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Screening of blue-green algae *Lyngbya* for its antimicrobial activities

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Abstract

This study aimed to assess the effect of extracts of *Lyngbya* strain, MZUCC066, prepared in different solvents (methanol-100%, ethanol:water-3:7 and dichloromethane:isopropanol-1:1) against six microorganisms that comprise three chlorophycean algae (*Chlorella vulgaris, Scenedesmus quadricauda* and *Selenastrum capricornatum*), one cyanobacterian alga (*Anabaena variabilis*), and two bacterial strains (*Bacillus subtilis,* ATCC11774 and *Bacillus pumilus,* ATCC14884). Among the algal test organisms, the highest inhibition was found in *S. quadricauda* (70%) by dichloromethane:isopropanol extract of *Lyngbya* followed by *A. variabilis* (41%) by the same extract. When tested on bacterial test organisms, the highest inhibition zone diameter of 15.67 mm was found in *B. subtilis* by dichloromethane:isopropanol extract and followed by 14 mm in *B. pumilus* by the same extract at 100 µl concentration. Minimum inhibitory concentrations of each extract on all the organisms were varied. The present study suggests that *Lyngbya* has specific bioactivities against different groups of microorganisms.

Key words: *Lyngbya*; bioactivity; antimicrobial activity; cyanobacteria; chlorophyceae.

Introduction

Cyanobacteria are diverse group of Gram-negative prokaryotes, and are widely distributed microorganisms equipped with oxygenic photosynthesis. They can grow as free-living organisms or as symbionts in association with other plants and lichens. They are very primitive and they were 'main living organisms' for 1.5 billion years. During evolution they have evolved well diversified metabolic pathways to cope with changing environment. Many metabolites with a diverse range of bioactivities have been reported in cyanobacteria.[1] These metabolites probably originated in cyanobacterial mats and were presumably responsible for regulation of communities. Although they evolved secondary metabolite pathways to protect (allelopathic) themselves from competitors sharing same habitat, many metabolites of cyanobacterial origin have poten-

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tial pharmaceutical, agricultural and other applications.[2] Various strains of cyanobacteria are known to produce intracellular and extracellular metabolites with diverse biological activities, such as antialgal, antibacterial, antifungal and antiviral.[3] The medicinal and nutritional qualities of cyanobacteria were first appreciated as early as 1500 B.C., when *Nostoc* species were used to treat gout, fistula and several forms of cancer.[4]

Lyngbya is a filamentous cyanobacterium and has been found to be a rich source of bioactive metabolites. Compounds like pahayokolide A, isolated from freshwater *Lyngbya*, has pharmacological and toxicological effects. Pahayokolide A inhibited some species of *Bacillus* as well as yeast, *Saccharomyces cerevisiae* and it also inhibited several representatives of green algae. Extracts of *Lyngbya* species were shown to be cytotoxic, antimicrobial and ichthyotoxic.[5] Many bioactive compounds like sulfolipids[6] and lyngbyastain1[7] have been identified from *Lyngbya* for their antiviral (HIV-1) and cytotoxic effect. A number of bioactive compounds from *Lyngbya* are being currently investigated as potential anticancer drugs.[8] It has been suggested that most of cyanobacterial bioactive substances are targeted against a range of biochemical processes including photosynthesis, phosphorylation, and other energy yielding pathways. Cyanobacterial metabolite known as algicide acts as light reactions of photosynthesis.[2]

Cyanobacteria are well known for great phenotypic and genetic diversity, very likely the bioactive chemicals are still to be discovered in this microbial group. In present study, *Lyngbya* was isolated from a wetland and was screened for its bioactivities against diverse type of organisms including blue green algae, green algae and bacteria. The main goals of this study were to assess the potential bioactivity of cyanobacteria on other organisms' growth and it aimed to extend our understanding on the bioactive chemicals of cyanobacteria.

Materials and Methods

Culture and growth condition

The cyanobacterial species, *Lyngbya* was isolated from Tamdil wetland of Mizoram, India. It was purified on agar plates by using standard methods of isolation and purification and was grown axenically in Chu-10 medium[9] at pH 7.0 in an air-conditioned culture room under the photoperiod of 12 hrs. Cultures were hand-shaken thrice daily. The pure culture of *Lyngbya* is deposited at the Microalgal Culture Collection of Mizoram University (accession No. MZUCC066).

