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Studies on Antibacterial Activity of a Cyanobacterium *Tolypothrix fragilis* (Gardner) Geitler

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Abstract

Tolypothrix fragilis, a cyanobacterium was isolated from the collected soil samples from different locations of Ahmednagar district of Maharashtra state (India). Identification was carried out using morphological variation and taxonomical approaches according to Desikachary and Prescott. The axenic culture of *Tolypothrix fragilis* was obtained by using the method recommended by Bolch and Blackburn. The isolated *Tolypothrix fragilis* was grown autotropically in BG-11 medium and incubated at 30 ± 2°C. After 25 days, biomass was harvested by filtration through double layered muslin cloth and dried using air blower. The biomass of this *Tolypothrix fragilis* species was used for the assessment of antibacterial activity against *Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Micrococcus flavus and Proteus mirabilis*. The antibacterial activity was studied by disc diffusion method. Methanol extract of *Tolypothrix fragilis* showed the activity against all the tested bacterial strains. Maximum zone of inhibition (22 ± 1.4 mm) was recorded with methanol extract of *Tolypothrix fragilis* against Bacillus subtilis. The MIC for all the bacteria was in the range of 32 – 256 µg/ml.

Keywords: *Tolypothrix fragilis* • Antibacterial activity • BG-11 • *Escherichia coli* • *Bacillus subtilis* • *Staphylococcus aureus* • *Pseudomonas aeruginosa*

Introduction

Telomere abrasion is considered a seal of the ageing process [1]. Significant progresses have been made in understating the basic biology of telomere function through *in vitro* research, the rendition of this research to an *in vivo* perspective is limited. Though numerous techniques are there to label telomeres, most of these are toxic to cells and cause DNA damage or non-compatible for *in vivo* applications [2]. The CRISPR-Cas system has enabled the refinement of these regions by fusing Cas9 to a fluorescent protein, allowing telomeres to be visualised in living organism [3]. The success rate of CRISPR Cas 9 technique is a new promise for future genome editing therapeutics. Telomere length and rate of telomere shortening are directly related to aging and eventual death for any organism. This effect can potentially be reversed by increasing the telomere length of an organism. CRISPR Cas system is an effective tool that can be used in the insertion of telomeres in the DNA of any given organism without error [4].

An infectious disease is one of the reasons for increasing number of deaths in developing countries and world-wide. They hold the second position after heart diseases. The search for antibiotics began in the late 1800s; the scientist began to devote time for searching the drugs that would kill the disease-causing bacteria. The goal of such research was to find so called 'magic bullet or wonder drug' that would destroy microbes without toxicity to the person taking that drug [5,6]. Today, most of the diseases are caused by pathogens that can be cured with the help of available antibiotics. Still there is need to explore and develop new effective antibiotics against microbial pathogens because of resistance mechanism of the target organism. Taking this into consideration, there has been a global attention towards finding new chemicals, which led to the development of structure, which either directly or after some modifications can be used for development of new drugs [7-9].

The interests in the cyanobacteria, as generators of pharmacologically active and industrially important compounds have been stimulated by the recent year [10]. Therefore, an optimized production of relevant compounds under controlled conditions is conceivable.

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The first partly identified antimicrobial compound isolated from algae was obtained from unicellular green algae particularly, chlorella which contained a 'chlorellin' that exhibited inhibitory activity against both gram-positve and gram-negative bacteria, including *Staphylococcus aureus, Streptococcus pyogenes, Bacillus subtilis and Pseudomonas aeruginosa*. A pronounced reduction of gram-positive bacteria in lakes during the occurrence of cyanobacterial water-blooms was reported and the production of antibacterial substances may be one reason for this phenomenon [11-12].

Cyanobacteria are known to be able to survive under all kinds of environmental conditions, terrestrial, saline water and freshwater, and even under extremely competitive environments; moreover, they are exposed to a wide variety of predators and to microbial pathogens, such as bacteria, viruses, and fungi [13]. Their flexible metabolism underlies both their adaptation to a diversity of growth conditions and habitats and their capacity to respond to different environmental stresses and nutrients sources. This versatility can explain the diversity and the number of chemical compounds that have been isolated from them.

