



## Antibacterial properties of freshwater *Microcystis aeruginosa* (Kütz) to bacterial pathogen—a comparative study of bacterial bioassays

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### ABSTRACT

Antimicrobial activities and minimum inhibitory concentration (MIC) values of various solvent extracts of *Microcystis aeruginosa* were studied. When tested against 17 Gram negative fish pathogenic bacteria belonging to 4 strains of *Aeromonas hydrophila*, 2 strains of *Pseudomonas putida*, 2 strains of *P. aeruginosa*, 2 strains of *P. fluorescens*, 6 *Vibrio* species *Edwardsiella tarda* and 6 animal serotypes of *Escherichia coli*, all the solvent extracts showed positive response except aqueous extract. Hot ethanolic extract showed maximum zone size (19 mm) against *A. hydrophila* (AH1) and *E. coli* (O111). The crude ethanolic extract was partially purified employing thin layer chromatography (TLC) and silica gel column chromatography techniques. Both crude as well as polar fractions showed positive results against selected pathogens. The antibacterial activities support that freshwater *Cyanobacteria* are still a promising source of new bioactive natural products. It is possible to develop specific microalgal based biologically active compound for aquaculture and to avoid resistance development against antibiotics used in human medicine.

**Key words:** *Aeromonas hydrophila*, Antibiotics, *Cyanobacteria*, *Microcystis*, Secondary metabolites

Algae are now drawing a greater interest following the increase in demand for biodiversity in the screening programs seeking therapeutic drugs from natural products. *Cyanobacteria* (blue green algae) are one of the richest sources of biomedical relevant compounds with extensive therapeutic pharmaceutical applications (Tan 2007, Gademann and Portmann 2008, Martins *et al.* 2008). Diverse array of cyanobacterial secondary metabolites that can cause undesirable tastes and odors (toxic or odorous metabolites) or otherwise biochemically active (bioactive) metabolites were studied in marine and aquaculture systems (Smith *et al.* 2008). The ability of *Cyanobacteria* to synthesize peptides, depsipeptides, polyketides and alkaloids etc. (Rastogi and Sinha 2009, Nunnery *et al.* 2010) fascinates the researchers for their pharmaceutical and biotechnological exploitations (Thajuddin and Subramanian 2005, Sielaff *et al.* 2006, Spolaore *et al.* 2006). *Cyanobacteria* produce a wide range of secondary metabolites, including antialgal substances (Bagchi and Ray 2001, Volk and Furkert 2006). Compounds from *Cyanobacteria* were isolated which display

inhibitory effects on bacterial growth (Mian *et al.* 2003, Rao *et al.* 2007, Kaushik *et al.* 2009, Das and Pradhan 2010, Vijaya and Ashok 2010), fungal growth (Noaman *et al.* 2004, Kasinathan *et al.* 2009), cancer cells (Kinghorn *et al.* 2009).

The blue green algae *Microcystis aeruginosa* is a rich source of aeruginosins, microginins, anabaenopeptins, cyanopeptilins, microcystins, and microviridins and other peptide-like compounds (Neumann *et al.* 2000, Martins *et al.* 2009, Okello *et al.* 2010). It also produces the cytotoxic peptide microcyclamide (Ziemert *et al.* 2008). Properties of cyanobacterial peptides include proteinase inhibition, anticyanobacterial antialgal and antifungal actions, immunosuppressive and cell differentiation-promoting functions (Singh *et al.* 2001, Mian *et al.* 2003). Berry *et al.* (2008) studied allelochemical roles of cyanobacterial toxins, as well as the potential for development and application of these compounds as algaecides, herbicides and insecticides.

Very few of *Cyanobacteria* cultures available in collection centers in India and abroad have been screened for such effective metabolites (Skulberg 2000). Owing to hydrophobic nature of these compounds these could be easily extracted in a polar solvent (methanol, ethanol and water). Common steps of thin layer chromatography; silica gel column chromatography can be used for partial purifications shown for a large number of secondary metabolites. Antimicrobial effects from cyanobacterial

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aqueous and organic solvent extracts are visualized in bioassays using selected microorganisms as test organisms (Kellam *et al.* 1988).

