ASSESSMENT OF ANTIBACTERIAL AND FREE RADICAL SCAVENGING ACTIVITY IN PSYCHROPHILIC *ARTHROBACTER SP.*

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Summary

Present investigation was undertaken to examine a psychrophilic bacterial species isolated from the high altitude region of Leh (India) as a potential sources of antioxidant and antibacterial properties. Extraction of secondary metabolites was done by using Diaion HP-20 adsorbent resin and elution was done with methanol. The methanol extracts were evaluated for their anti oxidant activity by DPPH radical scavenging assay and antibacterial activity was measured by using Kirby bauer method (disk diffusion method). In this report, methanolic extracts of *Arthrobacter* sp. was tested against *Dietzia sp K44, Micrococcus flavus, Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus cereus, Salmonella typhi* and *Micrococcus luteus* for their antibacterial properties. Methanol extracts were further analysed by UV-Vis spectroscopy and high performance liquid chromatography (HPLC). To the best of our knowledge, this is the first report demonstrating the antibacterial and antioxidant properties in psychrophilic *Arthrobacter* sp.

Keywords: *Extraction of metabolites, Antimicrobial assay, DPPH antioxidant assay.*

Introduction

The scope of novel metabolites from terrestrial microorganisms is decreasing hence it is suggested to search of new environments. In this quest for the exploration of low temperature microorganisms has proved to be a rich source of secondary metabolites that shows antibacterial and free radical scavenging properties. The antibacterial produced in cold environments working at low temperatures gives a competitive advantage to the producing microorganisms during their growth cycle (1). Now a days, there is a need for new antibacterial agents due to the increase in drug resistance in many common bacterial pathogens along with the susceptibility of immune compromised to common disease which has become an alarming problem worldwide (2, 3). In the current scenario, microorganisms are still a dominant source for antibacterial compounds (4). This has encouraged the new strategies to search useful microbes from psychrophilic environments. In addition to antibacterial compounds, these psychrophilic microorganisms are also a good source of antioxidants. Antioxidants are reducing agents, may be defined as radical scavengers which protect the human body against free radicals that may cause a number of degenerative diseases such as premature aging, Parkinson's diseases, deoxygenation of ischemic tissues, atherosclerosis, gastric ulcer, diabetes and cancer (5, 6). Antioxidant agents terminate oxidative chain reactions by removing free radical intermediates, thereby preventing oxidation of cellular components. Antioxidant based drugs/formulations for the prevention and treatment of complex diseases like Alzheimer's disease, atherosclerosis, stroke, diabetes and cancer has attracted a great deal of research interest in natural antioxidants. Our previous papers have discussed about various natural products of microbial origin, which proved effective radical scavenger and antimicrobial properties (7, 8). For these reasons, there has been increasing interest in recent years for search of some exogenous natural antioxidant materials especially of microbial origin (7, 9, 10, 11).

Keeping this in view, the objective of present study was to evaluate the *in vitro* antibacterial potential and exogenous free radical scavenging properties of psychrophilic *Arthrobacter* sp. isolated from the high altitude region of Leh, Himalaya region, India.

Methods

Sample collection and bacterial identification

The bacterial strain was isolated from the high altitude region of Leh, Himalaya region at a distance of 7135 m from the sea level. Bacterial identification of the isolated strain was determined according to the methods described in Bergey's manual of determinative bacteriology (12) and 16S rRNA sequence analysis (13). DNA sequencing was performed by The Centre of Genomic Application (TCGA), New Delhi (India) and homology search of the sequences obtained were performed using BLAST search algorithm of NCBI (http://www.ncbi.nlm.nih.gov).

Screening for production of bioactive metabolites

Shake flask fermentation

In shake flask fermentation a single colony from the sub-cultured plate of bacteria was used to inoculate 1000 ml Erlenmeyer flasks containing 500 ml of nutrient broth. The flasks were then incubated on a rotary shaker at 200 rev.min^{-1} for 7 days at 4 °C for bacterial cultures. After 7

days of fermentation, the fermented broth was centrifuged at 8000 g for 15 min and then both supernatant and pellets were collected in separate flasks.

