

Exploring possibility of controlling *Microcystis* population using terrestrial plant extracts and indigenous technologies

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Abstract

Algaecide property of leaf aqueous extracts (LAEs) of three plants viz., *Azadiracta indica*, *Tridax procumbens*, *Calotropis procera* as well as two indigenous technologies (ITKs) viz., application of neem oil and cow urine were evaluated against wild *Microcystis* bloom and pure culture of *Microcystis aeruginosa* in laboratory. Five treatments were designated as LAE of *A. indica* (T-1), neem oil (T-2), LAE of *T. procumbens* (T-3), LAE of *C. procera* (T-4) and cow urine (T-5). Inhibitory effects of these agents were studied in terms of colonial morphological alterations in bloom and reduction of cell density in pure culture. Cell density of 2.0×10^5 cells ml^{-1} was achieved through inoculation of *Microcystis* from pure culture and each inhibitor was added at 50 ml l^{-1} to the flasks. All flasks were maintained for 14 days at 30°C with continuous aeration and illumination (2000 Lux). After 14 days, cow urine (T-5) showed the highest cell inhibition (87.3%) followed by T-1 (82.7%) and T-3 (61.6 %) ($p < 0.05$), whereas there were 82, 259 and 371% rise in the cell density in T-2, T-4 and control, respectively, compared to the base population seeded on day-0. Indirect measurement through daily record of absorbance in treatments at 680 nm also revealed the growth rate to decrease in the order, control > T-4 > T-2 > T-3 > T-1 > T-5.



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Introduction

The rising global fish demand for fish in recent years has driven a paradigm shift from traditional fish farming to intensive aquaculture practices. While this trend aims to enhance unit area productivity, it often results in the irrational use of fertilisers, manures and feeds in aquatic systems. This imprudent application of inputs coupled with unpredictable climate conditions, frequently leads to eutrophication, which subsequently triggers algal proliferation. Cyanobacterial blooms are a common consequence of eutrophication in lakes worldwide (Guo *et al.*, 2015). These prokaryotic organisms produce a variety of secondary metabolites with antibiotic, algicide, cytotoxic, immunosuppressive and enzyme inhibiting activities (Das *et al.*, 2013). Prominent genera such as *Microcystis*, *Anabaena*, *Oscillatoria*, *Nodularia*, *Planktothrix*, *Cylindrospermopsis* and *Nostoc*, are frequently

noticed in eutrophic waters, causing multiple detrimental effects (Paerl and Otten, 2013). The frequency and severity of cyanobacterial blooms have been increasing globally, especially in warm-water lakes due to increasing eutrophication in recent years (Ke *et al.*, 2007). Blooms of toxic cyanobacteria are gradually increasing worldwide in both frequency and severity (WHO, 2015). These bloom-forming cyanobacteria are generally considered as pest species due to their toxicity, high biomass build-up and their adverse negative impacts on aquatic food webs and human use of freshwater resources (Chorus and Bartram, 1999; Codd *et al.*, 2005). Hypertrophic conditions and elevated temperatures facilitate algal proliferation and with the ongoing eutrophication and climate change, the frequency of algal bloom event is expected to rise further (Kosten *et al.*, 2012).

Microcystis spp. are the most common bloom-forming freshwater cyanobacteria that exhibit high phenotypic plasticity. Their global success is partially due to the physiological characteristics of their colony morphology (Xiao *et al.*, 2018). These can initiate series of serious environmental and ecological events, causing blockage of drinking-water supply systems, production of unpleasant odours, reduced water clarity and removal of dissolved oxygen during decomposition (Qin *et al.*, 2010). *Microcystis* genus in general and *M. aeruginosa* in particular produce microcystins (MCs), which are hepatotoxins that severely affect aquatic animals and human health (Chorus and Bartram, 1999; Landsberg, 2002). Upon ingestion, toxic MC gets actively absorbed in fish body and cause adverse effect on its physiology (Codd, 2000; De Figueiredo *et al.*, 2004). The search for an effective method for mitigation of *M. aeruginosa* has become essential for better management of aquatic ecosystems in recent times.

