https://doi.org/10.48047/AFJBS.7.1.2025.8-28

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

Antarctic cyanobacterium *Nostoc* CCC537: A treasure trove of synergistically active antibacterials.

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Volume 7, Issue 1, Jan 2025

Received: 15 Nov 2024

Accepted: 25 Dec 2024

Published: 03 Jan 2025

doi:10.48047/AFJBS.7.1.2025.8-28

Abstract:
The cold desert of Antarctica is known for its extreme climate and living organisms has less survival rate than most places. There are some microbes like cyanobacteria aided by developing a strong defensive mechanism through secondary metabolite production can survive in such extreme condition. This metabolic pathway under an extreme environmental condition not only has a survival strategy but also has an impact on other microbes. The *Nostoc* CCC537, a slow-growing Antarctica strain (generation time 78 h and k= 0.0128), grows better at 20ºC and produces different types of biomolecules with characteristic antibacterial properties against both Gram positive and negative bacteria along with MDR strains. The methanolic extract of harvested biomass (60d) and its extracellular isolate (90d) showed antibacterial activity against *Enterobactor aerogens, Escherichia coli*, *Salmonellla typhi* and *Streptococcus pneumonia*. The isolated cellular active principle (AP_{ce}) 4-[(5-carboxy-2-hydroxy)-benzyl]-1, 10-dihydroxy-3, 4, 7, 11, 11-pentamethyloctahydrocyclopenta<a>naphthalene, extracellular active principle (AP_{ee}), and cellular Gamma-linolenic acid (GLA) from *Nostoc* CCC537 shows synergistic effects when applied with various combinations against *E. aerogens.* The MIC for AP_{ce}: GLA 3:1), AP_{ee}: GLA (1:2), and AP_{ce} : AP_{ee} (2:1; 3:1) were 0.25, 1.00 and 0.5 respectively. Significantly, *Nostoc* CCC537 would be a good source of unique antibacterial compound especially against an increasing number of mutant/MDR bacterial strains in future endeavors.

Keywords: Antibacterial compounds; Bioactive molecule; Gamma-linolenic acid; *Nostoc* CCC537; Synergistic effect

1. Introduction

In the present world, there is an alarming rate of decline in the efficacy of the available antibiotics due to the development of multi-drug resistant (MDR) bacterial strains (O'Neill 2016). This MDR gives a challenge to the medical world as no new antibiotic discovery was made in the last two decades. The need to identify and explore new resource to develop new antibiotics and antiviral compounds to face MDR become imminent. The World Health Organization has initiated a worldwide plan (WHO, 2015) to explore new resources for the development of new antibiotics, however, the effort to discover new antimicrobial compounds have faced many challenges due to MDR strains, unfavorable regulatory climate, multiple company mergers and low financial returns associated with the development of antibiotic (Jackson et al. 2018). The methods which were earlier used to discover antibiotics based on waste water/ soil-derived organism becomes unyielding and exhausted completely by 1970s due to the non-identification of new novel compounds (Alsenani et al. 2020).

Bacteria have also adopted various methods to survive antibiotic inhibitions by enzyme inactivation, cellular efflux of the antibiotics, target mutations which decrease the binding efficiency of antibiotics, overproduction of the target, bypass of the metabolic pathway to remove essentiality of the target and decrease in antibiotic uptake (Singh and Barrett 2006). Therefore, targeting the mechanism of resistance against prospective biomolecules to reverse the decrease in antibiotic potency becomes an alternative strategy for drug discovery. Scientists in their research are also exploring microalgae and cyanobacteria for its high resistant potential to produce new antibiotics. These microalgae and cyanobacteria are quite diverse and could be a strong commercial source of high value bioactive compounds. These organism defense mechanism is quite excellent; a single organism is capable of synthesizing more than one anti-microbial compound, which can be intracellular or secreted out in the growing media.

The bioactive molecules present in cyanobacteria produce a wide spectrum of biological components such as toxins, antibiotics, fungicides, algicides, and antivirals (Tiwari & Tiwari 2020). These molecules could be produce in large number like *Spirulina* (which is use as food supplement) and others, which are used as biofertilizers (Soni et al. 2017). Besides, cyanobacteria can also be further explored to manufacture drugs as the expected rate is far lower than the other better studies group of organisms (Olaizola 2003). Cyanobacteria also produce secondary metabolites along with alkaloids, polyketides, non-ribosomal peptides and potent toxins (Neilan et al. 2008). Further, the search for functional biomolecules has also been extended to the so-called extreme environments (Burns et al. 2008).

In this studies, we have selected the Antarctic environment (extremophiles) as the source of cyanobacteria with the logic that unique biochemical pathways could be uncovered and may lead to the discovery of niche-specific bioactive molecules that could favor the evolution of indigenous species through novel biochemical adaptations (Núñez-Montero & Barrientos 2018). This productive experience is also supported by phylogenetic analysis of Type 1 polyketide synthase (PKS) and non-ribosomal polyketide synthetase (NRPS) genes in Antarctica sediments. Adenylation (a domain clustering mainly within the cyanobacteria and proteobacteria group) shows great diversity and novelty of PKS and NRPS genes in Antarctica sediments (Zhao et al. 2008). However, secondary metabolites in cyanobacteria other than

those found in Antarctica are reported to be synthesized by polyfunctional enzyme complex (Burns et al. 2004; Dittmann et al. 2013) and also by bacteria and fungi (Finking and Marahiel 2004). The NRPS and PKS genes are reported to be present in *Nostoc* ATCC29133, *Nostoc* ATCC53789, Cyanothece WH8901, *Scytonema*, *Spirulina*, *Chamaesiphon*, *Microcoleus*, *Pleurocapsa*, *Anabaena*, *Calothrix*, *Dermocarpa*, *Gloeobacter*, *Trichodesmium*, *Fischerella* (Ehrenreich et al. 2005) and also in the secondary metabolite genes of cyanobacterial orders such as Nostocales, Chrococcales and Oscillatriales (Kaluzhnaya & Itskovich 2016). This finding substantiates the investigations on the production of bioactive molecules, especially from Antarctica cyanobacterium.