Secondary metabolites from cyanobacteria have been reported to have pharmaceutical potential belonging to a wide range of structural classes like alkaloids, aromatic compounds, peptides, terpenes, etc. all of which exhibit some biological activity. They are known to produce a wide variety of toxins which include 40% lipopeptides. According to the cyanobacterial lipopeptides include different compounds like cytotoxic (41%), antitumor (13%), antiviral (4%), antibiotics (12%) and 18% activity includes antimalerial, antimycotics, multi-drug resistance reversers, anti-feedant, herbicides and immunosuppressive agents. Isolation of bioactive compounds from cyanobacteria is done with two objectives. One is to discover new compounds for pharmaceutical, agricultural or biocontrol application. The other is to better understand the interactions of individual organisms within their natural communities [14-16]. For each of these purposes, there is a need to screen new culturable organisms to understand the frequency and distribution of bioactive strains. There are numerous review articles about marine, freshwater, and terrestrial cyanobacteria, belonging to different families, as a source of antibacterial molecules [17]. The present work describes the results of screening of *Tolypothrix fragilis* against pathogenic bacteria.

Materials and Methods

Collection, isolation and identification of cyanobacteria

Tolypothrix fragilis was isolated from the collected soil samples from different locations. The isolated *Tolypothrix fragilis* was grown in BG-11

medium as described by and incubated at 30 ± 2°C. Identification was carried out using morphological variation and taxonomical approaches according *Tolypothrix fragilis* was cultured in BG-11 culture medium for large scale biomass production. After 25 days biomass was harvested by filtration through double layered muslin cloth and dried using air blower. The biomass of *Tolypothrix fragilis* was used for the assessment of antibacterial activity [18].

Extraction procedure

Five g of finely powdered biomass of *Tolypothrix fragilis* was successively extracted in 50 ml of hexane, chloroform, methanol and water by using soxhlet app aratus at 40°C for 24 h. The filtered extract was concentrated in vacuo at 40°C. Final volume of the extract was made 1 ml with respective solvents [19].

Standard antibiotic

Standard antibiotic disc (10 µg/ml streptomycin) used in the present study was procured from Hi Media (India). These discs were kept on the nutrient agar media containing known volume of the bacteria [20].

Test organisms

Pure cultures of *Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Micrococcus flavus* and *Proteus mirabilis* were procured from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory (NCL), Pune and used for antibacterial assay. Cultures were maintained according to guidelines of NCIM, NCL, Pune.

Preparation of culture medium

The chemicals required to prepare the nutrient agar media was procured from Hi-media Laboratories; Pune (India). Composition of the medium is as follow.

Ingredient	g L-1
Peptone	10.00
Beef	10.00
NaCl	5.00
Agar	20.00

pH was adjusted to 7.5 using 0.1 N HCL or 0.1 N NaOH on standardized pH meter. The culture medium was sterilized in an autoclave at 1.06 kg cm^{-2} pressure for 20 minutes. Required appliances like Petri-dishes, conical flasks, forceps, Pipettes, etc. were also sterilized in an autoclave at 1.06 kg cm^{-2} pressure for 30 minutes.

Preparation of inoculum

The gram positive (*Bacillus subtilis, Staphylococcus aureus* and *Micrococcus flavus*) and gram negative bacteria (*Escherichia coli, Pseudomonas aeruginosa* and *Proteus mirabilis*) were pre-cultured in nutrient broth for overnight in a rotary shaker at 37°C, centrifuged at 10,000 rpm for 5 min, pellet was suspended in double distilled water and the cell density was standardized spectrophotometrically (A610 nm).

Antibacterial assay

Antibacterial assay was carried out by agar diffusion assay. Paper discs (Whatman No. 41) of 6.4 mm diameter were prepared and sterilized in autoclave. The 10 ml molten nutrient agar medium was allowed to cool to 45°C and to it 20 µl bacterial cultures at a concentration of approximately 1.5 X 10^8 colony forming unit (CFU) was added and poured in sterile petri-dish. This was allowed to solidify and then individual plates were marked for the organism inoculated. The bundles of discs (Four disc together) were prepared consisting 400 µg/ml of extracts [21]. Solvent was allowed to evaporate. After solidification, the discs were placed in petri plates at equal distance. By the same method, for each organism duplicate plate, standard plates and control plates (solvent) were prepared. For standard plates, antimicrobial substance streptomycin (10 µg/ml) was used. The plates were incubated at 4°C for 8 hours to allow the diffusion of the samples. After that the plates were incubated at 37°C for 24 hours. After 24 hours, the diameter of the zone of inhibition was measured to the nearest mm. Depending on diameter of the zone of inhibition; activity of test extract was compared with standard. All the tests were performed under sterile conditions and repeated for three times.