The conventional method of mechanical/manual removal of algal scum is energy, time and labour intensive and is mostly impractical (Chen *et al.*, 2012). Another most common method for elimination of cyanobacteria in aquatic ecosystems is the use of chemical agents (Yan *et al.*, 2011). Chemical agents such as copper sulfate (Han *et al.*, 2001) and hydrogen peroxide (Drabkova *et al.*, 2007) effectively control blooms within a short period. However, their applications are also associated with non-target effects that destroy natural productivity (Su *et al.*, 2011) and causes secondary chemical pollution. Use of biological agents, such as bacteria (Kang *et al.*, 2008), viruses (Yoshida *et al.*, 2006), planktonic ciliates (Mayali and Doucette, 2002) and fungi (Sigee *et al.*, 1999) have shown difficulty in application, and create disastrous ecological consequences (Yi *et al.*, 2012). While control of blooms is important, no standardised, environmentally safe and supreme method is in place. Applications of organic algaecides are contemporary and unparalleled among the existing algal control measures in view of environmental health. With the growing awareness, efforts have been made using extracts of many aquatic plants such as *Phragmites communis*, *Myriophyllum spicatum*, *Ceratophyllum demersum*, *Stratiotes aloides*, *Najas marina* (Meng *et al.*, 2015) and *Acorus calamus* (Zhang *et al.*, 2016) to demonstrate inhibitory effect on cyanobacteria. But terrestrial plants, the treasure house of important bioactive compounds, are still under carpet to investigate for their anti-algal properties. Neem plant (*Azadirachta indica*, Family: Meliaceae) is widely used in traditional medicines

and well investigated for its allelopathic property (Kato-Noguchi *et al.*, 2014). Similarly, *Tridax procumbens* (Asteraceae) is an invasive perennial plant found in tropical and subtropical regions. It thrives in pastures, meadows, agricultural fields, along the sides of highways and degraded areas (Kissmann and Groth, 1995). *Calotropis procera*, popularly known as milkweed is rich in allelochemicals and has been documented to have phytotoxic potential (Yasin *et al.*, 2012). Although these plants have been extensively studied for their pharmacological and pesticidal effects, their potential to inhibit noxious algal blooms in culture ponds has received less research attention. Similarly, neem oil and cow urine are often used as ITKs for the control of pests in agriculture. Present study intends to explore the possible inhibiting effect of leaf extracts of these plants and the two ITKs (neem oil and cow urine) on the noxious *Microcystis* bloom.

Materials and methods

Isolation and pure culture of *Microcystis aeruginosa*

Bloom of *Microcystis* was collected from a culture pond (Fig. 1a) from the farm of ICAR-Central Institute of Freshwater Aquaculture (ICAR-CIFA) using bolting silk plankton net (125 µm mesh). The monolayer macroscopic colonies were pipetted into 50 ml tubes for further microscopic identification and isolation. The cyanobacterium *M. aeruginosa* was identified based on the irregular shape of its colonies, distinct holes or gas vacuoles in the colony and diffuse mucilage structure (Fig. 1b) (Komarek and Komarkova, 2002; Guiry and Guiry, 2016). Subsequently, isolation and pure culture was carried out following the standard procedure using B-12 medium (Shirai *et al.*, 1989). Deionised distilled water was mixed with NaNO₃ (100 mg l⁻¹), K₂HPO₄ (10 mg l⁻¹), MgSO₄·7H₂O (75 mg l⁻¹), CaCl₂·2H₂O (40 mg l⁻¹), Na₂CO₃ (20 mg l⁻¹) and ferric citrate, (6 mg l⁻¹) followed by autoclaving. The solution was further mixed with disodium EDTA·2H₂O (1 mg l⁻¹) and vitamin B12 (0.1 mg l⁻¹) to prepare the B-12 medium. The pH of the medium was adjusted to 9.0. Isolation was conducted through a series of serial dilution, agar streaking and sub-cultures on solid and liquid media. The cultures were placed in an isolated room at 30°C under 2000 Lux illumination. The unialgal culture was scaled up and maintained in B-12 medium under the same culture conditions.