Further, we may also look into gamma-linolenic acid (GLA) in relation with the present studies as some of the antimicrobial activities of microalgae and cyanobacteria have been linked to unsaturated fatty acids, such as, gamma-linolenic acid (GLA) from *Fritscheralla* sp. (Asthana et al. 2006), *Nostoc* CCC537 (Deepali et al. 2021), chlorellin, from *Chlorella* sp. (Pratt et al. 1944). Direct chemical synthesis of GLA is not preferred as it gives both cis and trans-isomers an equal percentage ratio; the latter is not only inactive but may also block the active cis form. Thus, the need for alternative, natural sources of GLA (not common in the human diet) is imperative (Sinclair 1990). There are several reports on the allelopathic connections among organisms due to the actual interactions among the active molecules produce by interacting partners (Zuo et al. 2016). Arora and Bharti (2014) demonstrated that the isolated bioactive constituents from *Emblica officinalis* had a synergistic effect against two test pathogens (*Fusarium oxysporum* and *Rhizoctonia solani*) than separated one. Zhu et al. (2010) explained, the submerged macrophyte (*Myriophyllum spicatum*) could produce polyphenols, i.e., pyrogallic acid, gallic acid, ellagic acid, and (+)-catechin, which exhibited synergistic interactions against cyanobacteria. The algal growth inhibition observed by Park et al. (2006) when two or three phenolic compounds from rice straw were added.
Although, there has been less study till date on allelopathic interactions among the bioactive isolates. Furthermore, the synergistic interactions depend on many factors such as the receptor, the respective proportions of active molecules, and the abiotic or biotic conditions. In the current article, we are presenting standardization, isolation and purification of the isolated bioactive molecules from an Antarctic cyanobacterium *Nostoc* CCC537 along with synergistic potential of among its isolates.

2. Material and Methods

2.1. Organism and growth conditions

The Antarctica strain *Nostoc* CCC537 (Centre for Conservation and Utilization of Blue Green Algae, Indian Agricultural Research Institute, New Delhi -110012, India) a kind gift of Prof. P. A. Broady, University of Canterbury, New Zealand, was grown in Chu-10 medium (Gerloff et al. 1950) lacking combined N-source with heterocyst frequency (~ 9%).

The growth temperature was optimized by incubating culture at 10, 20 and 30° C in a culture vessel under low irradiance (20.1 µmol photons m^{-2} s^{-1}) and was shaken periodically. The purity of cultures was routinely checked by transferring aliquots to the 'Luria Broth' medium and incubation in dark (37° C, 24 h). As the Antarctica cyanobacterium was growing and the desired biomass had to be collected at an appropriate time, biomass was harvested after 60 d of growth for elucidation of the biomolecules in reference. According to Kratz and Myers

(1955), the specific growth rate constant (k) was calculated as: $k = 2.303 (\log N_2 - \log N_1)/ T_2 – T_1$ (where N_1 and N_2 are the initial and final cell density at time T_1 and T_2 respectively) (Kratz & Myers 1955).

2.2. Dry weight determination

Cells were concentrated by centrifugation, washed with deionised water and dried (60° C) to constant weight (expressed as g L^{-1}).

2.3. Extraction and isolation of bioactive molecules (non-lipid)

Intracellular extraction of cyanobacteria was carried out according to Doan et al (2000). The biomass (10 g fresh weight) was pelleted, washed twice with deionized water, and lyophilized (Christ Alpha 1-2, Germany). The lyophilized biomass (1g) was extracted twice with 100 mL methanol, centrifuged (20,000 x g, 30 min), the supernatant evaporated to dryness and the residue was redissolved in methanol (5 mL). This methanolic extract was subjected to TLC (Merck Silica Gel-60) using the solvent carbon tetrachloride: methanol (9:1, v: v).

UV-trans-illumination of the plates revealed 9 spots, and these were eluted individually with a minimum amount of methanol (1 mL). The elutes were bio-assayed for their antibacterial potential against non-pathogenic *E. aerogenes* MTCC2822 (IMTECH, India) using a slightly modified Kirby Bauer disc diffusion susceptibility method by Bauer et al (1966) on 3.8% Mueller-Hinton (1941) agar (HiMedia, India). The spot corresponding to the maximum inhibition zone was eluted with ethanol and re-chromatographed with a different solvent (hexane: ethyl acetate, 1:1, v:v). After obtaining resolution in 8 spots, each of these spots was independently eluted in methanol and tested for antibacterial potential as described above. The most effective spot was eluted in ethanol (1 mL) and subjected to reverse phase HPLC (Waters, USA) equipped with C_{18} column (300 x 3.9 mm) and solvent gradient of 60-100% methanol (1 mL min^{-1}, 20 min) followed by methanol (100 %) for 10 mins. The fraction (1 mL) collected every minute was bio-assayed to select the most effective one.

For extracellular metabolites, the culture filtrate was concentrated in a rotary evaporator (Prefit, India) and lyophilized. This lyophilized sample was extracted in methanol (1 mL) and processed according to Doan et al (2000) as mentioned above for intracellular extraction (Doan et al. 2000).

2.4. Spectroscopical analysis of HPLC purified extracellular product

The UV-spectrum was recorded on UV-VIS spectrophotometer (UNICAM-UV2-100, Cambridge, UK) and the IR spectrum on JASCO FT/IR-5300 (Easton, MD) as a film on a KBr-disc.

2.5. Extraction of lipids

Lipids from the lyophilized cyanobacterial biomass (1g) were extracted using chloroform: methanol (1:2). Fatty acid methyl ester (FAME) of lipid formed by heating (80° C, 2 h) in MeOH: HCl (10:1) was extracted in hexane by Bligh and Dyer method (1959). The FAME along with pure GLA standard (100 µg mL^{-1}) (Sigma-Aldrich) was applied to TLC plate (Merck Silica Gel-60) with solvent system hexane: diethylether: acetic acid (60:40:1; v:v:v). The plates were visualized in iodine vapour. The spot on the TLC plate that ran parallel to the GLA (standard) lane (same R_f) was eluted in methanol (1 mL) for further use.

2.5.1. Identification and quantification of Gamma Linolenic Acid (GLA)

The TLC eluate was processed through HPLC (Waters associate-501, USA) fitted with 25 cm x 4.6 mm Biophase ODS 5 µm particle reverse phase column. The mobile phase consisted of methanol and 1 mM phosphate buffer (95:5; v:v; pH 7.4). The flow rate remained constant at 1 mL min^{-1}. Out of all the fractions, the fraction collected at 4.72 mins had GLA as compared with the retention time of standard GLA. The quantity present therein was calculated relative to the peak area obtained for pure GLA.