Extraction of secondary metabolites using Diaion HP-20

The extraction of secondary metabolites was done by using Diaion HP-20 poly aromatic adsorbent resin from culture supernatant. Before usage, 15g of adsorbent resin was soaked and swelled in methanol for 12 h to remove the impurities. Solvent was removed by washing with distilled water sufficiently. The washed resin was then weighed at 7% level and then added to 500 ml of cell free culture supernatant (CFCS) and kept for shaking for 3 h at 150 rev.min^{-1} for adsorption of bioactive molecules (14). After shaking, the cell free culture supernatant with resin was centrifuged at 8000 g for 15 min. The culture filtrate was removed and the resins were packed in a column. The column was washed with double distilled water and then eluted with stepwise gradient of aqueous methanol (0%, 20%, 40%, 60%, 80% and 100% v/v). Each eluted fractions were concentrated under reduced pressure using a rotary evaporator at 35 °C and 150 rev.min^{-1}. These were then collected in pre-weighed vials, lyophilized and were stored at 4 °C.

Recovery of secondary metabolites from pellet

The cell pellet resulted by centrifugation of fermented nutrient broth was suspended in 25 ml of methanol and sonicated for 15 min in an ultrasonic bath (Elma Transsonic T460H) and the resulting homogenate was filtered. The filtered methanol fractions were concentrated in vacuum at 35 °C and 150 rev.min^{-1} to obtain crude extracts and then it was also lyophilized to obtain the crude extracts in powdered form which were stored at 4°C.

Antimicrobial activity test

Preparation of test organisms

Antimicrobial activity tests were performed by using disk diffusion assay, bioautography and micro-well dilution assay. Six test strains of gram-positive bacteria *Dietzia sp.* K44*, Micrococcus flavus, Bacillus subtilis, Staphylococcus aureus, Bacillus cereus, Micrococcus luteus* and three test strains of gram-negative bacteria *Escherichia coli*, *Pseudomonas aeroginosa,* and *Salmonella typhi* were used for antibacterial activity.

Kirby-Bauer disk diffusion assay

Antimicrobial activity was performed by using the Kirby-Bauer single disk susceptibility test (15, 16). Whatman No. 1 filter paper disk of 6 mm diameter were sterilized by autoclaving for 15 min at 121 °C. The sterile filter paper disks were loaded with different extracts (100 µg/disk) (17). Filter paper disks soaked with each extract were placed in the respective grid of each Mueller Hinton agar plates seeded with target test organisms and then kept for incubation at 30 °C overnight. If the test materials have any antimicrobial activity, it will inhibit the growth of the microorganisms giving the clear distinct zone around the disk called “Zone of Inhibition”. The results are recorded by measuring the zone of inhibition surrounding the disk. Gentamicin disk (10 µg/disk) and autoclaved double distilled water were used as positive and negative control

respectively for bacterial test strains. For the temperature and pH stability of antimicrobial compound, methanol extract were incubated at different temperatures (-20 to 80 ^{0}C) and pH (4 to 10) for 30 min. Each sample was then bioassayed for antimicrobial activity using same disk diffusion method. All the assays were carried out in duplicates.

Minimum inhibitory concentration (MIC)

Antibacterial activity was evaluated by estimating the minimum inhibitory concentration (MIC) of both extract (pellet and supernatant) on all test microorganisms using the micro-well dilution method developed by Eloff (18). Briefly, bacterial strains were cultured overnight at 37°C on nutrient broth (HiMedia Laboratories) and used as an inoculum. The extracts were redissolved in methanol to a final concentration of 10 mg/ml. Two-fold serial dilutions of the methanol extracts (5 mg/ml to 0.05 mg/ml) were prepared in 96-well microtiter plate. In the first well 100 µl of test organism was added (without methanolic crude extract) as a negative control. The wells in plate were inoculated with 100 µl of the relevant culture and incubated at 37 ^{0}C overnight. As an indicator of bacterial growth, 50 µl of 0.2 mg/ml iodonitrotetrazolium chloride (INT) (Sigma-Aldrich) was added to the wells and incubated at 37 ^{0}C for 30 min. Each culture was assayed in triplicates. The formation of a red colour signified microbial growth and where bacterial growth was inhibited, the solution in the well remained clear after incubation with INT.