Determination of specific growth rate, protein, chlorophyll a and carotenoid

The growth rate was determined by spectrophotometric method and the values were calculated according to Guillard.[10] The protein content of the *Lyngbya* was determined using the standard BSA solution by Lowry's method.[11] Quantitative estimation of photosynthetic pigments, chlorophyll a and carotenoid were performed by using UV-VIS spectrophotometer (Systronics, India, model: 117) at 665 and 480 nm, respectively.[12,13]

Estimation of lipid content

The algal culture of 0.02 g fresh weight was washed with 0.2% NaCl. Then, the pellet was collected and suspended in hot isopropanol and boiled for 3 mins. After cooling, the suspension was mixed with chloroform and kept for 12 hrs at room temperature. 10 ml of water was added to the mixed chloroform extract and separated into two phases. The lower chloroform phase was collected and heated at 100-110°C to evaporate the chloroform completely within 15 mins. 2 ml of stock acid dichromate reagent was added and heated again for 30 mins and 10 ml of water was added. It was mixed well and the absorbance was recorded at 430 nm against water

blank.[14]

Estimation of sugar content

0.01 g dry weight of *Lyngbya* was mixed with 3 ml of 80% hot ethanol and kept at room temperature for 30 mins. Then, it was centrifuged at 5000 rpm for 10 mins. The supernatant was collected and used for estimation of soluble sugar. Equal amount of the extract (supernatant) and water were mixed with 4 ml of anthrone and kept for 10 mins in a water bath at 60°C. After that the absorbance was recorded at 550 nm and sugar content was calculated with its standard dextrose curve.[15]

Test microorganisms

Three green algae, *Chlorella vulgaris*, *Scenedesmus quadricauda* and *Selenastrum capricornatum*, one blue green alga, *Anabaena variabilis* and two gram positive bacteria *Bacillus subtilis* (strain ATCC11774) and *Bacillus pumilus* (ATCC14884) were employed as the target organisms for testing of bioactivities. Algae were grown axenically in Chu-10 medium whereas bacterial strains were incubated in nutrient broth medium. Unless specified throughout the study actively growing cultures were used for all the bioactivity tests.

Preparation of extracts

Algal mass from axenic stationary phase culture of *Lyngbya* growing in Chu-10 medium was harvested by centrifugation at 3000 rpm for 10 min and washed four times with sterile distilled water in order to remove extracellular impurities. The pellets were collected in a flask containing sterile distil water and kept in natural condition (at room temperature) for 48 hrs in order to stimulate synthesis of secondary metabolites. Subsequently, the biomass was freeze dried at -20°C for 24 hrs.[16] A 0.5 g freeze dried biomass was suspended into 10 ml of methanol and cell were ruptured by ultrasonication (Sartorius, Germany; model: BBI 8535108) for 30 sec and residues were separated by passing through glass wool. The filtrates were used as methanol extract and residues were further extracted first with ethanol: water 3:7, v/v and then with dichloromethane: isopropanol (DCM:ISO) 1:1, v/v in a column packed with Sephadex G.

Antialgal test

Diluted cultures of *Chlorella vulgaris*, *Scenedesmus quadricauda*, *Selenastrum capricornatum* and *Anabaena variabilis*, in growth phase were used for their bioactivity test. Different concentrations ranging from 25-100 µl of the extracts were added to 3 ml culture of *C. vulgaris, S. quadricauda, S. capricornatum* and *A. variabilis* (in three replicates each) and after 4 days of incubation in light, the absorbance was recorded at 684 nm for *C. vulgaris* and 440 nm for *S. quaricauda* and *S. capricornatum* and 417 nm for *A. variabilis* using spectrophotometer (Systronics, India, model: 117). Specific growth rate was calculated for treated and untreated cultures.

Antibacterial test

The antibacterial activities of different extracts were tested using the agar well diffusion assay method.[17] Briefly, bacterial strains were inoculated in nutrient broth medium and incubated at 35°C for 24 hrs. The 0.5 ml of bacterial suspensions were poured on nutrient agar plates in sterile condition and spread uniformly by using L-shape spreader. Plates were punched to make a well of 6 mm diameter with the help of sterile cork borer. Different concentrations (25 µl, 50 µl, 75 µl and 100 µl) of the extracts were pipette into the wells and plates were incubated overnight at 35°C in an incubator. All the tests were done in triplicates. Along with the treatment, positive controls were also considered with extraction media. The plates were observed after 48 to 72 hrs for the determination of the inhibition zones.