2.6. Antibacterial assay using selected Gram-positive and Gram-negative bacteria targets

2.6.1 Qualitative

A preliminary antibacterial assay was performed using non-pathogenic *E. aerogenes* MTCC 2822 and other bacterial targets *S. aureus* ATCC25923, *E. coli* ATCC25992, *Salmonella typhi* MTCC3216 and *P. aeruginosa* ATCC27853, along with three clinical isolates of multi-drug resistant (MDR) strains of *E. coli* (GS 2003/01, 02, 03) usinf. The inoculum derived from LB-grown cells (37° C, 18 h) was suspended in 0.85% NaCl and turbidity adjusted to 10^8 CFU mL^{-1} that corresponded to 0.5 MacFarland standard according to Clinical and Laboratory Standards Institute (CLSI), 9^{th} edition (2012). Each sample solution was inoculated on MH agar with a sterile, non-toxic cotton swab and incubated for 20 minutes at 37° C. Thereafter, 15 µL of each extract (crude) in different solvents (methanol, DMSO and water) or TLC eluates were spotted on plates (MH agar) keeping solvent as negative control and Rifampicin and Streptomycin as positive control, incubated (37° C, 18 h). The expression of antibacterial potential was based on the inhibition zone size (mm) on MH agar plates.

2.6.2. Quantitative

The minimum inhibitory concentration (MIC) was determined by agar dilution method described in the CLSI (2012). The lowest concentration of active principle (AP) that prevented bacterial growth was considered to be the MIC. To obtain the range of 0.002-512 µgmL^{-1}, the 'active concept' (1 mL) was serially diluted 2-fold and applied to 19 mL of MH-agar. These plates were dried and divided into sectors based on the number of test organisms. Bacterial cells grown overnight were adjusted to a population density of 10^8 CFU mL^{-1} in normal saline (0.85%) corresponding to 0.5 M $BaSO_4$ turbidity standard and applied to sectors with a sterile cotton swab. The inoculants were allowed to dry and incubated at 37° C.

2.7. Synergistic effect of isolated AP_{ce}, AP_{ee} and Gamma Linolenic acid (GLA) against target bacteria

According to CLSI guidelines 2012, the synergistic effect was performed by using same bacterial targets as mentioned for qualitative analysis [30]. The combination used for this study was in AP_{ce}: GLA, AP_{ce}: GLA, AP_{ce}: AP_{ee} and ratios such as 1:1, 2:1, 3:1, 1:2, 1:3. Expression of antibacterial potential was based on the inhibition zone size (mm) on MH agar plates.

2.8. Statistical analysis

Statistical analysis was carried out by one-way ANOVA and Student-Newman-Kuels (S-N-K) test using the SPSS software (SPSS Inc., version 12.0). ANOVA test has been used to test the significant difference among the mean level of bacterial count at different time intervals. If this resulted as significant then multiple comparison (S-N-K test) has been applied to find out the pair-wise significant difference. All the experiments were carried out in triplicates with standard deviation (SD) represented in bars.

3. Results

3.1. Growth condition and Biomass age to harvest

It was necessary to determine the optimum growth temperature for the desired biomass cultivation of the cyanobacterium *Nostoc* CCC537. The photoautotrophic growth of target cyanobacterium was in variably grown at 20^{o} C. For *Nostoc* CCC537, 60 d culture was selected for assessing the intracellular antimicrobial activity and 90 d old culture for extracellular extraction (Fig 1).

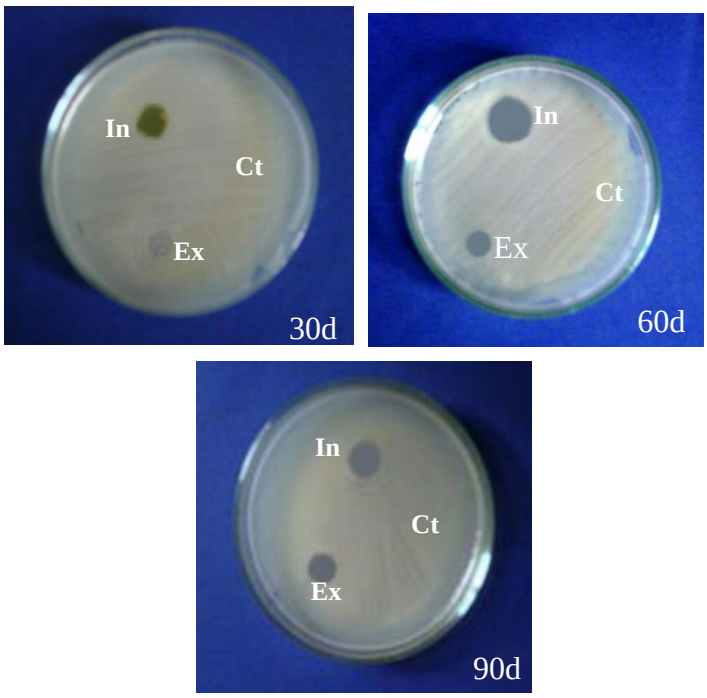


Fig. 1. Inhibition zones on the lawn of *E. aerogenes* by intracellular (In)and extracellular (Ex) biomolecule(s) from *Nostoc* CCC537 along with control (Ct).

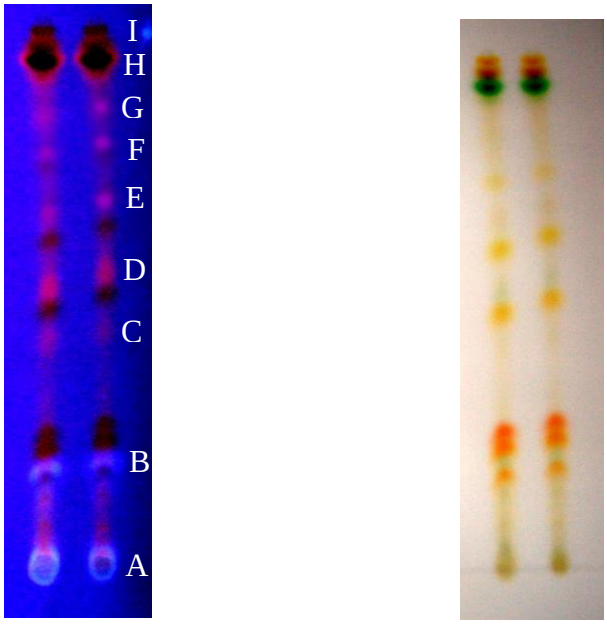
3.2. Antibacterial property of Nostoc CCC537 under different solvents

Antibacterial property of the crude extract of *Nostoc* CCC537 was also examined using three different solvents, namely water, DMSO and methanol (15 µL each) against non-pathogenic bacterium *E. aerogenes.* The diameter range of the inhibition zone as achieved by various extracts was in the sequence: 7 mm (methanol) > 3 mm (DMSO) (Deepali et al. 2014) while no inhibition zone was observed in the case of water. Therefore, the methanolic extract of *Nostoc* CCC537 was selected for further study.

