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RESEARCH ARTICLE

SPECTRAL ANALYSIS OF BIOLOGICALLY ACTIVE COMPOUND AND ANTIBACTERIAL ACTIVITY OF *SCYTONEMA OCELLATUM* ISOLATED FROM SUB-AERIAL HABITATS

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Glossary of Abbreviations
CIP- Ceproflaxin
CLSI – Clinical and Laboratory Standards Institute
FTIR- Fourier Transform Infra Red Spectroscopy
GC- Gas chromatography
MHA – Muller Hinton Agar
MDR- Multi drug Resistance
MTCC- Microbial Type Culture Collection and **Gene Bank**
NCCLS- National Committee for Clinical Laboratory Standards guidelines
R_f – Retention factor
TLC- Thin Layer Chromatography
U.V – ultraviolet

ABSTRACT

Background: Screening of antibacterial compounds from natural sources for social benefit is emphasized in recent years with the view that pathogens have developed resistance against antibiotics. **Objectives:** The present investigation has been carried out to screen sub-aerial cyanobacteria thriving in an extreme environment as they are supposed to be a potential source of natural bioactive compounds. **Methods:** *Scytonema ocellatum* isolated from building facades were used for antibacterial activity against certain reference human pathogenic bacteria after subjected to TLC purification twice by altering the solvents. **Results:** The acetone extract of *S. ocellatum* showed highest and moderate inhibition zone against *Escherichia coli* and *Pseudomonas aeruginosa* respectively. Chloroform extracts also exhibited significant zone against *Escherichia coli*; no activity was observed against *Bacillus subtilis* and *Staphylococcus aureus*. The UV illuminated bands with potential effect were subjected to GC and FTIR analysis. GC analysis showed 98% and 95.6% purity in each solvent extracts and FTIR spectra showed stretching bands containing various biologically active functional groups like alcohol, phenol, alkanes, carboxylic acids groups, amines etc respectively revealing antibiosis. **Conclusion:** The above screening is the first record of antibacterial activity enhancing the importance of sub-aerial cyanobacteria that possess a scope for producing biologically active compounds, pointing towards brilliant candidates for Pharmaceutical application.

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INTRODUCTION

Cyanobacteria are ubiquitous in the environment. Research of microalgae and cyanobacteria has mostly been directed towards freshwater and marine cultures with significant biologically activity *in vitro* and *in vivo*. During the recent years, the emphasis has been shifted to cyanobacteria which inhabit the extreme environments like rocks, roofs, tree barks, caves, mortar, city wall and other surfaces that are in contact with the atmosphere (Neustupa and Škaloud, 2008; Karande et al., 2012; Vasiliki et al., 2015; Keshari and Adhikary, 2014). It appears that the extracts of sub-aerial cyanobacteria posses a great capacity for producing biologically active compounds due to their ability to endure extreme condition such high solar irradiations, high temperature and nutrient depleted condition than marine and aquatic species. Some scientists believe that their special growing condition, survival and adaptation mechanisms leads to a wider amplitude of metabolic extremities and possibilities, which causes the production of a most diverse range of more or less, specific substances, revealing and pointing towards brilliants candidates for biotechnological application (Dobretsov et al., 2011; Uzair et al., 2012). On this basis, in the present study, search is on for biologically active constituents in sub-aerial cyanobacteria isolated from biofilms on building facades considering as extreme environments, and their exploitation for new natural products as antibacterial agents against resistant pathogens is

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very important for clinical medicine and public health, and a limited number of new antimicrobial classes have been developed by the international pharmaceutical industry in the last 20 years (Infectious Diseases Society of America, 2007). It is noted that however, no studies have yet indentified the antibacterial potential of cyanobacteria isolates from building facades. This study was designed to investigate sub-aerial cyanobacteria *Scytonema ocellatum* in the production of the potential antibacterial bio molecules of partial purified acetone and chloroform extracts using agar well diffusion methods and subjected to spectroscopic analysis for identification of biomolecules.