Bioautography

The antibacterial activity of methanolic extracts was evaluated against *Micrococcus flavus* by direct bioautography technique and was done according to Begue and Kline (19). Briefly, aluminium backed TLC plate (Merck, silica gel 60 F_{254}) was loaded with 10 mg/ml of each of the extracts dissolved in methanol. The plate was developed in BAW (4:1:5, butanol:acetic acid:water) and left to dry for 2 h at room temperature under a stream of air. The TLC plate was observed under UV light (254 and 366 nm) after its development. The test culture was sprayed on plate and the plates were incubated overnight at 37 ^{0}C with 100% humidity. The next day plates were sprayed with 2 mg/ml iodonitrotetrazolium chloride (INT) (Sigma-Aldrich) and incubated for 1 h. Clear zones on the plate indicates area of growth inhibition.

Free radical scavenging assays

The free radical scavenging activities of the crude methanol extracts were assessed by measurement of the scavenging ability on 1,1-diphenyl-2-picrylhydrazyl (DPPH). DPPH was prepared at a concentration of 80 µg/ml by dissolving 2 mg of DPPH in 25 ml of methanol. All the extracts were first tested in qualitative assays and the extracts which showed activity were further tested in quantitative assay. Quercetin, a well-known natural antioxidant, was taken as positive standard (20).

Qualitative assay

Free radical-scavenging activity was determined, using a rapid qualitative TLC plate method which is based on the reduction of a methanolic solution of the coloured free radical, 1,1-diphenyl- 2-picrylhydrazyl (DPPH). The crude methanolic extracts were loaded on aluminium backed pre-coated silica gel TLC plate (Merck, silica gel 60 F_{254}) and then plate was air dried at

room temperature. The plate was sprayed with 0.5% 1,1-diphenyl-2-picrylhydrazyl (DPPH) using an atomizer and system was left undisturbed for 30 min for development of plate.

Quantitative assay

DPPH radical scavenging assay

DPPH provides a convenient and accurate method for titrating the oxidizable groups of natural or synthetic antioxidants (21). The assay was carried out in 96 well microtitre plate. The stock solution of the extract and positive standard was prepared using methanol to achieve a concentration of 500 µg/ml. Dilutions were made to concentrations of 50, 5, 0.5, 0.05 and 0.005 µg/ml. 100µl of each diluted solutions were mixed with 100µl of DPPH (80 µg/ml) and allowed for 30 min of incubation in the dark at room temperature. The same procedure was followed for the quercetin which was taken as standard. The DPPH solution without sample solution was treated as control and 95% methanol was used as blank. The absorbance of the mixtures was measured spectrophotometrically at 517 nm. This activity was given as % DPPH radical-scavenging which was calculated by the following equation:

$$\text{Radical scavenging (\%)} = \left(\frac{\text{Absorbance of control - Absorbance of sample}}{\text{Absorbance of control}} \right) \times 100$$

Then % inhibitions were plotted against respective concentrations of methanol extracts used and from the graph IC_{50} was calculated. The IC_{50} of each extracts is defined as concentration sufficient to obtain 50% of a maximum scavenging activity. All the assays were carried out in triplicates.