3.3 Isolation of biomolecule from Nostoc CCC537 and its Bioassay

3.3.1. Thin layer chromatography of the methanolic crude intracellular extract

The methanolic extract of *Nostoc* CCC537 was subjected to TLC fractionation and UV illumination. The observed nine spots were arbitrarily designated as A, B, C, D, E, F, G, H and I, and their respective R_f value and inhibition zone size are illustrated in **Table 1.**



(i) **(ii)**

Fig. 2a. TLC pattern of methanolic extracts of lyophilized *Nostoc* CCC 537 (i) under UV and (ii) without UV-illumination

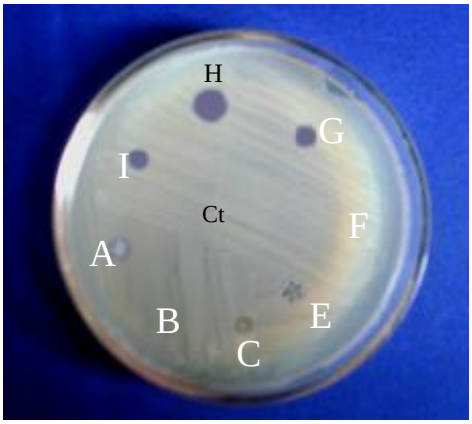


Fig. 2b. Antibacterial activity of TLC separated spots (A-I) against *E. aerogenes* and methanol as control (Ct)

The non-UV-illuminated TLC plate loaded with the same methanolic extract shown as green/orange spots reflected the presence of photopigments. The individual UV-illuminated spots were eluted in methanol and bioassayed for the antibacterial potential against *E. aerogenes*. The inhibition zones on the test plate showed that G, H, and I spot eluate did possess antibacterial activity in the sequence: H (5.5 mm) > G (3.0 mm) > I (2.5 mm) (Fig 2a, b). Out of these, the most potent spot 'H' was selected for further experiments.

Table 1. Bioassay of different designate bands from 1st TLC against *E. aerogenes*

Designated spots	R_f	Inhibition zone (mm)
A	0.00	-
B	0.14	-
C	0.28	-
D	0.39	-
E	0.54	-
F	0.67	-
G	0.77	3.0
H	0.87	5.5
I	0.96	2.5

3.3.2. TLC of 'H' eluate

'H' spot eluate in ethanol was subjected to 2nd TLC with solvent system hexane: ethyl acetate (1:1). UV-illumination of the TLC plate revealed a total of 8 spots designated as J, K, L, M, N, O, P and Q (Fig 3; i-iii). Individual spots got eluted in methanol (1 mL) and bioassayed against *E. aerogenes*. The R_f values of different spots and the inhibition zone size are represented in Table 2. Among these, only spot 'N' proved potent (inhibition zone 7 mm; R_f 0.58). Therefore, this spot was selected for further analysis and designated as $AP_{ce.}$

Table 2. Bioassay of different designate bands from 2nd TLC against *E. aerogenes*

Designated spots	R_f	Inhibition zone (mm)
J	0.00	-
K	0.24	-
L	0.40	-
M	0.48	-
N	0.58	7
O	0.76	-
P	0.85	-
Q	0.90	-

3.3.3. HPLC purification of potent spot (N) and Structural elucidation of the intracellular 'active principle' (APce)

The 'N' spot was eluted in ethanol (1 mL) and subjected to HPLC for purification. The potent fraction was further analyzed through various spectroscopic techniques such as UV, IR, NMR, EIMS and ESIMS to elucidate the molecular structure. The identified structure was "4-[(5-carboxy-2-hydroxy)-benzyl]-1, 10-dihydroxy-3, 4, 7, 11, 11-pentamethyloctahydrocyclopenta<a>naphthalene" and data have already been published (Asthana et al. 2009).

3.4. Antibacterial assay

3.4.1. Potency equivalence test

The comparison of AP_{ce} with selected antibiotics, such as streptomycin and rifampicin taking *E. coli* as the target is represented in Table 3. MIC being the least (2 μg mL^{-1}) for AP_{ce} relative to rifampicin and streptomycin (28 and 15 μg mL^{-1}, each) indicated the superiority of the AP_{ce}. It was noteworthy that a prominent inhibition zone (12 mm) was evident only for AP even at 30 μg mL^{-1}, which is in contrast to a complete lack of inhibition zone at the equivalent dose of streptomycin or rifampicin. The size of the inhibition zone formed by AP_{ce} is higher (18 mm) among the three test agents (45 g mL^{-1}). For the highest concentration (60 μg mL^{-1}) also, the overall effectivity of the three test agents maintained the same trend with the AP_{ce} on top (37 mm) followed by rifampicin (26 mm) or streptomycin (21 mm).

Table 3. Relative affectivity of the AP_{ce}, streptomycin and rifampicin against *E. coli*

Agents	Inhibition zone (mm) (μg mL^{-1}) 30	45	60	MIC (μg mL^{-1})
Active principle	12	18	37	2.0
Rifampicin	n.d.	12	26	28
Streptomycin	n.d.	15	21	15

n.d.: not detected

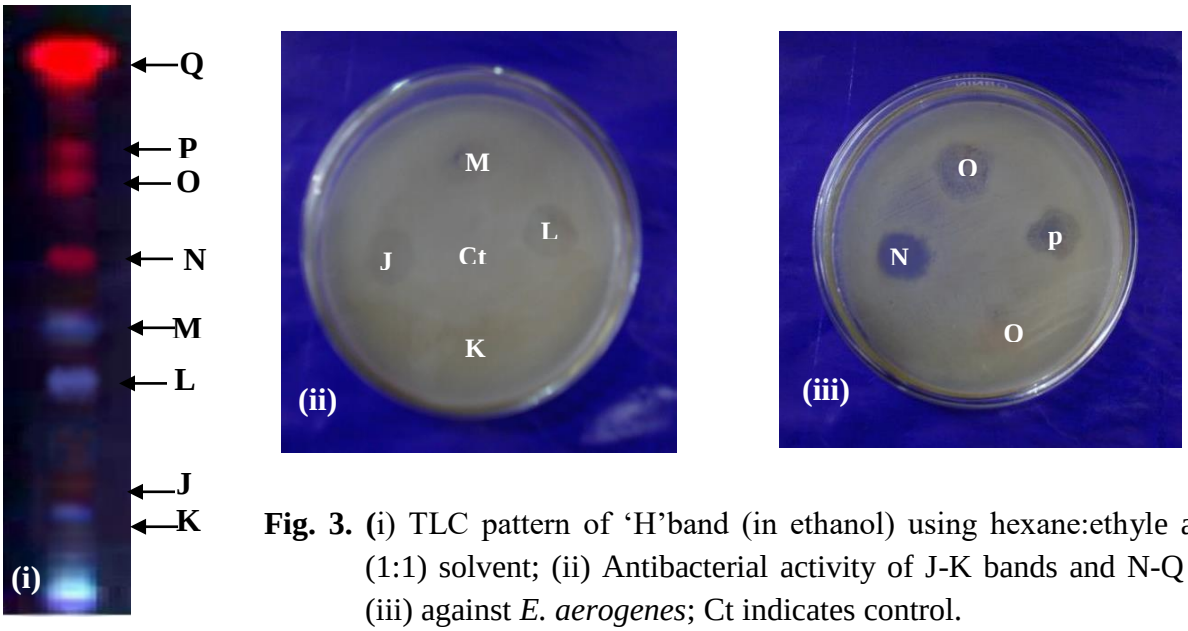


Fig. 3. (i) TLC pattern of 'H'band (in ethanol) using hexane:ethyle acetate (1:1) solvent; (ii) Antibacterial activity of J-K bands and N-Q bands (iii) against *E. aerogenes*; Ct indicates control.

