL 17 shows maximum activity 71.24 ± 1.11 nM pNA min-1 (mg protein)-1 and shows 92.61 % enzyme inhibition against chymotrypsin. Six extracts of cyanobacteria species Calothrix sp. DL 01 shows 75.10 % inhibition, Anabaena sp. DL 03 shows 87.80% inhibition, Oscillatoria sp. DL 08 shows 90.40% enzyme inhibition, Microcystis sp. DL 09 shows 91.50% enzyme inhibition, Nostoc sp. DL 20 shows 77.29% enzyme inhibition and Microcystis sp. DL 22 reduced enzyme activity and shows greater than 91.20 % chymotrypsin inhibition. Extracts of sixteen cyanobacteria sp. shows enzyme activity between 30-70% inhibitions compared as a control. Out of twenty five two extracts of cyanobacteria species Oscillatoria sp. DL 18 and Nostoc sp. DL 21 was unable to inhibit this enzyme.

Papain assays- The papain (control) activity shows 381.23 ±1.40 nM pNA min-1 (mg protein)-1. Extracts of Gloeocapsa sp. DL 17 inhibit papain enzymes and shows greater than 90 % inhibition activity. Gloeocapsa sp. DL 17 shows the maximum enzyme inhibition activity 37.01 ± 2.79 nM pNA min-1 (mg protein)-1 and observed 90.29 % papain inhibition. Extract of cyanobacteria species Oscillatoria sp. DL 02, Lyngbya sp. DL 07, Oscillatoria sp. DL 08, Oscillatoria sp. DL 12, Oscillatoria princeps DL 15, Lyngbya sp. DL 16 and Nostoc sp. DL 20 shows enzyme activity between 70-90% inhibition compared as a control. Single extract of cyanobacteria sp. Oscillatoria sp. DL 18 was unable to inhibit papain enzyme.

The inhibitory activities by all cyanobacterial strains were significantly different from control (one-way ANOVA, Dunnett's multiple comparison test, $p < 0.05$) during end point and kinetic assays. Three extracts of Calothrix sp. DL 01, Anabaena sp. DL 03 and Fischerella sp. DL 05 not significantly different from control with trypsin during endpoint assays. However, activity with Oscillatoria princeps DL 15 and Anabaena sp. DL 19 were not significantly different from control with trypsin during kinetic assays. Oscillatoria sp. DL 18 was not significantly different from control with chymotrypsin during endpoint assays. The inhibitory activities by all cyanobacterial strains were significantly different from control with chymotrypsin during kinetic assays and papain during both assays.

4. DISCUSSION

The present study is an attempt to identify the potential of protease inhibition by different genera of cyanobacteria from Central India. The study was based on the fact that a high number of cyanobacteria metabolites, including protease inhibitors have been isolated from a variety of

natural samples and cultured strains [26]. More than 80 structural archetypes of compounds belonging to more than 30 genera of all five sub sections of cyanobacteria have been defined. However, this distribution reflects the ability of stains and exploitable biomass from natural habitats. In this way, the potential to produce a protease inhibitor by theses genera often not comparable.

Central Indian part of Jabalpur and the surrounding is known for its sub tropical climates with plenty of rainfall. The ability of day light is throughout the year with temperature ranging from 20°- 25°C during most of the year. These climatic conditions promote the growth of cyanobacteria for all possible habitats. Further a large number of water bodies ensure the presence of planktonic and benthic cyanobacteria in this region. In order to make a comparative analysis of protease inhibitors from different genera of diverse habitats, the study took a survey of the Jabalpur town of Central India to collect cyanobacteria from all possible habitats. Cyanobacteria are known to produce biologically active metabolites; some of them can affect both wildlife and human. Among the secondary metabolites are cyclic and linear peptides, depsipeptides, proteins, alkaloids, lipids, glycolipids and fatty acid type of compounds. Peptides and depsipeptides are more intensively study group of these metabolites. Theses peptides are of general interest not only by their structural biological active relationship, but also by a possible pharmaceutical interest.

Previous data report that the peptide produced by the cyanobacteria has the ability to act as protease inhibitors. A large number of protease inhibitors belonging mainly to the class of serine and cysteine proteases, Theses protease inhibitors reported for regulating important biological processes [27]. Likewise Leung et al [28] used serine protease inhibitors in the treatment of a wide variety of human diseases. Previous data reported the central part of India including Jabalpur region is a rich source of cyanobacteria [29] and known for production of antimicrobial compound. Apart from this, a large list of non-toxic peptide is constantly growing to an interesting awareness of their broad distribution within the five sections of cyanobacteria [16]. Toxin and other compounds, i.e., enzyme inhibitors have been shown to affect the freshwater ecology, mostly in a negative manner. Agrawal et al [11] were the first report that zooplankton Moina macrocopa was negatively affected by the cyanobacterial bloom extract that contain protease inhibitors and not the microcystin, although earlier it was believed that microcystin are able to deter zooplankton community.

Apart from the other activities protease inhibitory activity pronounced and, in resent protease inhibitors from cyanobacteria has attracted worldwide attraction. These protease inhibitors have shown to cause inhibition of proteases necessary for digestion and assimilation, and hence can be related to sub acute toxicity [24], [30]. Among hundreds of protease inhibitors, most have been shown to inhibit trypsin, chymotrypsin, elastase, thrombin, plasmin and papain from mammalian or plant sources. The present study used their major proteases i.e., trypsin, chymotrypsin and papain. Trypsin and chymotrypsin is representative of the serine protease family and represent major digestive proteases. Papain is a member of cysteine protease family, available mainly from plant sources but has remarkable similarities with it is animal counterpart, cathepsin. Similar to trypsin and chymotrypsin, cysteine protease plays a major role in digestion in lower animal and zoo plankton.