Determination of minimum Inhibitory concentration (MIC)

Minimum inhibitory concentrations (MIC) of different extracts were evaluated using different concentrations (25 µL, 50 µL, 75 µL and 100 µL) of extracts against the test organisms. These tests were done to determine the lowest concentration of algal extracts that inhibit the growth of other organisms.

Statistical analysis

The data obtained from the study were analyzed statistically using the analysis of variance (ANOVA). The test was performed using statistical software, STATISTICA version 5.0 at $p<0.05$ significant level.

RESULTS

The growth pattern of *Lyngbya* was shown in Figure 1A by recording the absorbance of culture at 440 nm in alternate days. The changes in the level of protein, chlorophyll a and carotenoid content in its growing phase were also observed and plotted in Figure 1 B, C and D, respectively. The general biochemical profile including fresh weight, dry weight, moisture content, lipid content and sugar content were given in Table 1.

In the present investigation, *Lyngbya* species has been screened for their antialgal and antibacterial activities. Results showed that *Lyngbya* has diverse type of bioactive compounds affecting each test organisms in different ways.

Out of all the organisms tested, highest antialgal activity was found to be associated with dichloromethane:isopropanol extract of *Lyngbya* on *S. quadricauda* by 70% followed by methanol extract which was found to exhibit 64% growth inhibition and ethanol:water extract showed inhibitory effect (54%) on the same test on *S. quadricauda*. While testing on *S. capricornatum*, only methanol extract showed the inhibition of its specific growth rate. However, ethanol:water and dichloromethane:isopropanol extracts enhanced the growth of *S. capricornatum.* Tested on *C. vulgaris*, ethanol:water extract of *Lyngbya* exhibited the growth inhibition, while the other extracts did not show any inhibition. The specific growth rate of *A. variabilis* was inhibited around 36-41% by all the three extracts of *Lyngbya* (Figure 2).

Among the cyanobacterial extracts tested, antibacterial activity was found to be associated only with dichloromethane:isopropanol extract on both the bacterial test organisms. Maximum

Table 1. Some characteristic profile of the cyanonobacterium, *Lyngbya* species.

Weight in gram per litre (gl^{-1})		Percentage content		
Fresh weight	**Dry weight**	**Moisture content**	**Lipid content**	**Sugar content**
3.086	0.366	88.139	2.850	5.200

Table 2. Minimum inhibitory concentrations of extracts of Lyngbya on test organisms after statistical analysis (ANOVA).

Test organisms	MIC of *Lyngbya* extracts		
	Methanol extract	**Ethanol:H_2O extract**	**DCM:ISO extract**
C. vulgaris	75 µl	100 µl	-
S. quadricauda	50 µl	25 µl	25 µl
S. capricornatum	50 µl	-	-
A. variabilis	25 µl	25 µl	50 µl
B. subtilis	-	-	25 µl
B. pumilus	-	-	50 µl