## MATERIALS AND METHODS

*Microcystis* blooms were collected from Bindusagar, Bhubaneswar, India, in January 2009. Collected samples were washed 3 times in millq water to separate potential contamination and finally filtered. The samples were identified as *Microcystis aeruginosa* as per Botanical Survey of India (Biswas 1949). Harvested *Microcystis* (85 g wet weight) was dried under room temperature for 2–3 days. The dried weight of the algae was 55g and then it was powdered. For extraction of metabolites, 5 g of dry *Microcystis* powder was taken for each sample.

Resulting powder was submitted to lipid soluble polar solvents (ethanol, methanol, chloroform and ratio of chloroform and methanol) as well as aqueous medium for extraction, using a soxhlet extractor at 55–60°C. All samples were refluxed until saturation (24 h) and the respective extracts were dried in rota-evaporator. Subsequently the residual extracts were suspended in the respective solvents to a final concentration of 10 µg µl<sup>-1</sup> (Das *et al.* 2005, Das and Pradhan 2010). The active crude ethanolic extract (0.5g) was fractionated using silica gel (SRL, 100–200 mesh size) column chromatography (Motl and Novotny 1979). The solvent system was fixed by a preliminary thin layer chromatographic (TLC) study (Stahl 1969).

*Aeromonas hydrophila* (AH1, AH2, AH3 and AH4),

*Pseudomonas putida* (PP1, PP2), *P. aeruginosa* (PA1, PA2), *P. fluorescens* (PF1, PF2), *Vibrio alginolyticus* (VA), *V. anguillarum* (VAN), *V. fluvialis* (VF), *V. parahaemolyticus* (VP), *V. harveyi* (VH), *V. fischeri* (VFS), *E. coli* (O1, O115, O156, O164, O111 & O109) and *Edwardsiella tarda* which was maintained in the Aquatic Animal Health Division, CIFA, Bhubaneswar, were taken for the antibacterial sensitivity study. Pure cultures of different bacterial strains were taken and inoculated in brain heart infusion (BHI) broth and incubated at 37°C for 18 h.

Antibacterial potency of various extracts of the *Microcystis aeruginosa* extract was investigated against selected pathogenic Gram negative bacteria by the agar disc diffusion method (Chabbert 1963). Each extract 10 mg / 1 ml concentration was applied to sterile filter paper discs (6 mm in diameter). The presence of clear zones around discs and diameters of inhibition zones were measured in mm (Izzo *et al.* 1995). The zone of inhibition of the *Microcystis* extracts against microorganisms were determined by measuring the diameter of the halo around the discs (average of 3 experiments) and the assay was scored double positive (++) if  $\geq 5$  mm<sup>3</sup>, triple positive (+++) if  $\geq 10$  mm<sup>3</sup> and negative (-) if there was no inhibition of microbial growth. The antibacterial activities of *Microcystis* extracts were compared with inhibition zones around 3 commercial antibacterial discs i.e. streptomycin (S), tetracycline (T) and bacitracin (B) that were used as references. The minimal inhibitory concentration (MIC) value of the extracts of *Microcystis* (Mc1, Mc2, Mc3, Mc4, Mh1, Mh2, Mh3, Mh4, Mr1 and

Table 1. Antibacterial activity of various extracts of *Microcystis aeruginosa* against different strains of *Pseudomonas* sp.

| Solvent Extracts        | Microorganisms   |     |                      |     |                       |     |
|-------------------------|------------------|-----|----------------------|-----|-----------------------|-----|
|                         | <i>P. putida</i> |     | <i>P. aeruginosa</i> |     | <i>P. fluorescens</i> |     |
| Cold                    | PP1              | PP2 | PA1                  | PA2 | PF1                   | PF2 |
| Mc1(EtOH)               | ++               | ++  | ++                   | +++ | -                     | +   |
| Mc2(MeOH)               | +++              | +++ | +++                  | +++ | +++                   | ++  |
| Mc3(Chlo)               | +++              | ++  | +++                  | +++ | +++                   | +++ |
| Mc4(Chlo:MeOH)          | +++              | +++ | +++                  | ++  | ++                    | ++  |
| Hot                     |                  |     |                      |     |                       |     |
| Mh1(EtOH)               | +++              | +++ | +++                  | +++ | +++                   | +++ |
| Mh2(MeOH)               | +++              | +++ | +++                  | +++ | +++                   | +++ |
| Mh3(Chlo)               | +++              | +++ | ++                   | +++ | +++                   | +++ |
| Mh4(Chlo:MeOH)          | +++              | ++  | +++                  | +++ | +++                   | +++ |
| Rotavapour              |                  |     |                      |     |                       |     |
| Mr1(EtOH)               | +++              | +++ | +++                  | +++ | +++                   | ++  |
| Mr2 (Aqueous)           | -                | ++  | ++                   | -   | -                     | -   |
| Streptomycin(S), 10 mcg | +++              | +++ | +++                  | +++ | +++                   | +++ |
| Tetracycline(T), 30 mcg | +++              | +++ | +++                  | +++ | +++                   | +++ |
| Bacitracin(B), 10units  | +++              | +++ | +++                  | +++ | +++                   | +++ |