Fig. 1. (a) Typical *Microcystis* bloom in a farm pond at ICAR-CIFA, Bhubaneswar; (b) A natural colony of *Microcystis* spp. (x400) showing individual cells embedded inside sheath

Counting of cells from *Microcystis* colony

Separation of *Microcystis* cells is a primary requirement for cell counting as in nature, these cells exist in colony. Heat treatment method was used to separate the cells from the colony (Humphries and Widjaja, 1979). Five milliliters of sample were pipetted into 25 ml test tubes and loosely capped with aluminum foil. Tubes were immersed up to the sample level in a water bath for five minutes at 80°C. The hot samples were shaken at high speed in a vortex mixer for 30-60 s followed by few vigorous shakes by hand, ensuring complete separation of cells without disruption. Neubauer counting chamber (Marienfeld, Germany) was used to count the cells under the microscope, taking 10 µl from sample at a time which was repeated thrice. Each time, the cells in 25 squares were counted and total cells were enumerated subsequently.

Preparation of leaf aqueous extracts (LAEs)

In the present study, selection of three plants i.e. *Azadiracta indica*, *Tridax procumbens* and *Calotropis procera* were based on their anti-algal properties reported in earlier studies (Yasin *et al.*, 2012; Chia *et al.*, 2016; Mecina *et al.*, 2019). The leaf aqueous extracts (LAEs) were prepared with little modification of the protocol described by Tebaa *et al.* (2017). Fresh leaves of the selected plants (*A. indica*, *T. procumbens* and *C. procera*) were washed in flowing water and rinsed three times with ultrapure water to remove the debris and possible microorganisms attached. Fifty grams of these prewashed fresh leaves were cut into small pieces, crushed with 500 ml of sterilised water and the macerate was filtered through a sieve. The solution was again filtered through a filter paper (Whatman GF/C, 0.22 µm) and the filtrate was subsequently adjusted to 500 ml volume adding sterile distilled water and stored at 4°C as leaf aqueous extract (LAEs) for further use.

Phytochemicals screening

Phytochemicals screening of the extracts were carried out using standard qualitative phytochemical methods (Raphael, 2012). Tests for alkaloids (Mayer's test), flavonoids (lead acetate test), saponins (Frothing test), tannin (Ferric chloride test) and phlobatannins (1% HCl) were conducted to examine the presence of these phytochemicals in the LAEs.

Collection of cow urine and neem oil as ITKs

The first urine of cow was collected in the early morning in a sterile container to avoid external contamination, while commercially available neem oil was procured for the study.

Experimental design

Microcystis is a commonly available noxious algal bloom in freshwater culture ponds in India and as such there are no ethical issues involved in its control. The study was divided into two parts. In Experiment-I, inhibitory effect of the above-mentioned controlling agents (treatments) were evaluated against the wild *Microcystis* bloom collected from a pond of ICAR-CIFA farm, while in Experiment-II the inhibitory effects was studied against pure culture of *M. aeruginosa*.

In Experiment-I, conducted for 7 days, the five treatments were designated as LAE of *A. indica* (T-1), neem oil (T-2), LAEs of

T. procumbens (T-3), LAEs of *C. procera* (T-4) and cow urine (T-5). All treatments including control were maintained in triplicates. Cell density in the collected wild bloom was determined by counting under a microscope using a Neubauer counting chamber. Required amount of inoculum from the collected bloom sample was added to a one-liter conical flask containing 700 ml of B-12 liquid media, to get a cell density of 3.0×10^6 cells ml⁻¹. The flasks were provided with continuous aeration and 2000 Lux illumination and maintained at controlled room temperature of 30°C. The control agents were applied to the respective replication (flask) at 50 ml l⁻¹ and subsequently the changing colonial morphology of *Microcystis* in treatments were observed using a microscope (OLYMPUS BX 51) on 0, 3rd and 7th day of exposure. In the Experiment-II, conducted for 14 days, the experimental set up, treatments and the concentration of the controlling agents were kept identical to those of Experiment-I, except the test organism here which was *M. aeruginosa* drawn from the pure culture established in the laboratory.