MATERIALS AND METHODS

Study sites, collection, isolation and maintenance of sub-aerial culture: Sub-aerial cyanobacteria, blackish-dark greenish biofilms were collected from cement wall surface of modern building, Bhubaneswar (Temperature: 21°C, Latitude /Longitude: 20°25'36.82"N and 85° 48' 10"E) of Odisha and stored in pre-sterilized sampling bottles. The biofilms were soaked in sterile distilled water and incubated under fluorescent light for up to 72hrs and observed microscopically. Since the morphologically features needed for identification were not distinct even after prolonged soaking (up to 7 to 10 days), a small amount of sample were transferred to BG-11 agar plates medium with and without nitrogen (Rippka et al., 1979) (1.5% agar in same medium) with the help of inoculation needle. Cultures were incubated at 25 ± 1°C under continuous light from fluorescent tubes at an intensity of 2000lux. After a period of 10 to 14 days of incubation, cyanobacteria appeared in the culture were isolated and maintained in the laboratory. The cultures were microscopically identified by following standard monographs (Desikachary, 1959). The axenic culture were diluted and subculture to 50ml of culture media in 100ml conical flasks, and incubated under illumination of 2000lux with 16:8 hrs light and dark regime.

Preparation of sub-aerial Cyanobacteria crude extracts: After 28 days of incubation, biomass were harvested through Buchner funnel with Whatman No.1 filter paper and dried in shade. Then dried biomasses were grinded to fine powder with the help of glass homogenizer and finally weighed. 2gms of the powdered samples of *Scytonema ocellatum* were extracted with two different organic solvents, acetone (200ml) and chloroform (200ml) using soxhlet apparatus for 48 hours maintaining at temperature 45°C. The crude extracts were collected in air tight containers, dried in a rotary evaporator at 40°C (Buchi, USA) redissolved in 3ml of each acetone and chloroform and finally were stored at 4°C for further antibacterial assay.

Antibacterial bioassay

Partial Purification of crude extracts of isolates by thin layer chromatography: Each crude extracts of acetone and chloroform were further fractioned by Thin Layer Chromatography (TLC) on TLC silica gel plates (H-60 Merck, Darmstadt, Germany). Separations of acetone and chloroform crude extracts were done using silica gel as stationary phase, carbon tetrachloride and methanol (9:1) as mobile phase. Fractions developed on TLC plates were observed under UV illumination. The illuminated orange spots were eluted separately and redissolved in each 3ml acetone and chloroform respectively. Each elutes were again subjected to 2nd TLC purification using hexane: ethyl acetate (1:1). Each solvent showed one prominent fraction after 2nd TLC purification. Now each fraction of different organic solvents obtained at second stage was observed under UV illumination and the R_f values of the coloured spots were recorded as well as bio assayed for antibacterial activities. All reagents and chemicals were of analytical grade and supplied by Merck (Darmstadt, Germany).

Screening ***of partial purified fraction of sub-aerial cyanobacteria isolates for antibacterial activity:*** The potential antibacterial activity was tested in sub-aerial cyanobacteria strains against the bacteria were assessed using the National Committee for Clinical Laboratory Standards guidelines (NCCLS) for agar well diffusion assay and Mueller-Hinton II Agar (OXOID, UK) according to CLSI guidelines. Each partial purified designated fraction obtained in each solvent at 2nd TLC was redissolved in each acetone and chloroform (3ml). The MHA plates were swabbed with 0.1 ml of each target bacterium strain aseptically adjusted to turbidity of 0.5 McFarland.

The wells of 6mm diameter were made to the surface of the inoculated agar with sealing off bottom by soft agar (1%) and were loaded with a total amount of 50µl of each extract solution. The standard antibiotic disks (BIORAD, UK) Ciprofloxacin 30mg (CIP) were used as positive controls depending of the bacterial species. Both acetone and chloroform was used as a negative control since a volume of 50µl pure acetone and chloroform was inhibitory to bacterial growth. The plates were left to dry for 15 min and were incubated at 37°C for 24 hrs thereafter. For all agents the diameters of zones of inhibition were measured to the nearest millimetre in triplicates and for the positive controls the results were interpreted according to CLSI (2012) breakpoints. Each partial purified fraction of A3 and C3 was tested *in vitro* for their ability to inhibit growth of the following four reference human pathogenic bacteria isolates: *Bacillus subtilis* (MTCC 121), *Escherichia coli* (MTCC 723), *Pseudomonas aeruginosa* (MTCC 741), and *Staphylococcus aureus* (MTCC 902). Data are reported as means ± standard deviation (SD).