3.5. Bactericidal action of the AP_{ce} in liquid culture

Liquid growth of *S. aureus* and *E. aerogenes* against graded concentrations of the AP_{ce} (10-30 µg mL^{-1}) was also compared (Fig. 4). The data maybe divided into three phases with respect to time: (i) growth between 30-60 min, (ii) 60-90 min and (iii) 90-120 min. In *S. aureus*, the extent of the decrease in bacterial cell count progressed with an increase in the incubation period when challenged with 10 µg mL^{-1} of AP_{ce} to attain 34.7 % of the initial value at 120 min. A doubling in concentration (20 µg mL^{-1}) brought about a 53.4 % decline relative to the starter cell number. For a further rise in concentration to 30 µg mL^{-1}, the ultimate cell yield was restricted to 71.8 % of the initial. However, *E. aerogenes* cells seemed resistant against the AP_{ce} even at the comparable initial dose (10 µg mL^{-1}) as evident from only a 7.8 % decline in final cell count (120 min). Noticeably, the bacterial cell count between 90 to 120 min was recorded as nominal rise (1.56 %). The bacterial cells became sensitive to the highest dose (30 µg mL^{-1}) of the AP_{ce} as apparent from the time-dependent decline in cell count to reach the ultimate 40 % decline in viable cell count. The differential action of AP_{ce} towards two different bacterial strains was thus evident.

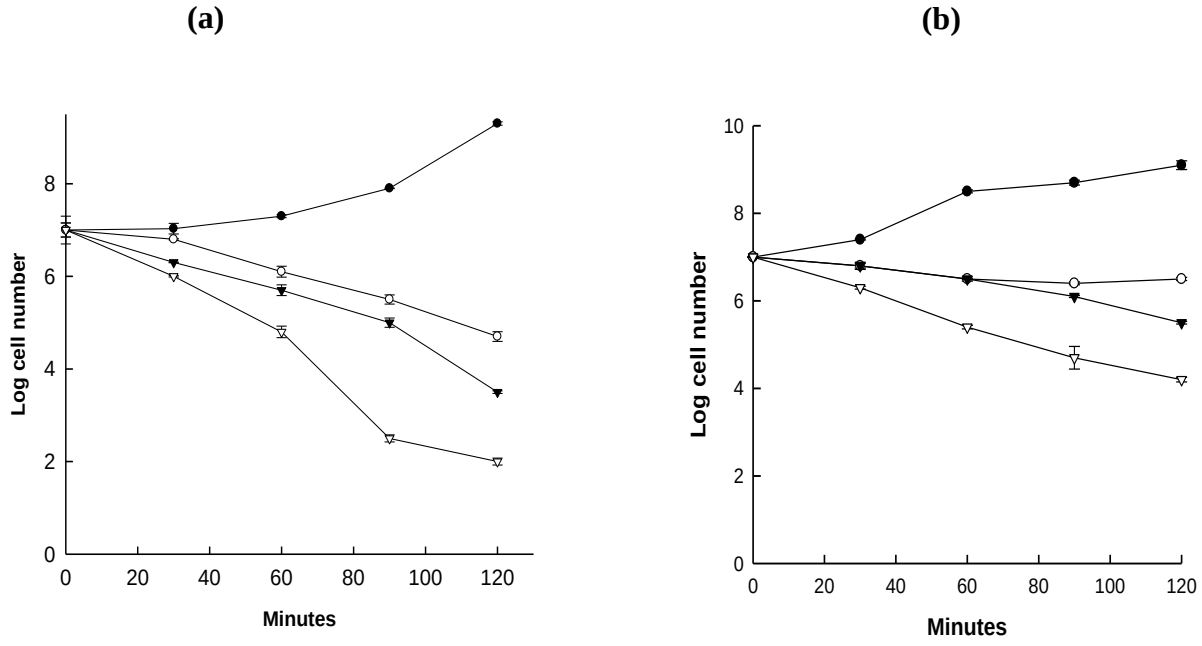


Fig. 4. Liquid growth response of *S. aureus* (a) and *E. aerogenes* (b) against 'active principle' at 10 µg mL^{-1} (o-o), 20 µg mL^{-1}(▼-▼), 30 µg mL^{-1} (∇-∇) along with control (●-●).

The ANOVA values also indicate a highly significant difference between the group of S. *aureus* (**Table 4**) and *E. aerogenes* (**Table 5**). S-N-K test as applied for pairwise group comparison with respect to growth inhibition of *S. aureus* and *E. aerogenes* by different concentrations of isolated AP. The difference between the log cell numbers indicates bactericidal property (**Table 6 and Table 7**) of AP.

Table 4. ANOVA (F) value and significance level between groups of 0,10, 20 or 30 µg mL^{-1} of the AP$_{ce}$ against *S. aureus* at different time interval (based on Fig. 4a)

Min	F	Significance
00	1.87	.213
30	186.66	.000
60	289.93	.000
90	2266.41	.000
120	6279.30	.000

Table 5. ANOVA (F) value and significance level between groups of 0, 10, 20 or 30 µg mL^{-1} of AP$_{ce}$ against *E. aerogenes* at different time interval (based on Fig. 4b)

Min	F	Significance
00	.190	.900
30	231.76	.000
60	3589.56	.000
90	461.81	.000
120	3528.22	.000

Table 6. Multiple comparisons (S-N-K test, q values) for growth of *S. sureus* in AP$_{ce}$ (10-30 µg mL^{-1}) (based on Fig. 4a)

	AP$_{ce}$ (µg mL^{-1})					
Min	0 vs 10	0 vs 20	0 vs 30	10 vs 20	10 vs 30	20 vs 30
0	1.74	2.18	0.87	0.44	0.87	1.31
30	5.00**	15.21***	21.25***	10.21***	16.25***	6.04**
60	13.37***	17.79***	29.10***	4.42*	15.73***	11.31***
90	35.76***	45.41***	81.52***	9.65***	45.76***	36.11***
120	81.25***	103.57***	129.29***	22.32***	48.04***	25.71***