Following application of the control agents, changes in the cell density of *M. aeruginosa* in the control and treatments were monitored and quantified every day. The cells were counted using Neubauer counting chamber under microscope (Olympus BX51TF, Japan) and density was also measured spectrophotometrically (VARIAN-50 BIO UV visible spectrophotometer) at 680 nm. The % change in cell population and inhibition rate (IR) were computed using the following formula:

$$\% \text{ change in cell density} = (N_t/N_0) \times 100,$$

where, N_t and N_0 are cell populations at tth day and the beginning respectively.

Inhibitory rate (IR) was determined by the following formula:

$$\text{IR \%} = (1 - N_t/N_0) \times 100$$

where, N_0 and N_t are the seeded population and cell population on tth day, respectively.

In spectrophotometric measurement, algal samples were taken from the culture media every day at same time for measurement of optical density at 680 nm (OD 680) as the algal density indicator. % Growth Rate (GR) was calculated using following formula (Liang, 2009):

$$\% \text{ GR} = (\ln \text{OD}_t - \ln \text{OD}_0) / t \times 100,$$

where, OD_0 is the initial optical density (after seeding) and OD_t is the optical density measured on tth day.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) using SPSS v20.0 (Windows 2010). One-way ANOVA and Tukey's test were used to test the differences between treatments at 5% level.

Results

Phytochemical analysis

Results of screening of phytochemical constituents in different leaf extracts are presented in Table 1. Alkaloids were found in all the three LAEs while tannin, flavonoid and saponin were absent in *A. indica*, *T. procumbens* and *C. procera*, respectively. No phlobatanin was found in any LAE.

Table 1. Phytochemical analysis of different aqueous leaf extracts

Tests	<i>A. indica</i>	<i>T. procumbens</i>	<i>C. procera</i>
Alkaloids	+	+	+
Tannins	-	+	+
Flavonoid	+	-	+
Phlabotanins	-	-	-
Saponin	+	+	-

Note: As per Ejoba (2012); '+' indicates presence, '-' indicates absence

Visual and morphological changes in *Microcystis* colony

Marked visual changes in colour of the wild *Microcystis* colony were observed with exposure to treatments T-1, T-3 and T-5. Visually, much effect in terms of change in colour of the colony was observed with cow urine application (T-5) than the other two. Therefore, *Microcystis* population in the cow urine treatment was further studied for morphological changes under microscope. Treatment applied with cow urine showed intense yellowing and depigmentation of the colony after seven days of exposure unlike that in control where green colour was persistent (Fig. 2a). *Microcystis* colony in the control group were clear with intact mucilaginous matrix covering

loosely arranged cells under microscope (Fig. 3a). In contrast, colonies in cow urine treated groups had lost their regular form and cell intactness; and were completely distorted with shrunken and empty sheaths (Fig. 3b and c) after 7 days exposure. The visual changes observed in the pure culture of *M. aeruginosa* (Fig. 2b) were also similar to the observations in the wild population (Fig. 2a) indicating T-1, T-3 and T-5 to have their inhibiting effect on the algal population.

