Indian Journal of Applied Microbiology

ISSN (Online): 2454-289X, ISSN (Print): 2249-8400

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<https://doi.org/10.46798/ijam.2021.v23i01.005>

Volume 23 Number 1

January - April 2021, pp. 39-48

Study of Anti-inflammatory Potential of Whole Cell Extracts of Cyanobacteria by Membrane Stabilization & Trypsin Inhibition Assay

Shraddha Ratnaparkhe*, Devyani Mali and Dr. Bela Nabar

Smt CHM College, P.O. Box no. 17, Opposite Ulhasnagar Railway station, Ulhasnagar-421003,

Abstract: Inflammation is a complex mechanism in response to any infection, injury, or irritation. The prolonged inflammation leads to tissue damage and loss of function result in various disease conditions like osteoarthritis, and autoimmune diseases like lupus, rheumatoid arthritis, asthma and Crohn's disease. Anti-inflammatory drugs are used to control inflammatory response and prevent tissue damage. Cyanobacteria are rich sources of phytochemicals like phenolics, flavonoids are known to have anti-inflammatory activity. Because of ease of cultivation and faster growth rate than plants, they are preferred candidates over plants. This study focuses on screening of cyanobacterial isolates for their anti-inflammatory activity. The human erythrocytes (HRBC) membrane stabilization assay indicated the potential of whole cell extracts of cyanobacteria to stabilize lysosomal membrane and thereby prevent tissue damage by lysosomal chemokines and enzymes. The trypsin inhibition assay is an indicator for potential to inhibit proteinases and decelerate tissue damage. The whole cell extracts of 10 cyanobacterial isolates of different genus namely *Weistellopsis*, *Pseudophormidium*, *Oscillatoria*, *Nostoc*, *Phormidium*, *Chlorella*, *Hapalosiphon* under study showed 85%-94% membrane stabilization in hypotonicity induced hemolysis and 92% to 97% membrane stabilization in heat induced hemolysis. The test extracts also showed 48% to 52% inhibition of trypsin. Thus, the isolates under study have application as anti-inflammatory agent.

Keywords: Cyanobacteria, trypsin inhibition, azocasein, anti-inflammatory, HRBC membrane stabilization assay

*Author for Correspondence. E-mail: shraddha.rp1@gmail.com

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Introduction:

Inflammation is body defense mechanism against various stimuli like infection by pathogens, irritants, environmental pollution and injury, or provocation by few cellular components and metabolites. Recent publications also suggest that increased glucose levels, obesity, aging and few metabolites induce auto antibodies which aggravate and prolong inflammation [1]. Inflammation is multifaceted biological response involving heat, pain, swelling, redness and loss of function and results in increase in vascular permeability, protein denaturation, alteration of membranes [2]. Inflammation can be acute or chronic involving series of reactions which result in tissue injury and some immune mediated diseases[3]. Acute response subsides gradually whereas chronic inflammation occurs in response to various stressors like UV rays, burns, heat, chemicals, pollution, toxins, injury, infection and conditions like lack of sleep, unhealthy diet, alcohol consumption, and smoking.

Cyanobacteria are ubiquitous in nature, easy for cultivation and can adopt to stress environments very fast. They are preferred over plants. The products derived from cyanobacteria are free from herbicides, pesticides, other toxic compounds[4]. Cyanobacteria produce various metabolites which have cosmetic and therapeutic applications. These bioactive metabolites include proteins, polysaccharides, lipids, phenolics, flavonoids, carotenoids etc. These metabolites are used as antimicrobial, antioxidant, anticancer agents. They are rich source of essential amino acids and various vitamins such as vitamin A, E, B₁₂ and vitamin C. Compounds like amino acids, proteins, polysaccharides, lipids, carotenoids, flavonoids are also known for their anti-inflammatory property[3]. The anti-inflammatory action may be due to scavenging of free oxygen radicals, manipulation of chemokines, reduction in generation and activity of pro-inflammatory cytokines, cell interaction receptors [5][3]. Thus, compounds having ability of radical scavenging, membrane stabilization, protein kinase inhibition activity play significant role in prevention of inflammation. Therefore, this study focuses on screening of different cyanobacterial isolates for their potential to stabilize lysosomal membrane and to inhibit proteinases using in vitro human erythrocytes membrane stabilization assay and trypsin inhibition assay.