Screening and identification of prospective compounds: The antibacterial potential partial purified fraction (A3 and C3) obtained at 2nd TLC analysis were subjected to Gas chromatography on a Clarus 680GC-HS+AS comprising an auto injector and RTX - 5 columns. The analysis was further extended to identify the functional groups of the antibacterial purified fraction of the each extracts by FTIR (Fourier-transform infrared spectroscopy, Nicolet 6700, USA) spectrophotometer in the range of wave number 500 to 4000 cm^{-1}.

RESULTS

Sub-aerial habitats are characterized by a dominance of biofilms comprising a diversity of prokaryotic organisms, principally cyanobacteria has long attracted the attention of mycologists because of their unique adaptation to these extreme environment. The crude extracts from these sub-aerial cyanobacteria are generally contains both active and non-active constituents, which can be screen by thin layer chromatography separation technique. Our results showed that the blackish-dark greenish biofilms tightly adhering to the

Table 1. Bioassay of TLC purified bands against selected human bacterial pathogens of two different extracts of *Scytonem ocellatum.*

Test Organisms	Extract Type	1st TLC Bands Mobile phase - Carbon tetrachloride : methanol (9: 1)	2nd TLC band Mobile phase - Hexane: ethyl acetate (1: 1)	R_f value	Effective zone of inhibition (mm)			
					Negative Control (50µl/well)	1st TLC partially purified fraction (50µl/well)	2nd TLC Partially purified fraction (50µl/well)	Positive Control Ciprofloxacin (30mg/disc)
Bacillus subtilis (MTCC 121)	Acetone	A1 – A4	A3	0.82	-	-	-	15.3 ± 0.08
	Chloroform	C1 – C4	C3	0.79	-	-	-	
Escherichia coli (MTCC 723)	Acetone	A1 – A4	A3	0.82	-	15.0 ± 0.4	20.0 ± 0.0	16.5 ± 0.2
	Chloroform	C1 – C4	C3	0.79	-	17.9 ± 0.8	18.5 ± 0.2	
Pseudomonas aeruginosa (MTCC 741)	Acetone	A1 – A4	A3	0.82	-	11.6 ± 0.2	12.6 ± 0.2	15.0 ± 0.4
	Chloroform	C1 – C4	C3	0.79	-	-	-	
Staphylococcus aureus (MTCC 902)	Acetone	A1 – A4	A3	0.82	-	-	-	18.5 ± 0.2
	Chloroform	C1 – C4	C3	0.79	-	-	-	

Table 2. Functional group assignments to the partially purified acetone and chloroform extracts (Band-A3, C3) using FTIR Spectra.

Band number	Functional group	Assignments	Band wave number (cm^{-1}) 2nd TLC partially purified band A3(R_f value 0.82)	Band wave number (cm^{-1}) 2nd TLC partially purified band C3(R_f value 0.79)
1	Alcohols, Phenols	O-H stretch, H-bonded	3366	-
2	Carboxylic acid	O-H stretch	-	3019
3	Alkanes	C-H stretch	2945, 2832, 1364	1362
4	Aliphatic amines	C –N stretch	1229, 1093, 1022	1214
5	Aldehydes	C=O stretch	1704	1710
6	Aromatics	C-C stretch, C-H stretch	1420	670, 743
7	Alkyl halides	C-Br stretching	532	530

Fig. 1. Microphotographs of *Scytonema ocellatum* isolated from blackish-dark greenish biofilms of building facades, Odisha