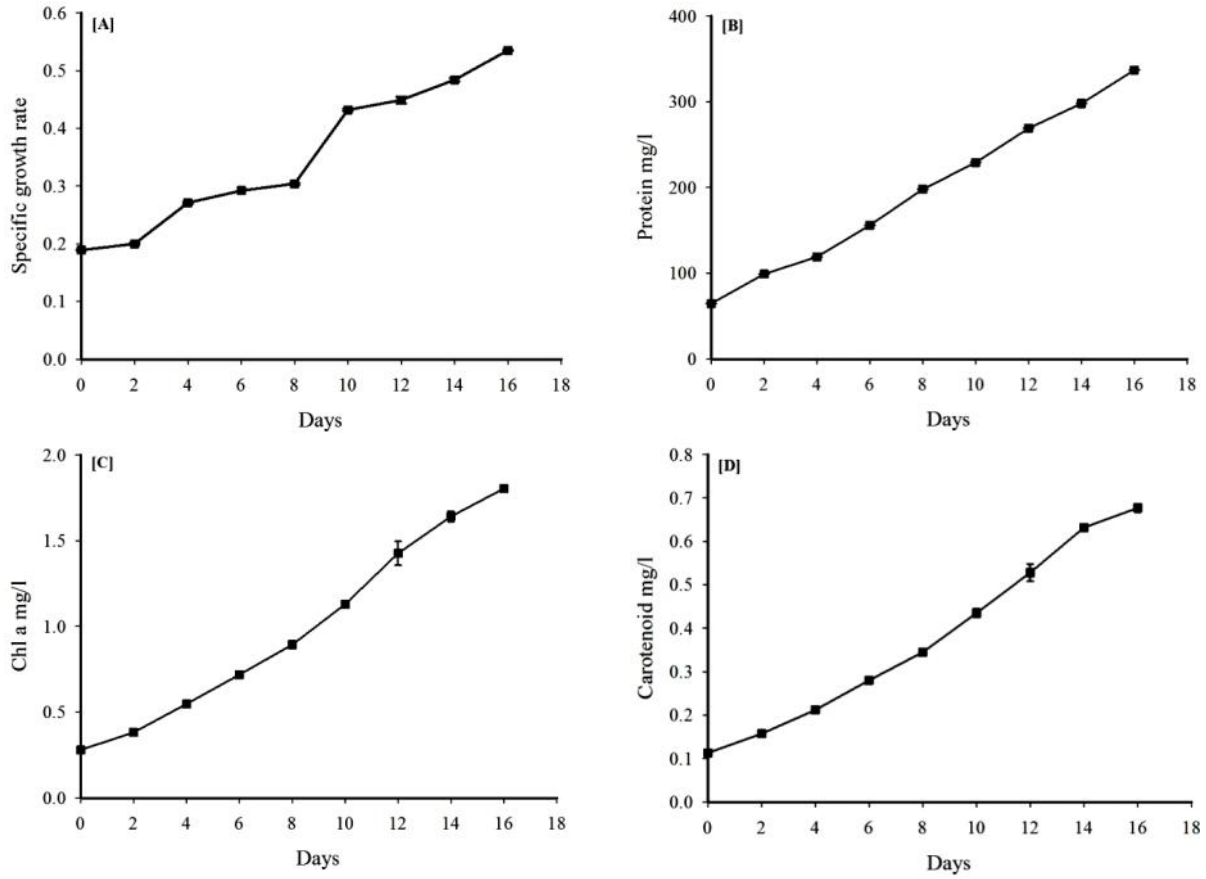

Figure 1 (A): Growth behaviour of *Lyngbya* species grown in Chu-10 medium and specific growth rates were calculated from absorbance recorded at different days. (B), (C) and (D): Protein, chlorophyll a and carotenoid content of *Lyngbya* cells in mg/l during the growth period of 16 days. Vertical bars indicate standard error of mean value of triplicates.

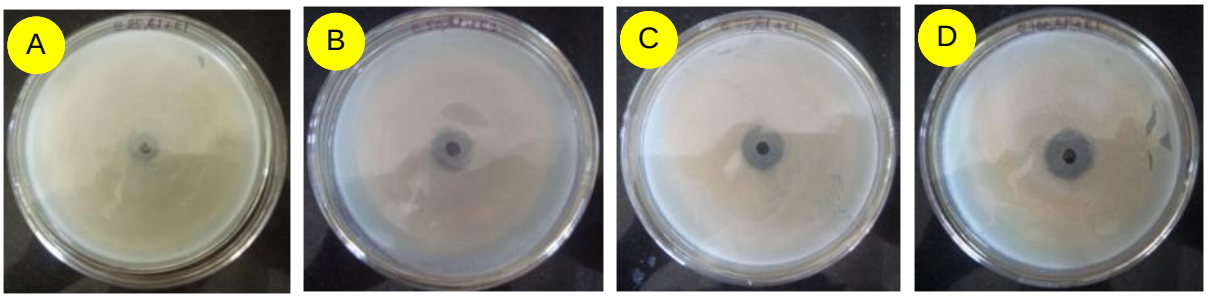

Plate 1. Inhibition zone in culture plate of *B. subtilis* after application of *Lyngbya* dichloromethane:isopropanol extract in 25 µl (A), 50 µl (B), 75 µl (C) and 100 µl (D) concentrations. The inhibition zone was measured after 48 hours of application of extract to plates.