Material and Methods:

The research work was carried out in Department of microbiology in Smt. Chandibai Himatmal Mansukhani College, Ulhasnagar-3.

Sampling sites:

Samples were collected from different ecological niche i.e., fresh water from Kalu river in Titwala region (19° 29' N & 73° 22' E) and Godavari and Kadhava river from Nashik region (19° 85' N & 72° 70' E). Biological crust samples from tree bark samples and garden soil from residential area in Thane (19° 17' N & 72° 95' E) and Industrial area in Taloja (18° 59' N & 73° 6' E) from Thane and Taloja region were collected for the study. The samples were maintained at 4°C till further processing. The tree bark samples were soaked in sterile distilled water[6].

Isolation and identification of cyanobacteria:

A pinch of crust sample soaked overnight in distilled water and river water samples were diluted serially using spread plate technique followed by repeated sub culturing [7]. The identification was done using microscopy and algae monographs by Desikachari under the guidance of Dr. Sukumar Bhakta, Botanical Survey of India, Pune [8][9].

Preparation of whole cell extracts of cyanobacteria:

Whole cell extracts from dry pellet were prepared using tetrahydrofuran (THF) and Methanol (2:8) with overnight incubation at 4°C [10]. The supernatant was separated by centrifugation. The extract was dried completely at room temperature and suspended in methanol to get final concentration at 1mg/ml.

Determination of anti-inflammatory activity of whole cell extracts of cyanobacteria:

Human erythrocytes (HRBCs) membrane stabilization assay:

During inflammation, different cells like leukocytes and neutrophils accumulate at the site of injury, leading to cellular infiltration. As the inflammation progresses, the cell lysis takes place releasing various lysosomal enzymes, including proteases resulting in tissue damage and subsequent inflammatory reactions. Various enzymes and chemical mediators are released due to lysis of lysosomes which lead to generation of reactive oxygen species and further tissue damage. The damaged cell membranes are more susceptible to secondary damage by free radical mediated lipid peroxidation. Damage of cell membrane loses permeability resulting in cell lysis. As the erythrocyte membrane is similar to that of lysosomal membrane, inhibition of red blood cell hemolysis may provide insights into the inflammatory process [21]. Stabilization of these cell membranes may retard or inhibit the lysis and subsequent release of the cytoplasmic contents which, in turn, minimize the tissue damage and, hence the inflammatory response [12]. The exact mechanism is unknown. In 2018, Gunathilake et al reported that phytochemicals in plant extracts have membrane stabilization property. They interfere with early phase of inflammation i.e., release of phospholipase which induce synthesis of different anti-inflammatory mediators [11]. The substances that contribute significant protection of cell membrane against injurious substances are important in the event of inhibiting the progression of inflammation.

Membrane of human erythrocytes is analogous to membrane of lysosomes therefore membrane stabilization assay of erythrocytes is used as a measure to study anti-inflammatory activity [12]. Lysis of human erythrocytes (HRBCs) generally occurs on exposure to any injurious substance like hypotonic saline, heat, methyl salicylate and phenyl hydrazine [13]. In this study, membrane stabilization activity of methanolic extracts of cyanobacterial isolates was studied through heat induced as well as hypotonic saline induced lysis of HRBCs.

Preparation of human erythrocytes (HRBC) suspension

Blood samples were collected from individuals who are not undergoing treatment with NSAID drugs at least for 15 days. Heparin was used as anticoagulating agent. The blood was mixed with

equal volumes of the Elsevier's solution (dextrose 2%, sodium citrate 0.8%, citric acid 0.05% and sodium chloride 0.42%) and centrifuged at 3000rpm for 10 min. The packed cells were washed three times with 0.85% isosaline having pH 7.2. Then, 10% v/v stock was prepared for the assay. Aspirin at a concentration of 1mg/ml was used as standard[14].