*significant level (*p < 0.05, **p < 0.01, ***p < 0.001)

Table 7. Multiple comparisons (S-N-K test, q-values) for growth of *E. aerogenes* against AP_{ce} (10-30 μg mL^{-1}) (based on Fig. 4b)

	AP_{ce} (μg mL^{-1})					
Min	0 vs 10	0 vs 20	0 vs 30	10 vs 20	10 vs 30	20 vs 30
30	15.02***	14.78***	26.25***	0.24	11.25***	11.46***
60	65.08***	66.19***	101.64***	1.12	36.56***	35.45***
90	21.89***	24.09***	36.55***	2.20	14.66***	12.46***
120	52.96***	72.65***	99.24***	19.69***	46.27***	26.58***

* indicates significant level (**p < 0.01, ***p < 0.001)

3.6. Isolation of Extracellular Biomolecules from Nostoc CCC537 and its Bioassay

3.6.1 TLC of extracellular methanolic extract

The methanolic extract of *Nostoc* CCC537 cellular media was subjected to TLC fractionation and UV illumination. The observed six spots were arbitrarily designated as A, B, C, D, E, F and their respective R_f value and inhibition zone size are illustrated in Table 8. Individual UV-illuminated spots were eluted in methanol and bioassayed for the antibacterial potential against *E. aerogenes*. The inhibition zones on the test plate showed that B, C, and E spot eluate did possess antibacterial activity in the sequence: C (2.5 mm) > B (2.0 mm) = E (2.0 mm). Out of these, the most potent spot 'C' was selected for further experiments.

Table 8. Bioassay of different designate bands from 1st TLC against *E. aerogenes*

Designated spots	R_f	Inhibition zone (mm)
A	0.0	-
B	0.18	2
C	0.22	2.5
D	0.40	-
E	0.54	2
F	0.69	-

3.6.2. TLC of 'C' spot eluate in a different solvent

'C' spot eluate in ethanol was subjected to 2nd TLC with solvent system hexane: ethyl acetate (1:1). UV-illumination of the TLC plate revealed a total of 4 spots again designated as G, H, I, and J. Individual spots got eluted in methanol (1 mL) and in bioassayed against *E. aerogenes*. The R_f values of different spots and the inhibition zone size are represented in Table **9**. Among these, only spot 'I' proved potent (inhibition zone 5 mm; R_f 0.52). Therefore, this spot was selected for further processing and designated as AP_{ee}.. The nature of AP_{ee} biomolecule is still yet to be identified.

Table 9. Bioassay of different designate bands from 2nd TLC against *E. aerogenes*

Designated spots	R_f	Inhibition zone (mm)
G	0.20	-
H	0.36	-
I	0.52	5
J	0.56	-

3.7. Spectroscopical analysis of AP_{ee}

The maximum UV absorption [λ (ε) 206] in methanol corresponded to an alkaloid chromophore. The IR spectrum indicated N-H (3431 cm^{-1}), C=C (1670 cm^{-1}) alkene, C=O (1640 cm^{-1}) amide, N-H (1525 cm^{-1}), C-N ((1214 cm^{-1}), and aromatic moieties (1555, 1604, and 3065 cm^{-1}).

3.8. Qualitative and quantitative analysis of AP_{ee}

The extracellular TLC purified extract at 15 μg mL^{-1} produced a maximum inhibition zone (18 mm) on *S. aureus* followed by *P. aeruginosa* (12 mm), *S. typhi* (12 mm), *E. coli* (06 mm) or *E. aerogenes* (10 mm) with the exception of *E. coli* MDR strains. Upon doubling the concentration (30 μg mL^{-1}), the inhibition zone sizes were arranged as: *S. aureus* (28 mm), or *E. coli* (10 mm) or *S. typhi* (16 mm) or *P. aeruginosa* (22 mm) or *E. aerogenes* (12 mm). MDR strains of *E. coli* (clinical isolates) were also more susceptible as reflected from the inhibition zone 07, 12 or 9.5 mm for GC 2003/01, 02 and 03, respectively. MICs for different bacterial species as determined by the agar dilution method are represented in Table 10 with the lowest (1.5 μg mL^{-1}) for *S. aureus*, followed by 2.5, 5.0 μg mL^{-1} each for *P. aeruginosa*, *S. typhi*, respectively. However, *E. coli*., *E. aerogenes* and *E. coli* MDR strains GC 2003/01, 02, 03 had raised MICs to 7.0 μg mL^{-1} to 18 μg mL^{-1}, respectively, thus reflecting their resistance.

Table 10. Inhibition zones and MICs of AP_{ee} from *Nostoc* CCC537 for different bacterial strains

Target	Inhibition zone (mm)		
	15 μg mL^{-1}	30 μg mL^{-1}	MIC (μg mL^{-1})
S. aureus ATCC 25923	18	28	1.5
P. aeruginosa ATCC 27853	12	22	2.5
E. coli ATCC 25992	06	10	7.0
S. typhi MTCC 3216	12	16	5.0
E. aerogenes MTTCC 2822	10	12	7.0
E. coli GS 2003/01	[b]n.d.	07	15.0
[a]*E. coli* GS 2003/02	[b]n.d.	12	18.0
[a]*E. coli* GS 2003/03	[b]n.d.	9.5	18.

[a]MDR strain, [b]n.d.: not detected, [a]Antibiotics for comparison.

3.9. Antibacterial Activity of Gamma Linolenic Acid (GLA)

The isolated GLA from *Nostoc* CCC537 has been purified by HPLC and shown antibacterial activity when bio-assayed against both Gram positive and negative bacteria (Pratt et al. 1944).

3.10. Synergistic effect of AP_{ce}, AP_{ee} and GLA against bacteria

All the three isolated biomolecules were subjected to synergistic activity against *E. aerogenes* by using different combinations i.e, AP_{ce}: GLA, AP_{ce}: GLA, AP_{ce}: AP_{ee} and ratios such as 1:1, 2:1, 3:1, 1:2, 1:3 (Table 11). The significance of this experiment showed combinational chemistry gave better results in the form of inhibition zones as compared to isolated biomolecules alone (Asthana et al. 2009). With a combination ratio of AP_{ce}: GLA (3:1), as the concentration of the applied mixture increased by 15, 25 and 35 μgmL^{-1}, there was a maximum increase in the size of the inhibition zone from 18, 29 and 36 mm, respectively with MIC 0.25. Although, all combinations of AP_{ce}: GLA showed a significant effect on bacteria. The applied combination of AP_{ee}: GLA (1:2) has maximum efficacy of inhibition as inhibition zone (12mm) in 15 $\mu g\ mL^{-1}$ and increased by 1.75, 2.33 times in 25, 35 $\mu g\ mL^{-1}$ applied mixture concentration with MIC 1. AP_{ce} : AP_{ee} combination in 3:1 ratio also increased efficacy against test organism along with increasing concentration with MIC 0.2.