The protease inhibitory activity was assayed using to enzyme assay techniques i.e., end point and kinetic assays. While endpoint methods used a natural substrate and presenting the natural phenomena of digestion while the kinetic assays provide a real time inhibition studies with the use of a chromogenic substrate. Most of the studies that deal with pharmacological importance of cyanobacterial protease inhibitors used chromogenic substrate [31], [32], [33]. The other group of researchers which have the ecological point of view have used end point assays using natural substrate, mostly casein [34], [11], [23], [30]. The 50% inhibitory concentration (IC50) for trypsin endpoint assays ranged to from 8.15 to 1355 µg dry weight equivalent with Microcystis sp. DL 22 and Microcystis sp. DL 09 showing the most potent inhibitory against trypsin endpoint assays. Agrawal et al [23] showed the IC50 of Microcystis aeruginosa PCC 7806 as a 6 µg dry weight equivalent against the trypsin isolated from a zooplankton, Moina macrocopa. Our results are in complete agreement with the earlier reported result.

The IC50 values of crude cyanobacterial extract against chymotrypsin endpoint assays ranged between 4 to 280.9

µg dry weight equivalents. Most of the protease inhibitor of cyanobacterial origin has IC50 values for chymotrypsin are as micropeptin 103 to 1.0 µg dry weight equivalent, oscillapepetin 2.2 µg dry weight equivalent, microviridin in the range of 1.1 to 2.9 µg dry weight equivalent [28] and cyanopeptolins in the range of 29.5 - 42.9 µg dry weight equivalent [34]. Various reports show that the recovery of pure metabolites from cyanobacteria is between 0.01 to 0.03 % [35] considering this, 4 µg dry weight equivalent Microcystis sp. DL 22 should posses the active metabolites in the range of 40-120 ng. Considering these facts the IC50 values for Microcystis sp. DL 22, Microcystis sp. DL09 as well as Gloeocapsa sp. DL 17 have much lower IC50 value against chymotrypsin.The IC50 values for papain by the crude extracts of tested cyanobacteria ranged from 21.8 to 365.1. Agrawal et al [23] showed IC50 of Microcystis aeruginosa PCC 7806 against papain as 79 µg dry weight equivalent for 1 ml reaction mixture.

During kinetic studies the IC50 values for all the three enzymes did not vary much from those observed during endpoint assays. However, one of the key features observed were with Nostoc and Anabaena sp. where the crude extract showed a tenfold lower IC50 value during kinetic studies. Although, the reason for this is unclear at the moment, yet it is clear that these terrestrial cyanobacteria may possess protease inhibitor compound different to those present in truecoid cyanobacteria i.e., Microcystis and Gloeocapsa. These structurally different variants may have different modes of action against the proteases. Further investigation is needed in order to identify these protease inhibitors.

Complex physiology of cyanobacteria regarding to production of bioactive secondary metabolites, further complicated to understand the pattern of presence and occurrence of various protease inhibitors in cyanobacteria. Various research has to be done in this field, as there is a number of biologically active secondary metabolites especially, protease inhibitors, emerging day by day [27]. It may be tempting to speculate that different protease inhibitors are produced by cyanobacteria because of different biotic and abiotic pressures appeared in nature. It was previously proved and reported that most of the protease inhibitors have many pharmaceutical and industrial (e.g., fungicide) application. The group of scientist reported the use of protease inhibitors in the clinic, they showed structures for 108 inhibitors of enzymes, namely aspartic, serine, cysteine and threonine, and some still remain in this promising class of new therapeutic agents.

Now a day's protease inhibitor used as antiviral medicines they prevent HIV multiplying redacting the amount of virus in the body. Protease inhibitor has also anticancer activity there are different types of protease inhibitor available, which are used as antiviral drugs. Previously, all the research showed that protease inhibitor has great pharmaceutical and industrial value in all over the world. But in this direction, not much work has been done in this area. The present work is an initiative in this direction. The results clearly showed the possibility of exploitation of protease inhibitors and the isolated strain of cyanobacteria was capable of producing of protease inhibitor compound. Previous results reported that most of the inhibitors are as drugs to treat disease. Many of these inhibitors target a human enzyme and aim to correct a pathological condition. As well as many herbicides and pesticides are acting as enzyme inhibitors [36].

5. CONCLUSION

In the present study, we have isolated some important protease inhibitor from cyanobacteria. These protease inhibitors responsible for inhibition of proteases necessary which is for digestion and assimilation, and it can be related to sub acute toxicity previously reported. In several cases such as protease inhibitors have been shown to affect trypsin, chymotrypsin, and elastase not only from bovine sources but as well as from zoo plankton. A large number of protease inhibitors belonging mainly to the class of serine proteases. Theses serine proteases reported for regulating important diagnostic therapy and biological processes. Serine protease inhibitors are important in the treatment of a wide variety of human diseases.

In India, especially in M. P., The research in this direction has not been performed with such approach and only a few groups are working in the field of cyanobacterial secondary metabolites. The study could be a good development in this direction. The work comes out with novel ideas of cyanobacterial protease inhibitor use in various pharmaceutical industries. It could be established the role of protease inhibitor in a wider context. On the basis of our result, we just concluded that these protease inhibitors have

the ability to counter the effect of proteases and stop the hydrolysis of another protein. Our idea is to utilize and commercialize these enzyme inhibitors for the benefit of mankind.

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