TLC-DPPH free radical scavenging screening and chemical characterization

The methanol extracts were assayed for free radical scavenging activity by loading 10 µl of 10 mg/ml of each extracts onto aluminium backed TLC plate (Merck, silica gel 60 F_{254}). The TLC plate was kept in an air sealed chamber in BAW solvent (4:1:5, butanol:acetic acid:water). The system was run till the solvent reached the top and then the plates were air dried and was sprayed with 0.5% 1,1-diphenyl-2-picrylhydrazyl (DPPH) in absolute methanol to visualize the antioxidant compounds that may be present in the extracts (22). To check the nature of these antioxidants, ninhydrin assay were performed. For characterization of free radical scavenging compound, the crude extracts were prepared and spotted on the TLC plate just 1 cm above the lower base. The plates were developed in BAW (4:1:5, butanol:acetic acid:water) and later sprayed with ninhydrin (0.5% w/v) solution in absolute ethanol to visualize the nature of free radical scavenging compounds. Finally the developed plate was air dried and observed under visible and UV light.

UV/Vis spectroscopy and HPLC

The desired concentrations (25 mg/ml) were made by dilution of stock solutions of crude extract in methanol and then it was filtered through 0.22µ syringe filter. Rectangular quartz cuvettes with an inner width of 1 cm were used for spectroscopic analysis of filtered extract. The absorbance

spectrum was analyzed on Perkin Elmer Lambda 2 Spectrophotometer at a wavelength of 190 nm to 900 nm. The final analysis of bioactive compounds was carried out using high performance liquid chromatography (RP-HPLC) column (Waters 600 controler, USA) equipped with a waters 2996 photodiode array (PDA) and waters 2475 multi-wavelength fluorescence detector. The column used was SunFire C18 reverse phase column (4.6 mm interior diameter × 150 mm long) with a particle size of 5 µm. HPLC of 0.22µ filtered sample (20 µl) was performed at room temperature with isocratic CH_3OH: H_2O (40:60) containing 0.1% (v/v) tri-fluoro acetic acid as a mobile phase over a 20 min period. The elution system was isocratic and the flow rate was 0.5 ml min^{-1}. A wavelength of detection was set at 220 nm. The HPLC analyses were performed in duplicates.

Results and Discussion

A new bacterium *Arthrobacter* sp. HKG 115 was isolated from the high altitude region of Leh, Himalaya region, India. The partial sequence of 16S ribosomal RNA gene of *Arthrobacter* sp. HKG115 has been assigned GenBank (NCBI) Accession number GU549432.

Antimicrobial activity

Methanol extracts showed excellent antibacterial activity against *Micrococcus flavus, Bacillus subtilis* and *Salmonella typhi*. The antibacterial activity of methanol extracts of *Arthrobacter sp.* against different test organisms are shown in Table 1. The colourless tetrazolium salt acts as an electron acceptor and is reduced to a red-coloured formazan product by biologically active organisms. Studies on the minimum inhibitory concentration of the extracts on the test organisms showed that the lowest MIC were demonstrated against *Micrococcus flavus,* and *Bacillus subtilis* and the highest MIC was exhibited against *Micrococcus luteus* (Table 1). The minimum inhibitory concentration for the antibacterial, extracted from the supernatant extract was much more than that of pellet extract of *Arthrobacter* sp. which indicates that the antimicrobial agents from supernatant extract (comparatively lower MIC value) was more active than from pellet extract (comparatively higher MIC value). The lowest concentration of compound showing no growth was taken as its minimal inhibitory concentration (MIC).

Bioautography

The next day the inhibition zones developed on plate were noted and the R_f values of the antibacterial agent present in both extracts were determined. The spot given by the pellet extract of *Arthrobacter* sp. was a circular with R_f value 0.16 and that of supernatant extract of *Arthrobacter* sp. was an extended spot with R_f value 0.18. In bioautography the pellet extract of *Arthrobacter* sp. gave an inhibition zone of 10 mm diameter and the supernatant extract of *Arthrobacter* sp. gave inhibition zone of size 16 mm. According to the TLC separation, the two extracts yielded components with similar R_f values as visible on bioautogram along with greenish yellow spots which had been detected under UV radiation indicates that the same compounds are responsible for antibacterial activity of those isolates.

Table 1: Inhibition zones (mm) and Minimum inhibitory concentration (MIC) against several pathogenic microbial test strains caused by methanolic extracts of *Arthrobacter* sp. exhibiting their antibacterial activity.