Table 11. Inhibition zone (mm) observed for synergistic effect against *E. aerogens*

AP_{ce}: GLA	15 $\mu g\ mL^{-1}$	25 $\mu g\ mL^{-1}$	35 $\mu g\ mL^{-1}$	MIC
1:1	10	12	19	7
2:1	16	22	27	5
3:1	18	29	36	0.25
1:2	15	24	31	0.5
1:3	11.5	20	25.2	4
AP_{ee}: GLA				
1:1	7.0	10	18.5	6
2:1	10	14	22	6
3:1	12	21	25	4
1:2	12	21	28	1
1:3	14	16	28	1.2
AP_{ce} : AP_{ee}				
1:1	08	09	12	7
2:1	12	18	29	0.5
3:1	16	25	30	0.5
1:2	14	26	26	2
1:3	14	20	23	2

ce* cellular extraction
ee* extracellular extraction

4. Discussion

The isolated antibacterial molecules are chemically isoprenoid, alkaloid and polyunsaturated fatty acid in nature. The final extraction has been done from *Nostoc* CCC537 in methanol and tested positive against both Gram Positive and Negative bacteria. During initial

purification process, the observed colorful bands on TLC, didn't show any antibacterial properties. However, the UV illuminated bands shown antibacterial potential (Deepali et al. 2014). Even the efficacy of methanolic extract from *Nostoc* CCC537 was maximum as compared to that in other solvents. The polar nature of methanol makes it a good solvent for extraction and bioassay of a biomolecules (Bacon et al. 2017; Truong et al. 2019). The analyzed observations were well in agreement with previous reports of UV-illuminated TLC band of *Fischerella ambigua)* (Ghasemi et al, 2004) and *Fischerella* sps. (Asthana et al. 2006) as these natural extracts are secondary metabolites having chiral properties (Abegaz & Kinfe 2019). The presented work has been only focused on the selected illuminated bands of both intracellular (isoprenoid) and extracellular (alkaloid) band having maximum killing efficiency against the target bacteria. Therefore, the HPLC purified most potent fraction was processed for spectroscopical analysis (**Table 2**). The spectroscopical analysis of both intracellular products were published (Asthana et al. 2009; Deepali et al. 2021). One is isoprenoid having Naphthalene ring in its core. Another intracellular product was Gamma Linolenic Acid (a PUFA) having same R_f on TLC and retention time for HPLC as GLA standard (Deepali et al. 2021), confirmed the presence of GLA in *Nostoc* CCC537.The purified extracellular extract showed UV_{max} at 206 nm and its IR spectrum explained the major functional groups present were N-H, C=O, and C-H stretching groups, that confirmed an alkaloid nature of the target molecule. These three isolates from same organism showing three different chemical properties and all are efficient to inhibit the bacterial cells. The result explains, the cyanobacterium has unique biochemical pathways could be uncovered and may lead to the emergence of niche-specific bioactive molecules that could favor the evolution of indigenous species through novel biochemical adaptations (Núñez-Montero & Barrientos 2018). It also supported by the phylogenetic analysis of Type 1 PKS & NRPS genes and its adenylation that shows great diversity and novelty of PKS and NRPS genes in Antarctica sediments (Zhao et al. 2008). However, secondary metabolites in cyanobacteria other than those from Antarctica are reported to be synthesized by polyfunctional enzyme complex**.**

Any biomolecule/lead molecule once identified as antimicrobial has to be compared with selected known antibiotics to ascertain the potency equivalence. The lowest concentrations of streptomycin or rifampicin (30 µg mL^{-1}) were ineffective while the AP_{ce} at the comparable level produced a 12 mm inhibition zone (Table 3). The AP_{ce} maintained its superiority in terms of the inhibition zone produced even for higher concentrations of the target drugs. However, the known anti-tubercular drugs rifampicin and streptomycin generally inhibit RNA polymerase and the function of the 30S ribosome, the precise mechanism(s) of the action of AP_{ce} is yet to be worked out. The data observed for $AP_{ee,}$ at fairly low level (15 µg mL^{-1}) of was hypertoxic to *S. aureus* (Gram-positive) compared to the negative ones i.e., *S. typhi, P. aeruginosa, E. coli* or *E. aerogenes* **(**Table 10**).** Since the same level of the AP_{ee} left the MDR strains unaffected, it was increased two-fold (30 µg mL^{-1}) in the second testing round taking all the bacterial targets in reference. The antibacterial substances may be of two types, bacteriostatic i.e., those responsible for the arrest of bacterial growth, and bactericidal i.e., being lethal. AP_{ce} lysed the bacterial cells to produce the inhibition zones indicating the drug-bacterial membrane interaction (Fig. 4a, 4b). Bactericidal drugs are known to bind to their biochemical target sites very tightly; whereas bacteriostatic ones tend to bind reversibly so that cells recover once the inhibitor is removed (Madigan el al. 1997). The bacterial cell wall is the structurally complex barrier that varies considerably among organisms. Therefore, cell permeability properties, as well as the chemical configuration of special molecules therein, decide the bactericidal and/or

bacteriostatic properties. The AP_{ce} adopted (10, 20, 30 µg mL^{-1}) presently, invariably featured bactericidal potential towards selected bacteria i.e., *S. aureus* (the most sensitive) and *E. aerogenes* (the least sensitive) tested in liquid broth, although to a varying extent. Noticeably, the complete abolition of the bacterial population could not be achieved even after 120 min contact with the highest concentration of the bioactive molecule (30 µg mL^{-1}). The residual cells of the target bacteria might have escaped interaction with the bioactive molecule possibly because of the dilution factor in the liquid broth. The differential behavior of twin bacteria against the biomolecules in reference might be attributed to the permeability property as well as its chemical configuration that in turn decides the bactericidal and/or bacteriostatic potential. It will be worthwhile to extend these observations. This sort of study recommended a further evaluation of other bacteria too.