Zone size: 0, negative (-); 5–10, ++ and 10–20 = +++; Mc1, cold ethanolic extract; Mc2, cold methanolic extract; Mc3, cold chloroform extract; Mc4, chloroform: methanol (1:1) extract; Mh1, hot ethanolic extract, Mh2: hot methanolic extract, Mh3: hot chloroform extract, Mh4: hot chloroform: methanol (1:1) extract; Mr1, rotavapour ethanolic extract; and Mr2, aqueous rotavapour extract of *Microcystis aeruginosa*.

Mr2) were determined by serial dilute nutrient broth method (Alderman and Smith 2001).

## RESULTS AND DISCUSSION

Our goal in this study is to reveal the biological production of antibacterial substances by the *Cyanobacteria*, *Microcystis aeruginosa*. From the screening test of all the extractants of *Microcystis* it was observed that cold ethanolic (Mc1) and chloroform (Mc3) extract showed moderate to high antibacterial activities against all the strains of *P. putida*, *P. aeruginosa* and *P. fluorescens* with MIC value 20–44 mg/ml. Rotavapour extract (Mr1) of *Microcystis* shows low MIC

values (100–130 mg) against above selected pathogens (Table 5). Whereas both hot ethanolic (Mh1) and methanolic (Mh2) extracts of *Microcystis* showed comparatively high antibacterial activities against all the strains of *P. putida*, *P. aeruginosa* and *P. fluorescens*, than cold extracts (Table 1).

All the solvent extracts of *Microcystis* showed effectiveness against above selected strains of *A. hydrophila* except aqueous rotavapour extract (Mr2). Cold and rotavapour ethanolic (Mr1, Mc1) extracts showed maximum zone of inhibition (12, 13 and 14 mm) against 3 strains of *A. hydrophila* (AH1, AH2 and AH3) with lowest MIC value, i.e. 24 mg/ml (Tables 2, 5).

Table 2. Antibacterial activity of various extracts of *Microcystis aeruginosa* against different strains of *Aeromonas hydrophila* and *E. tarda*

| Solvent extracts        | Microorganisms       |     |     |     |                 |
|-------------------------|----------------------|-----|-----|-----|-----------------|
|                         | <i>A. hydrophila</i> |     |     |     | <i>E. tarda</i> |
|                         | AH1                  | AH2 | AH3 | AH4 |                 |
| Cold                    |                      |     |     |     |                 |
| Mc1(EtOH)               | +++                  | +++ | ++  | +++ | ++              |
| Mc2(MeOH)               | +++                  | +++ | +++ | +++ | +++             |
| Mc3(Chlo)               | +++                  | +++ | +++ | +++ | +++             |
| Mc4(Chlo:MeOH)          | +++                  | +++ | +++ | +++ | +++             |
| Hot                     |                      |     |     |     |                 |
| Mh1(EtOH)               | +++                  | +++ | +++ | +++ | +++             |
| Mh2(MeOH)               | +++                  | +++ | +++ | +++ | +++             |
| Mh3(Chlo)               | +++                  | ++  | ++  | +++ | +++             |
| Mh4(Chlo:MeOH)          | ++                   | +++ | +++ | +++ | ++              |
| Rotavapour              |                      |     |     |     |                 |
| Mr1(EtOH)               | +++                  | +++ | +++ | +++ | ++              |
| Mr2 (Aqueous)           | -                    | -   | -   | -   | ++              |
| Streptomycin(S), 10 mcg | +++                  | +++ | +++ | +++ | +++             |
| Tetracycline(T), 30 mcg | +++                  | +++ | +++ | +++ | +++             |
| Bacitracin(B), 10 units | +++                  | +++ | +++ | +++ | +++             |

(for abbreviations, see Table 1).