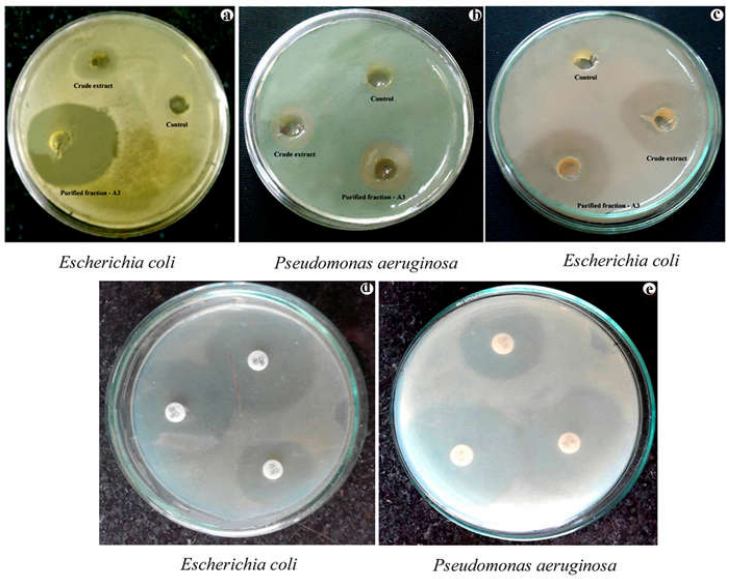

Fig. 2. Zone of inhibition exhibited by acetone and chloroform extracts of *Scytonema ocellatum* against *Escherichia coli* and *Pseudomonas aeruginosa* (a) acetone extract against *E. coli*, (b) acetone extracts against *Pseudomonas aeruginosa*, (c) chloroform extracts against *E. coli*, (d) Standard Antibiotics Ciprofloxacin (30mg/disc) against *E. coli* and (e) Standard Antibiotics Ciprofloxacin (30mg/disc) against *Pseudomonas aeruginosa*

upper surface of the cement wall, Building, *Scytonema ocellatum* appeared as a major cyanobacterium upon prolonged exposure to culture (Fig. 1). The antibacterial fraction of the crude extract extracted from two different solvent using acetone and chloroform were separated by TLC silica with alternating mobile phase. The R_f value of active fraction is shown in Table 1. On the TLC plates, four bands (A1 - A4) were noticed of acetone crude extracts by following autobiography which separated at R_f 0.67, 0.75, 0.79 and 0.82 values in carbon tetrachloride: methanol (9:1) solvents system. TLC plates were visualized under UV illumination orange colour spots were seen. The chemical nature of the biologically active compound was detected by spraying with Dragendroff's reagent for alkaloids and 10% Ferric Chloride for phenolic compounds and 5N potassium hydroxide solution for flavonoids.

Among four, one bands of R_f value 0.79 was flavonoids and other one R_f value 0.82 was alkaloids, whereas chemicals nature of two bands with R_f value 0.67 and 0.75 may be phenol which remained unclear. Similar results were also observed in case of partially purified chloroform crude extract with four bands (C1- C4). *In vitro* evaluation of the individual bands of 1st TLC and 2nd TLC partially purified bands of both extracts against selected human pathogenic bacteria strains such as *Bacillus subtilis* (MTCC 121), *Escherichia coli* (MTCC 723), *Pseudomonas aeruginosa* (MTCC 741) *Staphylococcus aureus* (MTCC 902) cultures is shown in table 1, and fig. 2. It has been observed that only one band (A3) of R_f value 0.82 retained activity against two strains *Escherichia coli* and *Pseudomonas aeruginosa* whereas partial purified C3 band showed specific activity against *Escherichia coli.* The 2nd TLC purification of the partially purified band A3 and C3 was done using hexane: ethyl acetate (1: 1), gave a major single fraction in each solvent of R_f value 0.82 and 0.79. The bioassay of the major single fraction A3 and C3 from each solvent along with activity profile with standard commercial antibiotics are presented in Table 1, Fig. 2. Concerning the antibacterial effects, the results depicted that 2nd TLC partially purified acetone extract (A3band) of *S. ocellatum* showed highest biological activities towards *E. coli* (20.0 ± 0.0) and moderate activities towards *Pseudomonas aeruginosa* (12.6 ± 0.2). On the other hand partially purified band C3 of chloroform extracts gave positive results towards *E. coli* only and negative results for other tested bacterial strains. Negative antibacterial effect was recorded towards the *Bacillus subtilis* and *Staphylococcus aureus.* The solvents (acetone and chloroform) used as negative control revealed no activity. *Scytonema ocellatum* were tested against four human bacterial strains: *Bacillus subtilis* (MTCC 121), *Escherichia coli* (MTCC 723), *Pseudomonas aeruginosa* (MTCC 741) *Staphylococcus aureus* (MTCC 902) by agar well diffusion method, show potent antibacterial activity for acetone extract and less effective for chloroform extract.