Membrane stabilization study in hypotonic solution induced hemolysis:

The whole cell extracts of 10 isolated cyanobacteria were assessed by HRBC stabilisation method in Hypotonic solution using modified procedure of Sakat et al[14]. The reaction mixture contained 400µL of Phosphate buffer (pH 7.4), 800µL of hyposaline (0.36%) and 500µl HRBC suspension and 500µl test extracts. Incubation was done at 37°C for 30 min followed by centrifugation at 3000 rpm for 20 min. Red colour in supernatant indicate lysis of RBCs membrane. The supernatants were collected, and absorbance was recorded at 560 nm using colorimeter (Model: Kanad Vidyut 0492). The experiments were performed in triplicates and results were expressed as mean value with standard deviation. Aspirin was used as standard, and methanol was used as blank. IC₅₀ value was determined using different concentration of test extracts (100,250,500 and 1000 µg/ml). The extent of membrane stabilisation was calculated using following formula

$$\text{Percent RBC membrane stabilisation} = (A_{\text{Blank}} - A_{\text{Test}}) / A_{\text{Blank}} * 100$$

Where A_{Blank}= Absorbance of blank A_{Test}= Absorbance of Test

Membrane stabilisation study in heat induced hemolysis:

The whole cell extracts of 10 test cyanobacteria were evaluated for anti-inflammatory activity at 56°C using modified procedure of Sakat et al[14]. This assay is based on the stabilisation of the RBC cell membrane by inflammatory drug during heat induced hemolysis.

The reaction mixture of 500µL test extract and 500µL of HRBC suspension was incubated at 56 °C for 30 min. in water bath. The tubes were cooled and centrifuges at 3000rpm for 5 min. The lysis of RBCs was monitored by measuring absorbance of supernatant at 560nm in colorimeter (Model: Kanad Vidyut 0492). Saline was used as blank, and aspirin (1mg/ml) was used as standard. The experiments were performed in triplicates and results were expressed as mean value with standard deviation. Percentage inhibition of hemolysis was calculated using same formula as above.

Inhibition of proteinase enzyme using azocasein method:

Lysis of lysosomal membranes is followed by release of proteinases which can degrade almost all kinds of proteins. Role of protease inhibitor is important as an anti-inflammatory agent. Therefore, all the 10 whole cell extracts of cyanobacteria under study were evaluated for their proteinase inhibition activity using trypsin inhibition assay with azocasein as substrate.

The assay was performed by modified method of Spelbrink et al and Yadav et al using Aspirin (1mg/ml) as standard and Tris buffer as blank.[15,16] The reaction mixture consists of 200µl of 0.06mg/ml trypsin in 20mM Tris buffer, 200µl of test extract. After incubation at 37°C for 5 min, 200µl of Azocasein (0.08 mg/1.5 ml) was added in the reaction mixture. Incubation was done at

37°C for 20 min. The reaction was stopped using 400µl of 5% TCA. The tubes were centrifuged at 13500rpm for 20 min and 400µl of 500mM NaOH was added. Greater the inhibition lesser will be the colour. Absorbance was measured at 430 nm using colorimeter (Model: Kanad Vidyut 0392). IC50 value was determined using different concentration of test extracts (100,250,500 and 1000µg/ml). The trypsin inhibition potential was determined using following formula:

$$\text{Percent Inhibition} = (A_{\text{Blank}} - A_{\text{Test}}) / A_{\text{Blank}} * 100$$

Where A_{Blank} = Absorbance of blank A_{Test} = Absorbance of Test

Results:

Sampling sites:

Samples were collected from different ecological niche of Maharashtra viz, Freshwater samples from rivers like Kalu river; Titwala, Godavari and Kadhava river from Nashik. Garden soil sample from Thane and biological crust samples from tree barks of *Albizia julibrissin* and *Syzygium cumini*. The six samples were processed further for enrichment and isolation of cyanobacteria as depicted in Table 1.

Isolation and identification of cyanobacteria:

After repeated subculturing, the unialgal isolates were obtained belonging *Weistellopsis* sp., *Pseudophormidium* sp., *Oscillatoria* sp., *Nostoc* sp., *Phormidium* sp., *Chlorella* sp., *Hapalosiphon* sp. and *Leptolyngbya* sp., the details of source and isolate are shown in Table 1. The identification was done using microscopy using algae monographs under the guidance of Dr. Sukumar Bhakta, Botanical Survey of India, Pune.