Effects on *M. aeruginosa* pure culture

Fig. 4 depicts the changes in the cell population of *M. aeruginosa* in different treatments during the course of 14 days study. While the control group showed a continuous rise in cell density all through the study period, T-1 (LAE of *A. indica*) and T-5 (cow urine) exhibited drastic reduction in cell count within the first six days and no subsequent cell revival was discernible. However, in T-3 and T-4, a brief increase in the cell density was observed during the initial two days followed by reduction up to 5th day. Thereafter, cell density reduced continuously in T-3, while it showed marked increase in T-4. In T-2, inhibitory effect of neem oil observed during the initial four days, but subsequently the cell revival occurred. Such reduction in cell count (Fig. 4) after 14 days in the treatments also corresponded

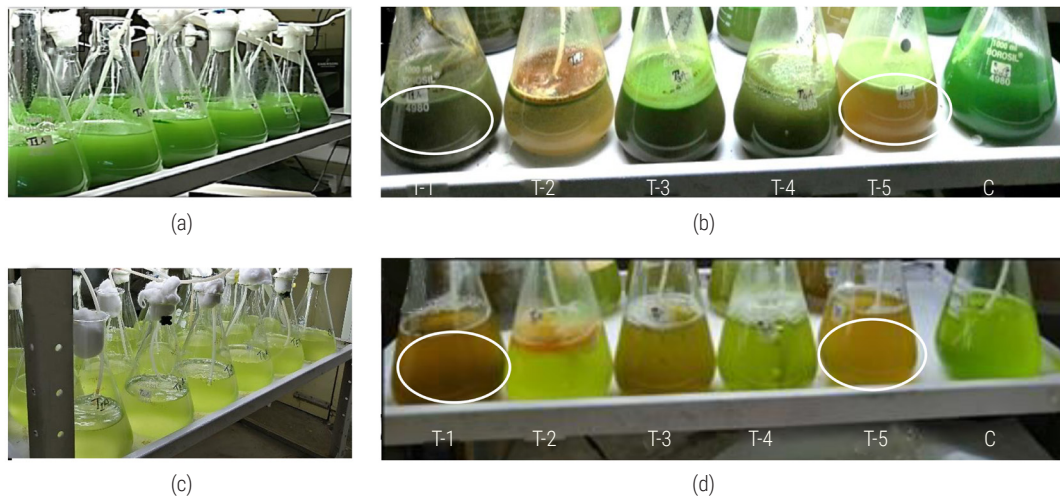


Fig. 2. *Microcystis* bloom collected from wild, (a) Before treatment; (b) Post-treatment effect; (c) Laboratory established *M. aeruginosa* before treatment; (d) After treatment with LAE of *A. indica* (T-1), Neem oil (T-2), LAE of *T. procumbens* (T-3), LAE of *C. procera* (T-4) and Cow urine (T-5). White circle shows yellowing and depigmentation of the algae indicating algal inhibition in treatments unlike green colour

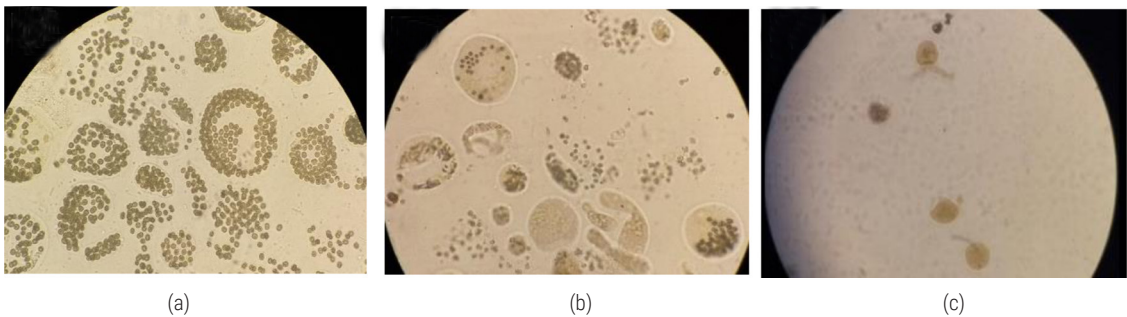


Fig. 3. Gradual deterring effect of cow urine on *Microcystis* colonies collected from wild (a) Control (natural colony); (b) Affected colonies on 3rd day; (c) Affected colonies on 7th day (40 X)