Table 3. Antibacterial activity of various extracts of *Microcystis aeruginosa* against *Vibrio* sp.

| Solvent extracts | Microorganisms                 |                                 |                             |                                    |                             |                           |
|------------------|--------------------------------|---------------------------------|-----------------------------|------------------------------------|-----------------------------|---------------------------|
|                  | <i>V. anguillarum</i><br>(VAN) | <i>V. alginolyticus</i><br>(VA) | <i>V. fluvialis</i><br>(VF) | <i>V. parahaemolyticus</i><br>(VP) | <i>V. fischeri</i><br>(VFS) | <i>V. harveyi</i><br>(VH) |
| Cold             |                                |                                 |                             |                                    |                             |                           |
| Mc1(EtOH)        | ++                             | +++                             | +++                         | +++                                | ++                          | +++                       |
| Mc2(MeOH)        | +++                            | +++                             | +++                         | +++                                | +++                         | +++                       |
| Mc3(Chlo)        | +++                            | ++                              | +++                         | +++                                | +++                         | +++                       |
| Mc4(Chlo:MeOH)   | +++                            | +++                             | ++                          | -                                  | -                           | ++                        |
| Hot              |                                |                                 |                             |                                    |                             |                           |
| Mh1(EtOH)        | +++                            | +++                             | +++                         | +++                                | +++                         | +++                       |
| Mh2(MeOH)        | +++                            | +++                             | +++                         | +++                                | +++                         | +++                       |
| Mh3(Chlo)        | +++                            | ++                              | ++                          | +++                                | +++                         | ++                        |
| Mh4(Chlo:MeOH)   | +++                            | +++                             | ++                          | -                                  | ++                          | ++                        |
| Rotavapour       |                                |                                 |                             |                                    |                             |                           |
| Mr1(EtOH)        | +++                            | +++                             | +++                         | +++                                | -                           | ++                        |
| Mr2 (Aqueous)    | -                              | ++                              | -                           | ++                                 | -                           | -                         |

(for abbreviations, see Table 1).

Table 4. Antibacterial activity of various extracts of *Microcystis aeruginosa* against different strains of *E.coli*

| Solvent extracts | Microorganisms |      |      |      |      |      |
|------------------|----------------|------|------|------|------|------|
|                  | E.coli         |      |      |      |      |      |
| Cold             | O1             | O115 | O156 | O164 | O111 | O109 |
| Mc1(EtOH)        | +++            | +++  | +++  | +++  | +++  | +++  |
| Mc2(MeOH)        | +++            | +++  | +++  | +++  | +++  | +++  |
| Mc3(Chlo)        | +++            | +++  | +++  | +++  | +++  | +++  |
| Mc4(Chlo:MeOH)   | +++            | +++  | +++  | +++  | +++  | +++  |
| Hot              |                |      |      |      |      |      |
| Mh1(EtOH)        | +++            | +++  | +++  | +++  | +++  | +++  |
| Mh2(MeOH)        | +++            | +++  | +++  | +++  | +++  | +++  |
| Mh3(Chlo)        | ++             | +++  | ++   | +++  | +++  | +++  |
| Mh4(Chlo:MeOH)   | +++            | +++  | +++  | ++   | ++   | +++  |
| Rotavapour       |                |      |      |      |      |      |
| Mr1(EtOH)        | ++             | +++  | +++  | +++  | +++  | +++  |
| Mr2 (Aqueous)    | +++            | -    | +++  | +++  | +++  | +++  |

(for abbreviations, see Table 1).

Mc1, cold ethanolic extract; Mc2, cold methanolic extract; Mc3, cold chloroform extract; Mc4, chloroform: methanol (1:1) extract; Mh1, hot ethanolic extract; Mh2, hot methanolic extract; Mr1, rotavapour ethanolic extract of *Microcystis aeruginosa*.