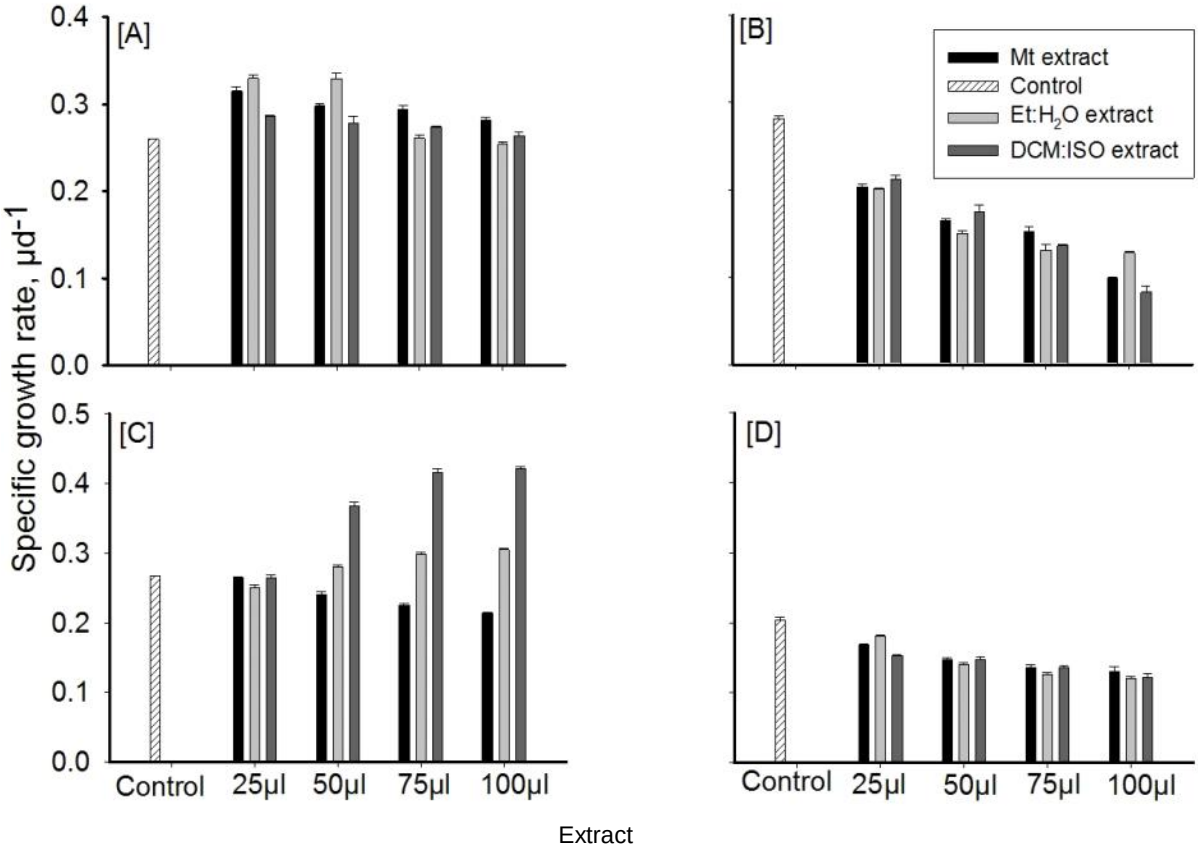

Figure 2: Effect of three extracts of *Lyngbya* on growth of [A] *C. vulgaris*, [B] *S. quadricauda*, [C] *S. capricornatum* and [D] *A. variabilis*. Values are the mean of triplicates and vertical bars represent standard error. Specific growth rates were calculated after 4 days of treatment.

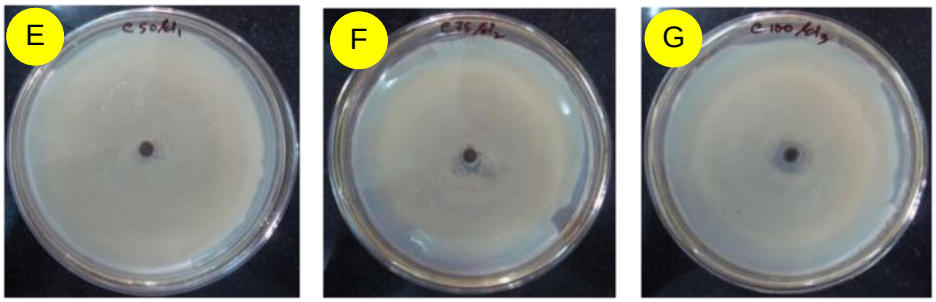

Plate 2. Inhibition zone in culture plate of *B. pumilus* after application of *Lyngbya* dichloromethane:isopropanol extract in 50 µl (E), 75 µl (F) and 100 µl (G) concentrations. The inhibition zone was measured after 48 hours of application of extract to plates.