For spectroscopic analyzed of 2nd TLC potential partially purified band (A3 and C3) obtained from each crude extracts which showed antibacterial activity was subjected to Gas chromatography to study the purity status. The results showed 98 % purity with a single peak and retention time of 2 min 18 sec in partial purified A3 band of acetone extract and 95.6 % purity with a single peak and retention time of 1min 98 sec in partial purified C3 band of chloroform extract (Fig. 3, 4). The Infra Red spectra of the single partially purified band of respective acetone (A3) and chloroform (C3) extracts of *S. ocellatum* were obtained to determine the surface functional groups by using FTIR Spectrophotometer (Nicolet 6700, USA). The presences of various kinds of functional groups revealed from each partially purified extracts are presented in (Table - 2, Fig. 5 & 6). The results of FTIR analysis confirm the presence of 7 functional groups and the expected phytocompounds are identified in the partially purified band (A3) of acetone extracts. The strong and broad instance peaks are identified at 3366.5 and 1704.7cm^{-1} which are assigned to the alcohols and phenols, aldehyde compound frequency vibration respectively. The medium peaks at 2945.5, 2832.7, 1420.3, 1364.8, 1229.6, 1093.8, 1022.5 and 532.5cm^{-1} which are assigned to the alkanes, aromatic compound, amines and alkyl halides has existed in these extracts, whereas in partially purified band (C3) of chloroform extract showed strong and broad instance peaks are identified at 3019.4 and 1710.1cm^{-1} which are assigned to carboxylic acid and aldehyde compound frequency vibration in addition to some other groups at 1362.8 cm^{-1}, 1214.2cm^{-1}, 743cm^{-1}, 670cm^{-1}, 530cm^{-1} indicates the presence of C-H group, C-N stretches and C-Br stretch in the extract.

DISCUSSION

The presence of the family Scytonemataceae in all the long established biofilms on the sub-aerial habitats confirm previous suggestion of this group as indicators of mature biofilms on building in Indian environments (Pattnaik and Adhikary, 2002; Keshari and Adhikary, 2014). The most frequent identifiable organisms on walls belong to the genera *Tolypothrix*, *Scytonema* and *Lyngbya* group. In present investigation, filamentous cyanobacteria *Scytonema ocellatum* with thick sheath layer around their trichome were major species occurring on the exposed cement wall surface of building. *Scytonema* is one of the several algal genera that have attracted special attention due to their unique adaptation to these harsh environments. In recent decades, interest in search for bioactive compounds has been more directed towards the exploration of new environment and the screening of less exploited microbial group endowed with more versatile secondary metabolites such as cyanobacteria due to the emergence of MDR strains of important pathogen like *B. subtilis*, *E. coli*, *S. aureus* and *P. aeruginosa* (Kluytmans et al., 1997). Investigations are needed for antibacterial agents that offer broad spectrum activity against resistant strains of microorganisms. Toxicity profiling of the cyanobacteria extract also need scientific validation. In the present investigation, the antibacterial activity of the *Scytonema ocellatum* were evaluated against reference human pathogenic bacteria such as *B. subtilis*, *E. coli*, *S. aureus* and *P. aeruginosa*. Although the antimicrobial effect of these genus of different species has already been reported by many workers, their activities against MDR strains are less evaluated (Pignatello et al., 1983; Ishibashi et al., 1986; Stewart et al., 1988;Carmeli et al., 1990 Miao et al., 1990;Prinsep et al., 1992; Falch et al., 1995; Nagatsu et al., 1995; Jaki et al., 1999; Ma and Led, 2000; Jaki et al., 2000; Hirata et al., 2003; Raveh and Carmeli, 2007; Gutiérrez et al., 2008; Asthana et al., 2009; Yadav et al., 2012; Dixit and Suseela, 2013; [6] Tyagi et al., 2014; Panigrahi et al., 2015). Moreover, the current study focused on the phytochemical nature of biologically active compound of active band. Two types of extracts, acetone and chloroform were listed for their possible antibacterial activity against MTCC bacteria strains after twice thin layer chromatography analysis.