Table 5. MIC values of various extracts of *Microcystis* against *Pseudomonas* sp., *Aeromonas hydrophila* and *Vibrio* sp.

| Exts | MIC values in mg |     |                      |     |                       |      |                      |      |      |       |                |      |      |      |       |
|------|------------------|-----|----------------------|-----|-----------------------|------|----------------------|------|------|-------|----------------|------|------|------|-------|
|      | <i>P. putida</i> |     | <i>P. aeruginosa</i> |     | <i>P. fluorescens</i> |      | <i>A. hydrophila</i> |      |      |       | <i>Vibrios</i> |      |      |      |       |
|      | PP1              | PP2 | PA1                  | PA2 | PF1                   | PF2  | AH1                  | AH2  | AH3  | AH4   | VAN            | VF   | VP   | VFS  | VH    |
| Mc1  | 34               | 30  | 44                   | 41  | 35                    | 30   | 25                   | 26   | 24   | 24    | 18             | 16   | 16.5 | 16   | 15    |
| Mc2  | 32               | 33  | 32                   | 32  | 22                    | 23   | 26                   | 24   | 25   | 24    | 32             | 32   | 30   | 31   | 35    |
| Mc3  | 31               | 30  | 32                   | 33  | 24                    | 22   | 24.4                 | 25   | 26   | 25    | 23             | 22.5 | 25   | 24.5 | 23    |
| Mc4  | 22               | 24  | 36                   | 30  | 30                    | 24   | 24.6                 | 25   | 24   | 24    | 32             | 31   | 30   | 31   | 31    |
| Mh1  | 32               | 32  | 30                   | 24  | 20                    | 20   | 24                   | 23   | 24   | 24    | 20             | 16   | 17   | 18   | 16    |
| Mh2  | 30               | 28  | 40                   | 36  | 34                    | 28   | 24                   | 25   | 22   | 24    | 31             | 30   | 32   | 33.2 | 32    |
| Mr1  | 0.1              | 0.1 | 0.1                  | 0.1 | 0.13                  | 0.11 | 0.1                  | 0.05 | 0.11 | 0.075 | 0.075          | 0.1  | 0.05 | 0.11 | 0.075 |

Mc1, cold ethanolic extract; Mc2, cold methanolic extract; Mc3, cold chloroform extract; Mc4, chloroform: methanol (1:1) extract; Mh1, hot ethanolic extract; Mh2, hot methanolic extract; Mr1, rotavapour ethanolic extract of *Microcystis aeruginosa*.

Similarly out of 6 selected strains of *Vibrio* three strains (VF, VAN and VP) were very sensitive (17, 16 mm) towards both cold and hot ethanolic extract (Mc1, Mh1) of *Microcystis*. The detail antibacterial activities of *Microcystis* against different strains of *Vibrios* and MIC values are shown in Tables 3, 5.

After screening, it was observed that, the effectiveness of hot ethanolic extract (Mh2) was highest (19 mm) than that of both cold (Mc1) ethanolic (15, 14, 13 mm) as well as rotavapour (Mr1) ethanolic (14, 12 mm) extract of *Microcystis* against 5 strains of *E. coli* (Table 4).

Out of seven polar fractions 4 fractions were effective against all the selective strains of *Pseudomonas*. Eluents of 1:4 ethylacetate/ hexane (Mf3, Mf4), 1:1 ethylacetate/ hexane (Mf6) and 1:1+4:1 ethylacetate/ hexane (Mf7) showed maximum zone size (14, 15, 16 and 18 mm) against *P. aeruginosa* (PA1, PA2) and *P. fluorescens* (PF1, PF2) (Fig. 1). The effectiveness of various fractions of *Microcystis* against

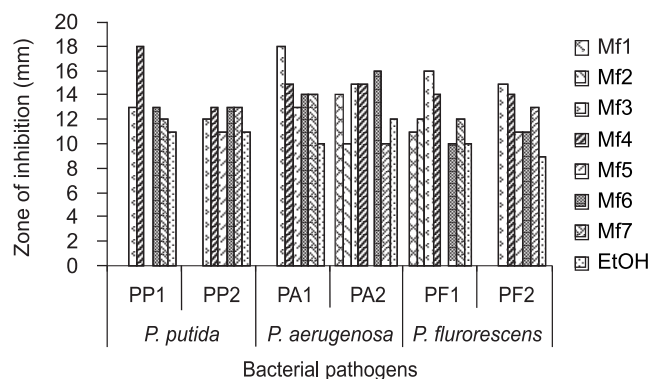


Fig. 1. Antibacterial activity of various fractions of ethanolic extracts of *Microcystis* against *Pseudomonas* species

*A. hydrophila* were cited in Fig. 2. Fractions like Mf3, Mf4 and Mf5 showed maximum zone of inhibition (14, 15, 16 mm) than crude ethanolic extracts of *Microcystis*.