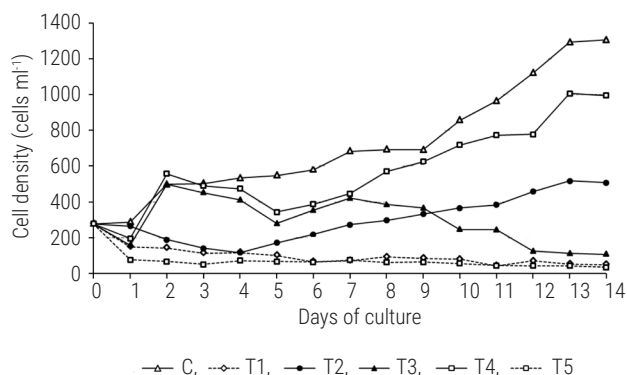


Fig. 4. Effect of different treatments on *M. aeruginosa* growth up to 14 days

to the visual observation of the colour changes in the colonies in T-1, T-3 and T-5 (Fig. 2).

Comparison of the cell densities in the treatments and control on 7th and 14th days with that of the seeded population at the beginning of the study is depicted in Fig. 5(a). While there was 146 and 371% rise in the cell population on 7th and 14th day in control group, similar rise was also observed in T-2 on day 14 (82.3%) and T-4 (61 and 259% on day 7 and day 14). In contrast, significantly higher cell inhibitions were observed in T-1 (74 and 83%) and T-5 (73 and 87%) ($p < 0.05$) on 7th and 14th day, respectively and the % inhibition was almost similar between T-1 and T-5 ($p > 0.05$). Algal growth rate (GR) was also quantified indirectly using absorbance at 680 nm every day and the cell densities on days 7 and 14 post-treatment were in the order: control > T-4 > T-2 > T-3 > T-1 > T-5 Fig. 5(b). The % growth rate of the cell population computed for the two weeks period is presented in Fig. 6.

Discussion

Among the bloom forming cyanobacteria, the genus *Microcystis* has been the biggest threat to the culture pond environment and invites

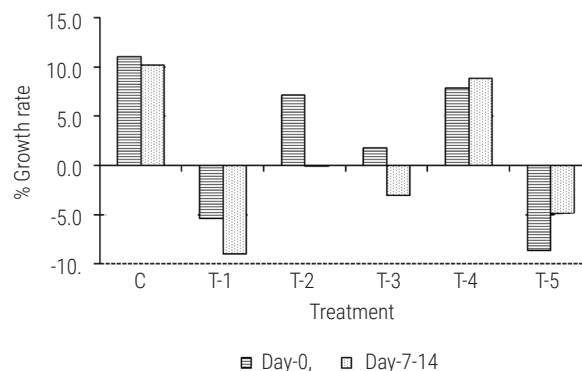
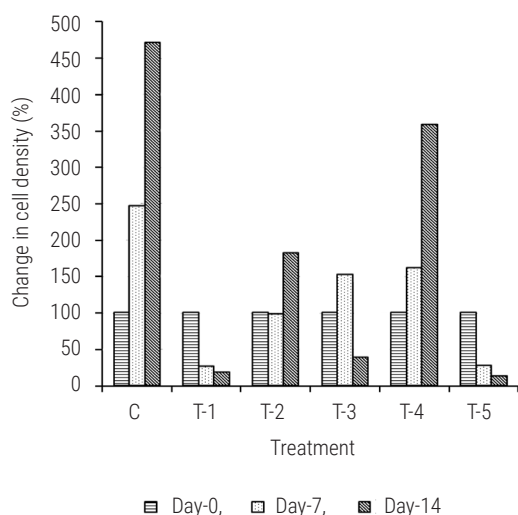