The antibacterial potential of an Antarctica cyanobacterium was first time reported by Asthana et al. (2009), although reports on bioactivities in cyanobacteria inhabiting the Antarctica existed earlier (Jaki et al. 1999). These workers prepared 126 extracts from 48 strains of cyanobacteria from the lakes there and found that extracts from 17 cyanobacteria were antimicrobial (the Gram-positive *S. aureus*), antifungal (*Aspergillus fumigatus)* and the yeast (*Cryptococcus neoformans*) and 25 were cytotoxic toward Hela cells . The present data on the hypersensitivity of Gram-positive bacteria are well in line with the reports of Biondi *et al* (2008) but quite in contrast with regard to the sensitivity of Gram-negative bacteria. This may be attributed to strain specific bioactivity of the AP in the Antarctic cyanobacterium. The apparent difference in the cell wall of Gram-positive and Gram-negative bacteria and interactions with the lipophilic biomolecules (as in the present case), may be attributed to the differential sensitivity of bacterial targets tested. Reports on the bioactivity of extracts from mesophilic cyanobacteria are in plenty. Jaki et al (1999) screened 86 methanolic (lipophilic) and hydrophilic extracts from 43 samples of cultured and field-collected freshwater and terrestrial cyanobacteria. Out of these, only 18.3% were active against Gram-positive and 5.8% against Gram-negative bacteria. Kamenarska et al. (2000) reported broad-spectrum antibiotics produced by *Nostoc* sp. that inhibited the growth of bacteria, notably the MDR strains of *S. aureus* and *P. aeruginosa*. Goud et al (2007) reported high antibacterial activity in extracts of *Nostoc, Lyngbya,* and even green algae (*Mougeotia* and *Pithophora* sp.). These investigators also observed high susceptibility of Gram-positive bacteria relative to their Gram-negative counterparts. Data on the bioassay of AP from *Nostoc* CCC537 compared well with the MICs for the bacterial targets. The hypersensitivity of *S. aureus* was also reflected from the lowest MIC (1.5 µg mL^{-1}) and the raised inhibition zone (18 mm) at 15 µg mL^{-1} or even more at (28 mm) for 30 µg mL^{-1}. Based on such parameters, the overall sensitivity sequence of bacterial targets towards the AP can be arranged as *S. aureus >P. aeruginosa >S. typhi >E. coli = E. aerogenes > E. coli* MDR strains (GS 2003/01, 02 and 03). Hospital-acquired infections invariably claim about 20,000 lives each year due to the ineffectiveness of drug-resistant strains including *S. aureus* (Peacock et al. 2001). Therefore, the increased susceptibility of *S. aureus* emphasizes the importance of bioactive molecule elucidation. It was interesting to note that MDR *E. coli* strains (GS 2003/01, 02 and 03) had low MICs of the AP_{ee} (15, 18 µg mL^{-1}).

The synergistic effect of antibiotics such as ampicillin, penicillin G, tetracycline, and chloramphenicol in combinations with Ethylenediaminetetraacetic acid (EDTA), against the resistant strain of *P. aeruginosa,* have been reported earlier as EDTA combination reverse resistant nature of bacterium (Weiser & Asscher 1968). According to Leive (1965) and Weiser & Asscher 1968, resistance against antibiotics can be a consequence of permeability barrier

development, which prevents antibiotics from reaching their site of action, and they suggested that the EDTA combinations broke that permeability barrier (Umerska et al 2018). EDTA had also been reported to act in synergy with a 12-residue cationic antimicrobial peptide ARVA (Peacock et al. 2001). Nakai et al. (2012) reported that the addition of the polyphenols and fatty acids would inhibit the growth of *M. aeruginosa*, and the interaction of the polyphenols and fatty acids was additive. Moreover, when the collective activity of a mixture of the polyphenols, i.e., ellagic, gallic and pyrogallic acids and (+) − catechin, was examined, the synergistic growth inhibition of *M. aeruginosa* occurred (Nakai et al. 2000). In the present study, we observed that a significant combination of AP_{ce}, AP_{ee} and GLA doses against *E. aerogens* showed an increase in inhibition zone as compared to applied separately (Table 11). It suggests that GLA as a lipophilic molecule increases the membrane permeability as large inhibition zone was observed against the *E. aerogens* by mixing it with AP_{ce} and AP_{ee}. Even the combination of AP_{ce} and AP_{ee} also showed a synergistic effect suggesting the secretory nature of AP_{ee} and to help APce to be more effective.

5. Conclusions

The experimental organism *Nostoc* CCC537 are growing in Antarctic region (below 0^oC) although its suitable growing temperature is 20^oC, as standardized during study. Their natural habit represented; this cyanobacterium evolves different coping mechanisms to endure in hostile environments. Such organisms showed vast morphological as well as biochemical diversity to cope with the situation. We also observed, it has different biochemical combinations that showed potential against bacteria and fungi (preliminary study, data not shown) targets. The isolated potent molecules in present study are intracellular a Naphthalene derivative (4- [(5-carboxy-2-hydroxy)-benzyl]-1, 10-dihydroxy-3, 4, 7, 11, 11-pentamethyloctahydrocyclopenta<a>naphthalene); a PUFA Gamma Linolenic Acid; and an extracellular Alkaloid. The present experimental results explain: the molecular diversity actually evolved by the Antarctic cyanobacterium to defend ourselves from adverse environmental conditions. The PKS and NRPS genes in the Antarctic sediments are one of the causes of these diversity, regulation and multiple bioactive molecules synthesis by the cyanobacteria. After the pandemic, the world demanding now the novel biomolecules with diverse active potential to fight new variant and MDR strains. The whole science academia is now looking towards unexplored habitats and species to find the such novel compounds. Keeping this important point we had chosen an unexplored strain *Nostoc* CCC537 from an unusual habit Antarctica. The selected bioactive isolates from the cyanobacterium showed characteristic antibacterial properties alone but their potential increased synergistically when we used them in various combinations with other active molecules. On the basis of present finding, we can conclude, these molecules can be proposed as a potent antibacterial agent against both Gram-positive and negative bacteria. It could be more potent molecules against MDR strains as we found its synergistic potential.

Acknowledgments

We are grateful the Head, Centre of Advanced Study in Botany and Institute of Medical Science, Banaras Hindu University, Varanasi, India, for providing us with laboratory facilities. The Department of Chemical Engineering, Institute of Technology, BHU, for HPLC facility.

Declarations

Funding: NO

Conflicts of Interest: N.A.

Availability of data and materials: N.A.

Code availability: N.A.

Author contributions: Deepali designed & performed the experimental concepts, Ravi K Asthana and Gopal Nath provided the strains and lab facilities to complete this research work. Elangbam Geetanjali and Rashmi Shakya helped in the compilation of data, and data analysis. Satish Kumar and Reeta Kumari helped in the manuscript writing and editing.

Consent for publication: The presented research work has not been submitted elsewhere and is not under consideration by any other journal. All authors have critically proofread the manuscript and approved it for publication in this Journal.

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