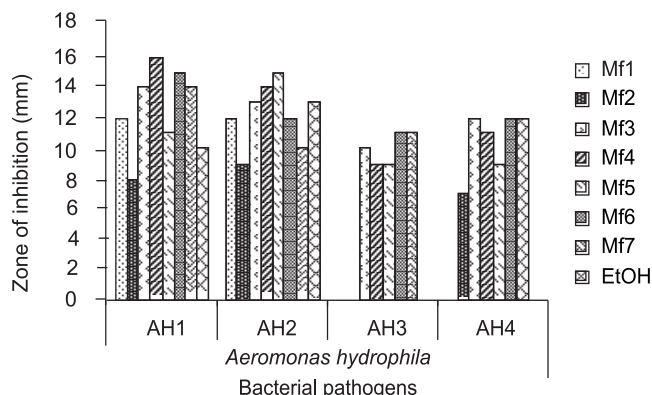


Fig. 2. Antibacterial activity of various fractions of *Microcystis* against *A. hydrophila* (AH1, AH2, AH3, AH4)

The present study revealed that most of the bacteria were negative to rotavapor aqueous extracts except *P. putida* (PP2), *P. aeruginosa* (PA1), *E. tarda*, *V. alginolyticus* (VA), *V. parahaemolyticus* (VP) and *E. coli* (O1, O156, O164, O111, O109). Some studies concerning the effectiveness of organic solvents that provide a higher efficiency in extracting compounds for antimicrobial activities compared to water based method (Lima-Filho *et al.* 2002). The present results are in agreement with those of Ghosh *et al.* (2008) who showed that aqueous extracts are generally less potent in their bioactivity than organic extracts. Differential antimicrobial activities in the rotavapor aqueous extract indicated its mode of action by which it regulates the growth of the microorganisms, or a variety of secondary metabolites that are released by the microalgae, that could inhibit the growth of particular bacteria. Several different organic solvents were used for screening algae for antibacterial activity. Recently, Das *et al.* (2005) reported the antibacterial activity in the chloroform, acetone, methanol and ethanol extracts of *E. viridis* against different fish pathogens. Antibacterial assay of some cyanobacterial species against the human pathogenic bacteria such as *Streptococcus mutants*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Klebsiella pneumonia* was studied (Vijaya and Ashok 2010). Singh *et al.* (2001) and Mian *et al.* (2003) reported antibacterial and antialgal activities from terrestrial and freshwater *Cyanobacteria*.

Antibacterial substances showed different solubility depending on the extract solvent used and the differences may due to the efficiency of the extraction methods to recover the active metabolites, solvent used (Ostensvik *et al.* 1998, Tuney *et al.* 2006) susceptibility of strains (Val *et al.* 2001), assay methods and seasonal variation (Sasidharan *et al.* 2009). They reported that the organic extracts with methanol combined with acetic acid expressed the greatest zones of inhibition of the test bacteria. Similarly Nurul *et al.* (2010) studied that methanol and ethyl acetate extract from solvent partitioning exhibited broader spectrum activity against *B. aereus* ATCC10876, *B. licheniformis* ATCC12759, *S. aureus*,

*P. aeruginosa* ATCC27853 and *Yersinia* sp. Flach *et al.* (1995) used successively extractions with solvents of increasing polarity: petroleum ether, dichlorometan, ethylacetate, methanol and 80% aqueous methanol or water. These extracts showed different antibacterial effects in bioautographic assays with *B. subtilis*, *E. coli* and *M. luteus*. In the present study we employed 3 types of extraction process i.e cold, hot and rotavapor and the solvents used were methanol, ethanol, chloroform and chloroform: methanol in equal proportions.

It was observed that cold and hot extracts with organic solvents produce more or less similar results to the wide range of microorganisms tested. Among all microorganisms tested, the crude hot methanolic and ratio of chloroform and methanol extracts of *Microcystis* showed maximum activity. Fractionation study revealed that chromatographic fractions (Mf1-Mf7) produced highly antibacterial activities against above selected *Pseudomonas* species, *A. hydrophila*, *Vibrios*, *E. coli* and *E. tarda* except little variations. These were compared with the standard antibiotic discs *e.g.* streptomycin (S), tetracyclines (T) and bacitracin (B), and found to be similar zone of inhibitions. This could be of potential for future drug development from this *Cyanobacterium*. Further study on bioassays and effect on the target organs is necessary to arrive at a final conclusion.

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