Determination of Minimum Inhibitory Concentration (MIC)

Crude extracts of biomass of *Tolypothrix fragilis* were screened for antibacterial activity against Gram positive and Gram negative bacteria using the micro broth dilution techniques. Dilution of the crude extracts was prepared in nutrient broth ranging from 1 to 400 µg ml^{-1} in dimethylsulphoxide (DMSO). The extract solutions were serially diluted in 96 well plates. Bacteria at a concentration of approximately 1.5 x 10^8 colony forming units (CFUs) ml $^{-1}$ were added to each well. Plates were then incubated at 37°C for 24 hours, and the final MIC was determined as the lowest concentration turbidity (by measuring absorbance at 600 nm). Streptomycin was used as positive control, and DMSO was used as a negative control.

Results and Discussion

The antibacterial activity of *Tolypothrix fragilis* was studied by disc diffusion method. After the measurement of the size of inhibition zone, the results of antibacterial activity of different extracts of *Tolypothrix fragilis* at 400 µg/ml concentration against gram positive and gram negative bacteria have been given in Tables 1 and 2. Streptomycin was used as a positive control. The antibacterial activity of the methanol extracts showed varying magnitudes of inhibition patterns with standard positive control depending on the susceptibility of the tested microorganism. Methanol extract of *Tolypothrix fragilis* showed the activity against all the tested bacterial strains. Maximum zone of inhibition (22 ± 1.4 mm) was recorded with methanol extract against *Bacillus subtilis*.

Chloroform extract of *Tolypothrix fragilis* showed the activity against all the tested bacteria. Chloroform extract of *Tolypothrix fragilis* showed more pronounced activity against Pseudomonas aeruginosa.

Hexane extract of Tolypothrix fragilis was moderately effective against *M. flavus* and *P. mirabilis* at 400 µg/ml concentration. Aqueous extract did not show activity against any bacterium.

Antibiotics are the most important weapons in fighting bacterial infections and have greatly benefited the health-related problems of human life. However, over the past few decades these health benefits are under threat as many commonly used antibiotics have become less and less effective against certain illnesses not only because many of them produce toxic reactions but also due to emergence of drug resistant bacteria. It is essential to investigate newer drugs with lesser resistance. Systematic studies among various pharmacological compounds have revealed that any drug may have the

Table 1: Antibacterial activity of different extracts of *Tolypothrix fragilis* at 400 µg/ml concentration against gram positive and gram negative bacteria.

Bacterium	Diameter of effective zone of inhibition (mm)				
	Methanol extract	Chloroform extract	Hexane extract	Aqueous(water) Extract	Streptomycin (10 µg/ml)
Escherichia coli	12 ± 1.3	12 ± 1.6	-	-	17 ± 2.4
Bacillus subtilis	22 ± 1.4	9 ± 1.5	-	-	25 ± 1.3
Staphylococcus aureus	12 ± 1.5	8 ± 1.2	7 ± 1.1	-	23 ± 1.7
Pseudomonas aeruginosa	10 ± 1.3	15 ± 1.6	-	-	22 ± 1.6
micrococcus flavus	10 ± 1.3	11 ± 1.5	14 ± 1.9	-	20 ± 1.2
Proteus mirabilis	12 ± 1.4	12 ± 1.6	11 ± 1.4	-	19 ± 1.2

Table 2: Minimum inhibitory concentration (MIC) of different extracts of *Tolypothrix fragilis* against tested pathogenic bacteria. Concentration of extracts is expressed in terms of µg/ml.