Microbial Test Strains	Diameter of zone of inhibition (mm)[a]				MIC (mg/ml)	
	Supernatant extract [b]	Pellet extract [c]	Distilled water [d]	Gentamicin [e]	Supernatant extract [f]	Pellet extract [g]
Dietzia sp. K44 (MTCC 7402)	10.0	9.0	ND	25.0	1.5	2.0
Micrococcus flavus (NCIM 2378)	18.0	13.0	ND	25.0	0.125	1.0
Bacillus subtilis (NCIM-2063)	20.0	8.0	ND	30.0	0.125	2.0
Escherichia coli (NCIM 2739)	11.0	9.0	ND	28.0	1.5	2.0
Pseudomonas aeroginosa (NCIM 2053)	8.0	8.0	ND	28.0	2.0	2.0
Staphylococcus aureus	8.0	7.0	ND	28.0	2.0	2.5
Bacillus cereus	9.0	7.0	ND	32.0	2.5	2.5
Salmonella typhi	13.0	10.0	ND	18.0	1.0	1.5
Micrococcus luteus	7.0	7.0	ND	28.0	3.0	3.5

[a]Diameter of zone of inhibition (mm) including disk diameter of 6 mm., [b]Methanol extract from supernatant (100 µg /disk), [c]Methanol extract from Pellet (100 µg/disk), [d]Distilled water as a negative control, [e]Gentamicin (10 µg/disk) was used as positive reference standards antibiotic disk, [f]MIC value (mg/ml) of methanol extract from supernatant of *Arthrobacter* sp., [g]MIC value (mg/ml) of methanol extract from pellets of *Arthrobacter* sp. ND: No activity detected.

Qualitative and thin layer chromatography analysis of antioxidants

The colour changes and pale yellow spots on purple background were observed due to the bleaching of DPPH (Fig. 1). Only zones where the colour turned from purple to yellow within the first 30 min (after spraying DPPH) were taken as positive results. After visual comparison with the intensity of bleached colour of the TLC spots of positive standard it was found that the free radical scavenging strength of methanol extracts from supernatant was more active than from pellet extract.

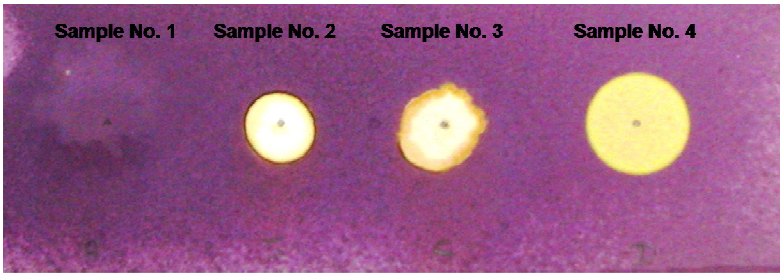

Fig. 1: Qualitative free radical scavenging assay on TLC sheet showing the presence of anti-oxidant activity in methanolic extracts of *Arthrobacter sp*. HKG 115. Sample 1: Methanol (Negative control), Samples 2: Methanol extract (25 mg/ml) from pellet, Sample 3: Methanol extract (25 mg/ml) from supernatant using diaion HP-20. Sample 4: Quercetin was taken as positive standard (1 mg/ml).

Further identification of active spots responsible for free radical scavenging activity in both extracts, TLC-DPPH free radical scavenging screening assay were performed (Fig 2 A). The spot given by the pellet extract of *Arthrobacter* sp. was with R_f value 0.45 and that of supernatant extract was with R_f value 0.46. The reference antioxidant, Quercetin was with R_f value 0.67. According to the TLC separation, the two extracts yielded components with similar R_f values indicates the presence of same bioactive compound responsible for free radical scavenging activity in both pellet as well as in supernatant extract. In addition, these free radical scavenging zones on TLC membrane were associated with a brown spot with similar R_f value (Fig 2 B) which indicates the presence of free amino acids and amines in identified antioxidant compound.