size of inhibition zone diameter of 15.67 mm was observed in *B. subtilis* (Plate 1D) with 100 µl/well concentration and followed by 14 mm inhibition zone diameter in *B. pumilus* with the same concentration by the dichloromethane:isopropanol extract (Plate 2G). Whereas, smallest zone of inhibition (7.33 mm) was occurred in *B. subtilis* with 25 µl/well concentration (Plate1A) but, no inhibition was found on *B. pumilus* with the same concentration.

Minimum inhibitory concentrations of each extract on all the test organisms have been shown in Table 2. The results indicated that MICs of methanol extract of *Lyngbya* were 25 µl against *A. variabilis*, 50 µl against *S. quadricauda* and *S. capricornatum* and 75 µl against *C. vulgaris*. While, the same for ethanol:water extract were 25 µl against *S. quadricauda* and *A. variabilis* and 100 µl against *C. vulgaris* and dichloromethane:isopropanol extract were 25 µl against *S. quadricauda* and *B. subtilis and* 50 µl against *A. variabilis* and *B. pumilus*. Increase in the inhibition of the test organisms occurred upon increasing concentration.

Discussion

The present study showed that extracts of *Lyngbya* has antimicrobial activity against tested algae and bacteria. Our results have shown inhibitory effect on the growths of *C. vulgaris with* ethanol:water and *B. subtilis* with dichloromethane:isopropanol extracts, respectively. Similarly, the growth of gram positive bacteria, *Bacillus* species, such as *B. megaterium* and *B. cereus* as well as strains of green algae, like *Chlamydomonas*, *Ulothrix* and *Chlorella* were also partially inhibited by *Lynbgya* extract due to the presence of a compound called pahayokolide A. This compound has toxicological and pharmacological properties.[5]

The growths of *B. subtilis* and *B. pumilus* were significantly inhibited with dichloromethane:isopropanol extract. However, methanol and ethanol:water extracts do not have any effect on the growth of bacteria. It suggests that dichloromethane:isopropanol extracts of this cyanobacterium have some bioactive chemicals that are effective against eukaryotic cells. Contrary to this study, Madhumathi *et al*., (2011) reported the antibacterial activity of methanol extract of *Lyngbya martensiana* on *B. subtilis.*[18] Cyanobacterial strains are known to exhibit extensive genetic and physiological variability, and the discrepancy observed in the present study can be attributed to such variation.

The extracts of *Lyngbya* exhibited antialgal activity against the cyanobacterium, *A. variabilis*. Similarly, extract of *L. majuscule* has shown anticyanobacterial activity, and this alga derive a toxin, lyngbyatoxin A (LTA) which is a specific activator of protein kinase C.[19] All these results showed very effectiveness, particularly inhibition of *Lyngbya* extracts on eukaryotic as well as prokaryotic target organisms. Further studies are required to purify and characterize bioactive chemicals of these algae.

We have found that some extracts of *Lyngbya* significantly stimulated the growth of *C. vulgaris, S. capricornatum, S. quadricauda* and *A. variabilis*. This suggests that they produce metabolites with differential actions in different targets. Similar to the present study, stimulation in growth of some phytoplankton in response to cyanobacterial extract was earlier reported.[20] The cyanobacterial stimulation can be attributed to nutritional effect of compounds present in extract and adaptation mechanisms of target organisms to bioactive compounds.[21] Stimulation can also be explained that target organism has evolved pathways to effectively metabolize and used the allelochemicals-like compounds.[22] Effect of phytohormones like auxins, gibberellins and cytokinins present in extract cannot be ruled out. Present study showed that composition of extraction solvent has strong effect on inhibition or stimulation of growth of target organisms. However, as shown in present study, stimulation or inhibition is target organism specific, but not the solvent system specific.

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