As depicted in Table 1, the 10 unialgal cultures were selected for further study. They were enriched and processed further to study the anti-inflammatory potential of the strains.

Table 1: The details of isolates obtained from various samples collected from different sources

Sr. No	Source	Sample ID	Isolate details	Isolate ID
1	Kalu river	KL01	<i>Weistellopsis</i> sp.	KL01A7
2	Godavari river	GR01	<i>Pseudophormidium batrachospermi</i>	GR01A1
3			<i>Leptolyngbya</i> sp.	GR01A3
4	Kadhava river	KDR01	<i>Leptolyngbya</i> sp.	KDR01A7
5	<i>Syzygium cumini</i> tree bark	JB01	<i>Oscillatoria</i> sp.	TG01A3
6	Garden soil	TG01	<i>Nostoc</i> sp.	TG01A4
7			<i>Phormidium priestleyi</i>	TG01A4
8	<i>Albizia julibrissin</i> tree bark	ALB01	<i>Chlorella</i> sp.	ALB01A2
9			<i>Pseudophormidium tenue</i>	ALB01A4
10			<i>Hapalosiphon welwitschii</i>	ALB01A5

Estimation of membrane stabilization using hypotonicity induced hemolysis method:

The whole cell extracts of 10 cyanobacterial isolates were evaluated for membrane stabilisation potential using hypotonicity induced hemolysis method. In case of hypotonic solution, accumulation of fluid in cells occurs followed by rupture of cell membrane and release of various chemicals and enzymes. Reactive oxygen species generation and tissue damage is observed after prolonged inflammation. In presence of anti-inflammatory compounds, the RBC membranes are stabilised, and no lysis is observed. The stability of RBC membrane was monitored by measuring absorbance at 560nm. All the test extracts showed membrane stabilisation in the range of 85% - 94% stabilisation as indicated in Figure 1. The highest membrane stabilisation of $94 \pm 0.08\%$ was observed in whole cell extract of KL01A7 among the group of 10 extracts under study. The whole cell extracts of two isolates JB01A2 and ALB01A5 showed membrane stabilisation of $92 \pm 0.33\%$ and $92 \pm 0.08\%$ respectively. Aspirin (1000 mg/ml) showed 98.5% stabilisation of HRBC membrane in hypotonicity induced hemolysis. The membrane stabilisation of all the test extracts incomparable with Aspirin. The lowest IC 50 value of $84 \pm 4.24\mu\text{g/ml}$ was observed in case of GR01A1 among the 10 whole cell extracts of cyanobacteria under study.

Figure1: Membrane stabilisation study of whole cell extracts of cyanobacteria using hypotonicity induced hemolysis method expressed in per cent stabilisation.

Estimation of membrane stabilisation using heat induced hemolysis method:

The whole cell extracts of 10 cyanobacterial isolates were evaluated for membrane stabilisation potential using heat induced hemolysis method. As shown in Figure 2, the highest membrane

stabilisation of 97 ± 0.13 %, 97 ± 0.12 %, 97 ± 0.22 % was observed in whole cell extract of GR01A1 of Godavari river from Nashik region, TG01A4 from garden soil of Thane region and ALB01A2 from tree bark of *Albizia julibrissin* from Thane region respectively in the study. As shown in Table 1, the lowest IC₅₀ value of 453 ± 2.62 µg/ml of whole cell extract of TG01A4 isolate from garden soil of Thane region.

Figure 2: Membrane stabilisation study of whole cell extracts of cyanobacterial using heat induced hemolysis method.

Inhibition of proteinase enzyme using azocasein method:

The whole cell extracts of 10 cyanobacterial isolates were evaluated for protease inhibition potential using trypsin inhibition assay using azocasein method. In this assay, azocasein casein coupled with diazotized aryl amines is a nonspecific protease substrate. The proteases digest azocasein with generation of chromophore which is soluble in Trichloro acetic acid. The red orange color is developed which is measured at 430nm. The intensity of color is directly proportional to the amount of azocasein digested as undigested proteins precipitate after addition of TCA. Proteinases released from lysosomal granules, mainly serine proteinases play significant role in denaturation of proteins during inflammation[11]. As shown in Figure 4, the highest trypsin inhibition of 52 ± 0.06 % in whole cell extract ALB01A4 of *Albizia julibrissin* from Thane region followed by 51 ± 0.19 % inhibition by whole cell extract of KDR01A7 from Kadhava river of Nasik region was observed. The test whole cell extracts of two isolates, namely KL01A7 from Kalu river of Titwala region and JB01A2 from tree bark of *Syzygiumcumini* of Thane region showed 48 ± 0.28 %, 48 ± 0.14 trypsin inhibitions respectively.