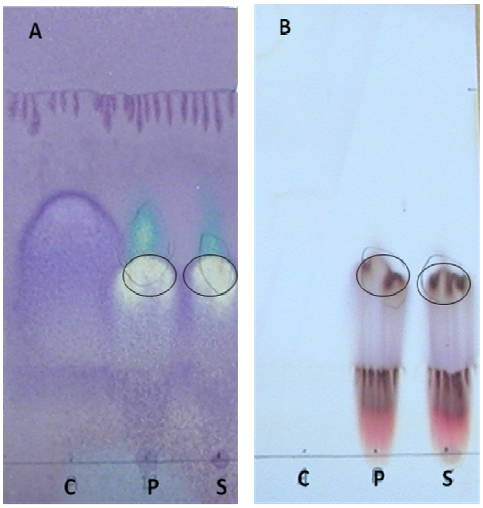

Fig. 2: Chromatogram of methanol extracts from *Arthrobacter* sp. developed in BAW, **(A):** The chromatogram was visualized by spraying with 0.5% 1,1-diphenyl-2-picrylhydrazyl (DPPH) in absolute methanol. Yellow zones (R_f = 0.45) indicates the compounds with free radical scavenging activity, **(B):** The chromatogram was visualized by spraying with 0.5% ninhydrin solution in ethanol. A brown spot with equal R_f value (R_f = 0.45) indicates the presence of free amino acids and amines in methanolic extracts responsible for free radical scavenging activity, [(C): methanol as negative control. (P): methanol extract from pellet (10 mg/ml) of *Arthrobacter* sp., (S): methanol extract from supernatant (10 mg/ml) of *Arthrobacter* sp.]

Quantitative analysis of antioxidants

The DPPH free radical scavenging capacity of the methanol extracts exhibited in a concentration-dependent manner (Fig. 3). The addition of an antioxidant results on a decrease of absorbance proportional to the concentration and free radical scavenging activity of the compound and it indicates an increase of the DPPH radical-scavenging activity (23). However, the rate of scavenging capacity was variable for methanol supernatant and pellet extract. The IC_{50} (the 50% inhibition) concentration of each extracts were calculated after observing the data. In the present experiment, the IC_{50} of the compounds were observed to be 9.5 and 29 µg/ml for quercetin and methanolic extract from supernatant respectively. On the basis of the calculated IC_{50} concentration and graphical analysis, DPPH free radical scavenging capacities of methanol extract from supernatant were found stronger than the pellet extract.