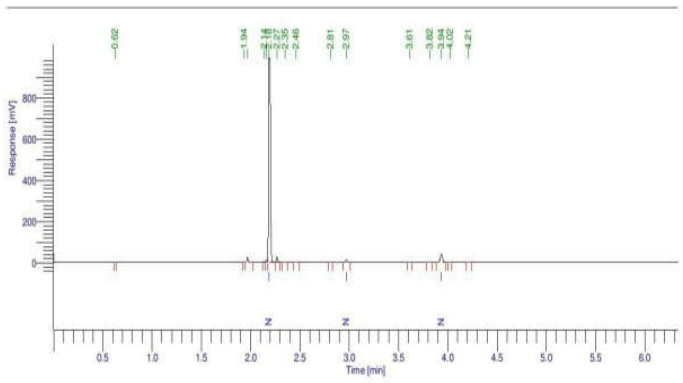

Fig. 3. GC analysis of the purified 2"" TLC spots (Band-A3) of the acetone extracts of *Scytonema ocellatum.*

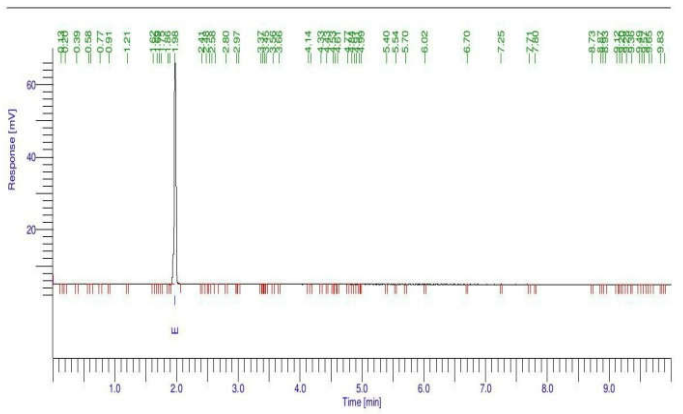

Fig. 4. GC analysis of the purified 2nd TLC spots (Band-C3) of the chloroform extracts of *Scytonema ocellatum*.

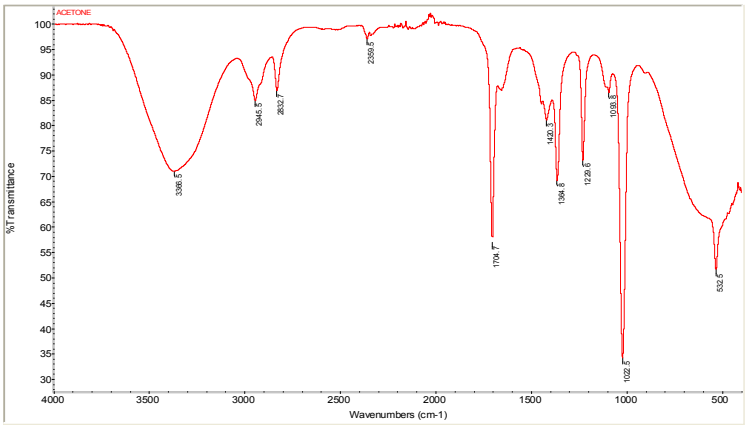

Fig. 5. Infra Red Spectrum of the purified 2nd TLC spots (Band-A3) of acetone extracts of *Scytonema ocellatum.*