Bacterium	Concentration of extracts in µg/ml.			
	Methanol extract	Chloroform extract	Hexane extract	Aqueous(water) Extract
Escherichia coli	64	>400	>400	>400
Bacillus subtilis	32	256	>400	>400
Staphylococcus aureus	256	325	325	>400
Pseudomonas aeruginosa	64	325	>400	>400
Micrococcus flavus	128	256	256	>400
Proteus mirabilis	256	256	325	>400

possibility of possessing diverse functions and thus may have useful activity in completely different spheres of medicine.

The antibacterial activity of *Tolypothrix fragilis* extracts were examined against six pathogenic bacteria. The extraction was carried out using hexane, chloroform, methanol and water. Out of six bacterial strains tested, three showed inhibition activity to all the extracts. The highest activity in terms of effective zone of inhibition (22 mm) was observed in *Tolypothrix fragilis* against *Bacillus subtilis*. The analysis of methanol extract of *Tolypothrix fragilis* showed a significant level of inhibition against *Bacillus subtilis*. On the other hand, comparatively less activity was observed in hexane extract of *Tolypothrix fragilis*. It can be understood that methanol extract is more potent, showing a higher degree of antimicrobial activity to pathogen in comparison to other extract and also reported that the methanolic extraction yields higher antimicrobial activity than hexane and other solvents, whereas others reported that chloroform is better than methanol and benzene [22] It is clear that organic solvents provide higher efficiency in the extraction of compounds for antimicrobial activity when compared to the water based methods.

Screened organic solvent extracts of different cyanobacteria for their antibacterial activity against *Escherichia coli, Bacillus subtilis, Staphylococcus aureus* and found activity in five cyanobacterial cultures.

In chloroform extract, the maximum activity in terms of effective zone of inhibition (15 mm) was recorded in Tolypothrix fragilis against *Pseudomonas aeruginosa*. Chloroform extract of *Tolypothrix fragilis* showed moderate activity against all tested bacteria. This means that the compound responsible for the antibacterial activity may be least in concentration. The chloroform extract was found less effective as compared to methanolic extract.

The hexane extract was observed less effective against the tested bacteria as compared to methanolic and chloroform extract of *Tolypothrix fragilis* biomass. In hexane extract, there was no activity against *E. coli* up to 400 µg/ml concentration made successive extractions with solvents of increasing polarity i. e. petroleum ether, dichloromethane, ethyl acetate, methanol, etc. These extracts showed different antibacterial effects in bioautographic assay with *B. subtilis, E. coli* and *Micrococcus luteus*. According to our experimental results, methanol caused better effect than chloroform and hexane against gram positive and gram negative bacteria [22].

Minimum Inhibitory Concentration (MIC)

The lowest concentration of compound that failed to show any visible macroscopic growth was considered as its MIC. The MIC values for a given isolate were either identical, or within one dilution. In the present study, the extracts of biomass were prepared in methanol, chloroform, hexane, and water and studied against different gram positive and gram negative bacteria. The MIC was found to vary from solvent to solvent of *Tolypothrix fragilis*. The MIC for all the bacteria was in the range of 32 – >400 µg/ml. The methanol extract of *Tolypothrix fragilis* showed lower MIC of 32 µg/ml against *Bacillus subtilis*. Chloroform extract showed maximum activity at MIC 256 µg/ml. Higher value of MIC was shown in hexane extracts. Aqueous extract of *Tolypothrix fragilis* did not show any activity at any MIC upto 400 µg/ml.

Screening procedures gave some indication about the nature of compound involved in antibacterial activity of *Tolypothrix fragilis* which gave positive results. During this study the best antibacterial metabolite producing strains *Tolypothrix fragilis* showed varied spectra of activity, inhibiting the growth of bacteria (*B. subtilis, S. aureus, P. mirbilis*). The presented results are consistent with finding of others that cyanobacteria can be a rich source of biologically active compounds [15-19,23].

Conclusion

A cyanobacterium *Tolypothrix fragilis* is a good source of bioactive antibacterial metabolites. Methanol extract is more effective followed by chloroform extract. Hexane extract of *Tolypothrix fragilis* is less effective against all the tested bacteria. The bioactive metabolites are insoluble in water therefore aqueous extract does not show any activity against any tested bacterium upto 400 µg/ml. The lowest value of MIC is found in methanol extract.

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