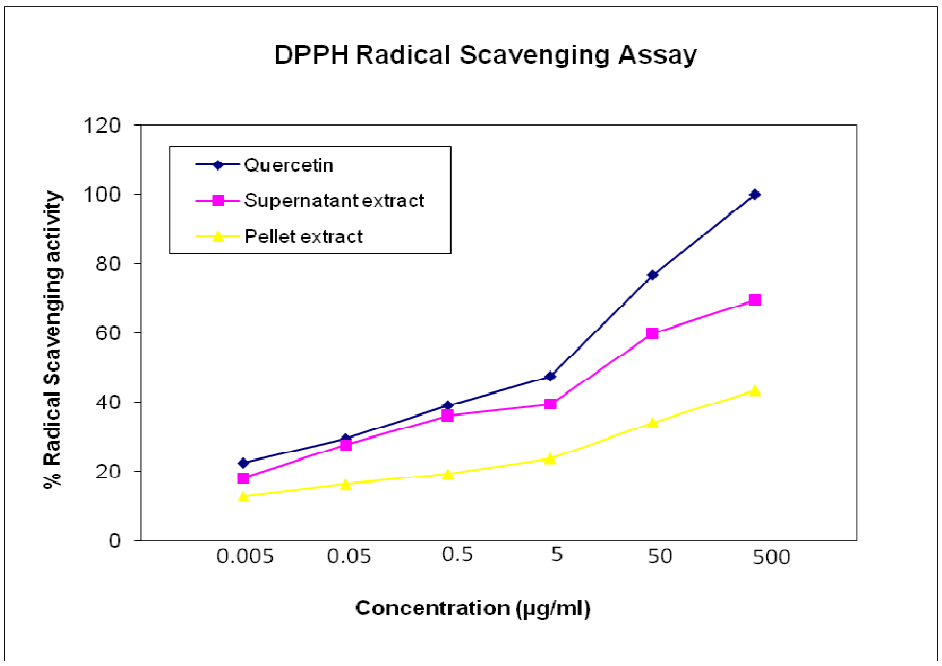

Fig. 3: Comparative % inhibition of DPPH showed by standard antioxidant (Quercetin), methanol extract of supernatant using diaion HP-20 and methanol extract of pellet from *Arthrobacter* sp. HKG 115. Each value is mean of three replicates.

UV/Vis spectroscopic and HPLC data analysis

The UV-Vis spectrum of filtered crude methanol extracts (Fig. 4) shows the presence of some bioactive molecules which can absorb the wavelength corresponding to 200- 350 nm at which major peaks were obtained**.** Since quartz is transparent in the UV region, the peaks are believed to be due to the compounds present in crude methanol extract. In bioautography assay it was clearly mentioned that the compound responsible for antimicrobial property was UV active compound and the fluorescence colour of active spots were greenish yellow. Although the UV-spectra is one of the basic evidences to identify any antimicrobial or free radical scavenging compounds, similarities in the UV spectra might explain that compounds produced by strains have similar structure. Therefore, further investigation is encouraged on the HPLC profile of the methanolic extracts of *Arthrobacter* sp. For HPLC chromatogram analysis, 20 µl of the clear and filtered methanol extract was injected into HPLC and their respective profiling is shown in Fig. 5. Three chromatographically distinct peaks were appeared in high performance liquid chromatography column. The active fractions were observed at 3.317 min, 4.132 min and 11.504 min indicating presence of three compounds in methanolic extract of supernatant. By comparing UV-spectra and HPLC chromatogram of known antibiotics and antioxidant compounds to that of active compounds in pellet and supernatant extract might explain the ability of the *Arthrobacter* sp. to produce those compounds.

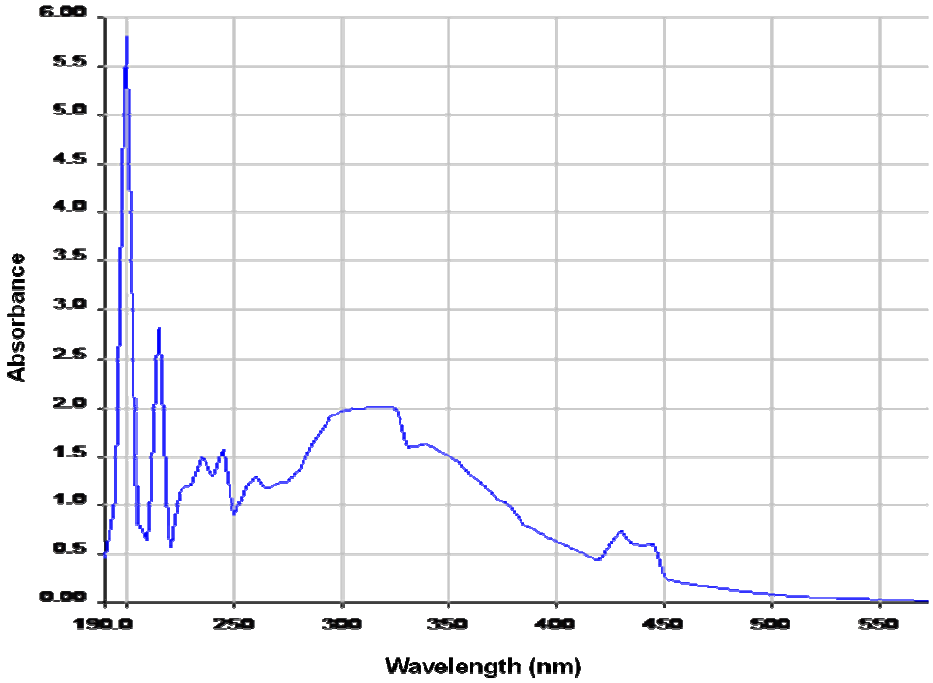

Fig. 4: UV spectra for the crude methanol extract from supernatant in which major peaks corresponding to 200- 350 nm**.**