Fig. 6. Percent (%) growth rate of the *Microcystis* population during two weeks post-application of the control agents using OD at 680 nm

various elimination approaches for sustainable aqua-production. Neem leaf has been reported to possess bioactive compounds which have anti-parasitic, anti-plasmodial, antibacterial, antifungal, antialgal and antiviral properties (Pillinger *et al.*, 1994; Ferrier *et al.*, 2005; Chia *et al.*, 2016; Kumari *et al.*, 2019). In the present study, the phyto-constituents such as alkaloid, flavonoid and saponin have been observed in LAE of neem (Kumari *et al.*, 2019). These phyto-constituents were likely to have effect on the *Microcystis* cells. Similarly, observation of alkaloids, saponins and tannins in *T. procumbens* is in agreement with earlier reports (Jude *et al.*, 2009; Agrawal and Talele, 2011). Pre- and post-emergence bioassays, found phytotoxicity of *T. procumbens* against seeds of *Lactuca sativa* L. and root cells of *Allium cepa* L. and attributed it to the rich flavonoid content present (Mecina *et al.*, 2016). In the present study, the observed changes in colony morphology in the wild bloom (Fig. 3) as well as cell inhibition (reduced cell count) in pure culture (Fig. 4) with application of LAEs of neem and *T. procumbens* revealed their anti-algal property against *Microcystis* which might be attributed to the presence of phytoconstituents. Earlier researchers have also attributed such cell inhibition effects to the presence of phenols, flavonoids and tannins (Macias *et al.*, 2007; Yan *et al.*, 2011;

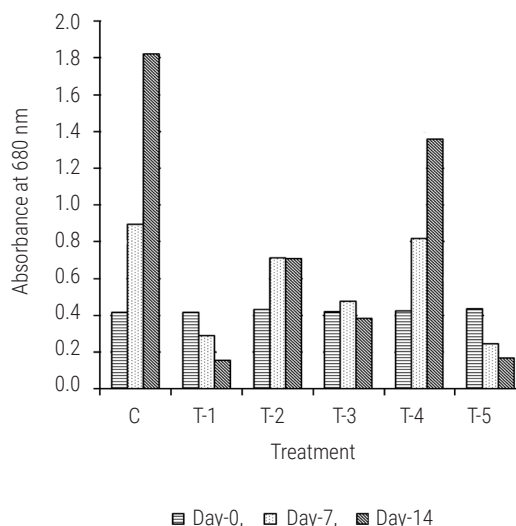


Fig. 5. Change in cell density (%) in *M. aeruginosa* on day 7 and 14 with reference to seeded population in different treatments (n=3) (a) Microscopic cell count; (b) Cell density measured at 680 nm

Chen *et al.*, 2017; Tebaa *et al.*, 2018). However, in the present study, the anti-algal effect of *C. procera* was not much pronounced despite presence of few phytoconstituents in its LAE (Table 1), signifying its ineffectiveness as a control approach against the *Microcystis* species.

The morphological changes as a function of inhibition, observed in the *Microcystis* colonies (Fig. 3) can be explained by the action of the aqueous extract on the polysaccharide compounds of colonial mucilage, inhibition at the biosynthesis level of this mucilage, or by an action on the phospholipids of the cell walls (Chen *et al.*, 2017; Naceradska *et al.*, 2017; Tazart *et al.*, 2019). These changes are certainly indicators of physiological alterations under a stressed environment (Yan *et al.*, 2011; Meng *et al.*, 2015; Li *et al.*, 2016) which implies weakening of colonies. In addition, evacuation of *Microcystis* colonies exposing cells out of the polysaccharide mucilaginous sheath and its subsequent shrinkage and disruption was also recorded in the treatment groups which provide an insight for possible way to control *Microcystis*. The disruption of bound extracellular polysaccharide that binds cells together can reduce floating velocities, increasing susceptibility of *Microcystis* to grazers and disaggregation of their colonies may be a potential approach to effectively mitigate the bloom (Xiao *et al.*, 2018).