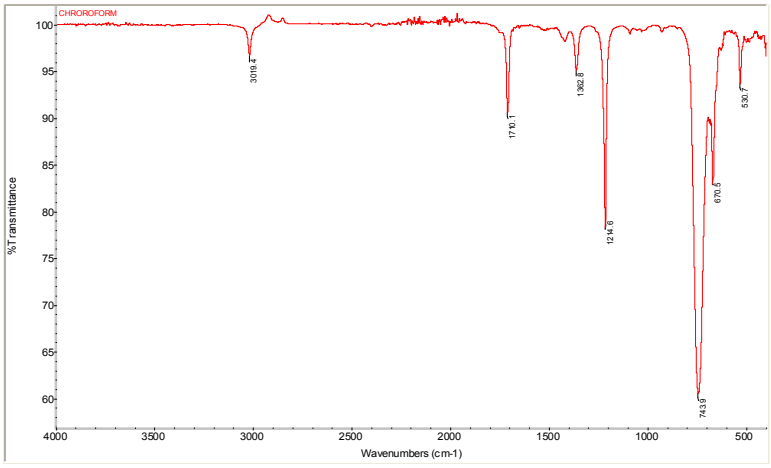

Fig. 6. Infra Red Spectrum of the purified 2nd TLC spots (Band-C3) of chloroform extracts of *Scytonema ocellatum.*

The partial purified bands (A3) of acetone extract were found to have maximum antibacterial activity against *E. coli* and moderate towards *P. aeruginosa*. While chloroform purified band (C3) were found to inhibit *E. coli*. The 2nd TLC purified band A3 with R_f value 0.82 was found to have variable effect on the reference strain *E. coli* showing greater sensitivity then to the 1st TLC partially purified. In contrast chloroform purified band C3 with R_f value 0.79 showed very little or less activity was observed against *E. coli* which might be due to less solubility of active component in chloroform. Both active and chloroform extracts, either 1st TLC or 2nd TLC purified did not show any zone of inhibition against *Bacillus subtilis* and *Staphylococcus aureus.* The current study indicates that it may be alkaloids or phenolic compounds that shows antibacterial activity; some other compounds of different nature are also bioactive and further studies are required for their separation and identification.

For Gas chromatography analysis of 2nd TLC purified band in both extract confirmed the presence of single peak which might be bioactive compounds showing antibacterial activity against *the growth* of the two human pathogens (*E. coli* and *P. aeruginosa*) might possess broad spectrum antibiotic activity. Further to determine if partially purified band is potential producer of biologically active compound in respective acetone (A3 band) and chloroform (C3 band) extracts, IR spectrum is used to recognize the functional groups of the active compound present in the extract based on the peak value in the region of IR radiation. The observation spectra of the samples are done and the associated functional groups are presented in the (Table - 2, Fig. 5 & 6). FTIR analysis confirmed the presence of alcohol, phenols, carboxylic acids, alkanes, amides, carbonyl, aromatic amines and alkyl halides of the partially purified band of acetone extracts (A3 band) and chloroform extract (C3 band) of *S. ocellatum* showed the presence of phenolic compounds. The FTIR spectroscopic analysis also revealed the presence of hydroxyl group due to O-H stretch at the 3366.5cm^{-1} of the partially purified band of acetone extracts. The presence of O-H stretch at the peak 3019.4cm^{-1} confirm carboxylic acids and at 1710.1cm^{-1} for C=O(stretch), C-H stretch at 1362.5cm^{-1} and 743.1cm^{-1}, C-N stretch at 1214.6cm^{-1} confirms the presence of Saponin type, terpenes, aromatic, primary, secondary and tertiary amines of the partially purified band (C3 band) of chloroform extract.

Conclusion

Keeping in mind the easiness and cost-effectiveness of culturing the cyanobacteria, coupled with rich biodiversity in tropical region, *S. ocellatum* produces a good opportunity toward the commercialized production of clinically important antimicrobial compounds. Such compounds may be used as a single compound or may be formulated with other compounds (cyanobacteria and/or synthetic origin) to get synergistic effects. The FTIR spectroscopic analysis revealed the presence of phenolic compounds and carboxylic acids, alkane, amines, aromatic and aldehyde compounds. *S. ocellatum* exhibits good antimicrobial activity with reference to standard this is due to the presence of O-H, -C-H, C-H, C=O, C-N and C-Br functional properties. This is the first report on the antibacterial activity of sub-aerial cyanobacteria *S. ocellatum* isolated from building facades. However, the active principals of *S. ocellatum* need to be isolated and identified before depicting further speculations could lead to the development of natural antibacterial agents.

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Conflict of Interest

The authors declare that they have no conflicting interests.

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