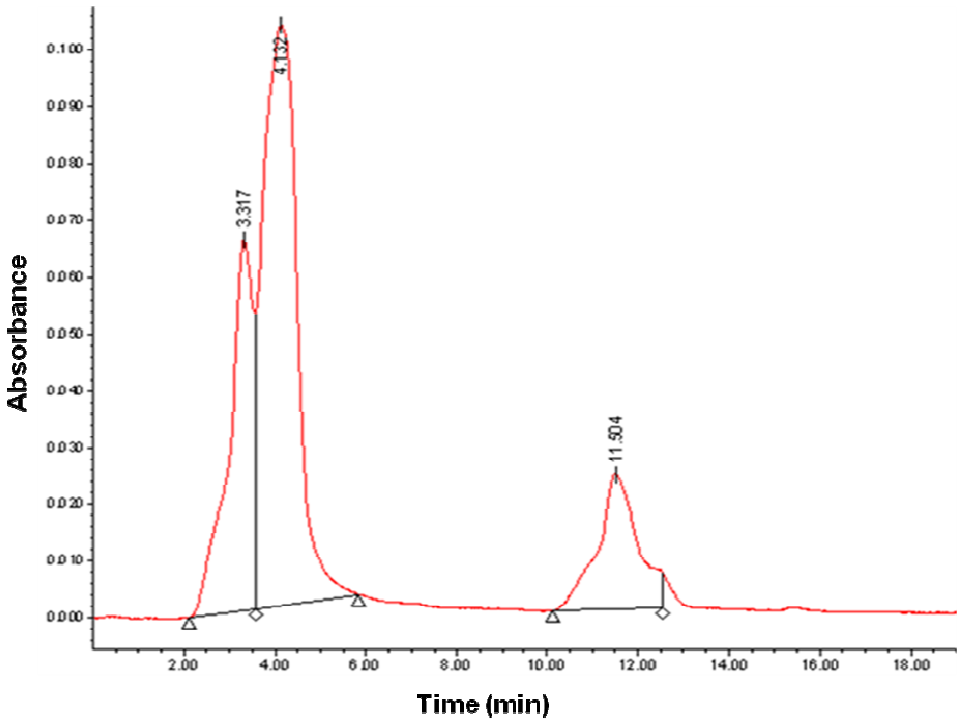

Fig. 5: RP-HPLC chromatogram of crude methanol extract from supernatant of *Arthrobacter sp.* HKG 115.

In this paper we have reported the antibacterial and free radical scavenging property in psychrophilic *Arthrobacter* sp. HKG-115 isolated from the high altitude region of Leh, Himalaya region, India. Methanol extract from supernatant of this bacterial strain showed excellent antibacterial activity against some bacterial pathogens, so it could be a possible source to obtain new and effective natural drugs to treat infections caused by multi-drug resistant strains of some microorganisms from community. In these results the antibacterial and free radical scavenging property of crude extract was less effective than Gentamicin and Quercetin, respectively. The reason behind this may be the presence of impurities in the crude extract whereas Gentamicin and Quercetin used was in pure form. This report may serve as a footstep regarding the biological and pharmacological activities of *Arthrobacter sp.* HKG 115 and other microorganisms from high altitude area.

Acknowledgements

The authors are sincerely grateful to the Director, Institute of Genomics and Integrative Biology, Delhi, India, for providing the infrastructural facilities and financial assistance (OLP-6101) to carry out this work. R. Pathak is thankful to University Grants Commission, India, for providing Junior Research Fellowship.

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