Significant algicidal effect of cow urine and LAEs of neem on *Microcystis* population was observed on day 7 when the reduction in cell density were 73 and 74% respectively, compared to the seeded cell density at the beginning. However, in these treatments, there were not much difference in the cell densities between day 7 and day 14 population which revealed inability of the cells to revive after day 7. Cell density on 14th day also showed 97.3% inhibitory rate in cow urine application, followed by T-1 (96.3%) and T-3 (91.8%) indicating persistence of their potential control effect than the cell population in control on the same day.

The spectrophotometric measurement of cell density in these treatments also revealed similar trend of reduction of cell population (Fig. 5b). In treatments T-2 and T-4, though there were a brief initial reduction in the cell density during the first five days (Fig. 4), the respective rise in cell population on 7th day were 0 and 82% higher as compared to the seeded population on day 0. Similarly, on day 14, cell count revealed 371, 82 and 259% rise in population in Control, T-2 and T-4, respectively compared to that on day 0. The inhibitory rates on day 14 for T-2 and T-4 were 61.3 and 23.8% compared to control which indicated ineffectiveness of *C. procera* and neem oil application in controlling the *Microcystis* population.

Fig. 6 depicts a sharp decline in the cell population in T-1 and T-5 during the 1st week unlike in the other treatments. The % growth rate further declined in these two treatments during the 2nd week revealing no cell revival. Further, a close look at the change in growth rate in these two treatments revealed a sharper decline in cell population in T-5 than that in T-1 during the 1st week, but *vice versa* occurred in the 2nd week. Such results revealed a quicker action of cow urine (T-5) in controlling the *Microcystis* bloom as compared to the LAEs of neem plant (T-1). The 2nd week also showed a decline in population growth in T-3 indicating a delayed effect of the LAE of *T. procumbens*. Ye *et al.* (2014) reported the effects of several Chinese herbal aqueous extracts on *M. aeruginosa* with a maximum inhibitory rate of 51-98% after 10 days. Similarly, *Ailanthus altissima* extracts resulted in 90% inhibitory rate against *M. aeruginosa* (Meng *et al.*, 2015). The observed inhibitory rate of the three control agents (T-1, T-3 and T-5) in the present study, being in the higher side of the

above reported ranges, indicated their potential to control *Microcystis* population. The information about the potential uses of cow urine as bio-pesticides, bio-enhancers, therapeutics as well as nitrogen booster in soil are well documented (Mohanty *et al.*, 2014). However, use of cow urine as an anti-algal agent in this study is probably a novel approach, which has also shown the highest cell inhibition on 14th day of the experiment against *M. aeruginosa* pure culture. Since this was a pilot study and no previous report elaborates such inhibitory effect of cow urine on *Microcystis* cells and bloom, the hypothesis needs to be deeply studied further.

Applications of organic algaecides are contemporary and unparalleled among the existing algal control measures in view of environmental health. Terrestrial plants, the treasure houses of important bioactive compounds, remain largely unexplored for their potential anti-algal properties. The present study clearly demonstrated the inhibitory effects of LAEs of *A. indica* and *T. procumbens* and also highlighted cow urine as a potential ITK solution in controlling *Microcystis* populations. Reduction in cell density and morphological alterations in *Microcystis*, recorded in the study, could be attributed to the allelopathic effect of phytoconstituents in the aqueous extracts, whereas effect of cow urine might be due to the presence of specific algaecide compounds, which needs further investigations. To the best of the authors' knowledge, this is probably the first study to explore on the potential algaecide properties of cow urine for effective control of *Microcystis*. However, the dosages of the various control agents used in this study were relatively high for field application, which highlights the need to isolate their specific active ingredients enabling the development of more concentrated formulations that can be applied in smaller quantities in the fish culture systems for effective control of *Microcystis* blooms. In addition, further studies are essential to evaluate the effect of these compounds/products on the water quality, microbial and algal communities, aquatic animal health and physiological conditions. Such studies are critical for formulating environmentally friendly measures to manage noxious algal blooms, especially when scaling up to macrocosm and field trials in freshwater ponds.

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