Figure 3: Protease inhibition study of whole cell extracts of cyanobacteria using trypsin inhibition using azocasein as substrate.

The lowest IC₅₀ value of $954 \pm 3.42 \mu\text{g/ml}$ of whole cell extract of KDR01A7 from Kadhava river of Nasik region was observed for inhibition of trypsin as shown in Table 1.

Table 1: IC₅₀ value of whole cell extracts of cyanobacteria for anti-inflammatory assay

Isolate ID	Membrane stabilisation assay		Trypsin inhibition assay
	Hypotonicity induced hemolysis	Heat induced hemolysis	
KL01A7	95 ± 3.40	468 ± 1.17	1122 ± 3.52
GR01A1	84 ± 4.24	479 ± 0.92	1095 ± 3.18
GR01A3	109 ± 1.22	459 ± 3.05	1331 ± 3.91
KDR01A7	93 ± 1.00	481 ± 1.51	954 ± 3.42
JB01A2	96 ± 1.13	493 ± 2.45	1074 ± 3.79
TG01A3	102 ± 4.26	489 ± 1.47	1357 ± 3.41
TG01A4	91 ± 2.74	453 ± 2.62	1334 ± 2.79
ALB01A2	94 ± 3.77	484 ± 2.45	980 ± 0.35
ALB01A4	108 ± 2.42	497 ± 2.52	1117 ± 1.13
ALB01A5	91 ± 6.68	485 ± 1.16	1220 ± 4.85

Discussion and conclusion:

Cellular infiltration is a key step in inflammation, as leukocytes release their intracellular enzymes leading to tissue damage and generation of free oxygen radicals. The free oxygen radicals induce lipid peroxidation, and further damage to cells. The cell membrane proteins are responsible for membrane integrity which in turn maintains homeostasis between sodium and potassium ions and permeability. Therefore, stabilisation of cell membranes is a key factor in regulation of inflammatory response. Considering the resemblance of cell membranes of human erythrocytes and lysosomes, the membrane stabilisation potential of whole cell extracts of cyanobacteria was assessed by hypotonicity induced hemolysis and heat induced hemolysis [11]. The whole cell extracts of all the 10 study isolates demonstrated significant membrane inhibition potential in dose dependent manner. This may be due to increase in surface area to volume ratio of the cells by expansion of membrane or shrinkage of cells or by interaction with cell membrane proteins which in turn prevent release of lysosomal constituents [17]. The precise mechanism of membrane stabilisation is unknown; it may be due to interaction of bioactive metabolites in the extract with constituents of the membrane or effect on the ratio of surface area/volume of the cells by the expansion or shrinkage of membrane. Neutrophils carry many serine proteinases in their lysosomal granules which play a key role in tissue damage during inflammation. As reported in previous studies, significant protection from tissue damage was observed by proteinase inhibitors. This activity is mainly attributed to polyphenols, flavonoids, and carotenoids [11]. The study demonstrated proteinase inhibition potential of all the cyanobacterial extracts in dose dependent manner.

This study was limited to assessment of the potential of whole cell extracts of cyanobacteria for anti-inflammatory activity. Previous studies also reported accelerated wound healing activity in presence of anti-inflammatory agent [16]. The wound healing activity also protects skin from invading of skin by pathogens. Thus, the whole cell extracts of study isolates have multiple applications in therapeutics for anti-inflammatory action, wound healing, skin protection. The wound healing assay will give more insights for the same. Further study is needed to study the active metabolites of whole cell extract for membrane stabilisation and anti-proteinase activity.

Acknowledgement: We are thankful to Botanical Survey of India, Pune for allowing us to use their facility for identification of cyanobacteria.

Conflict of interest: Conflict of interest declared none.

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