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Original Research Article

Antimicrobial Potential of Soil Isolates from Uttarakhand Region against Pathogens and Drug Resistant Strains

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ABSTRACT

Soil is the major repository of microbes that produce antibiotics. The soil is dominantly an ecosphere of different microbes comprising pathogenic bacterial and fungal strains along with beneficial and antibiotic producing microbes. The antibiotic producers are of great concern to the biotech and pharmaceutical industry. The antibiotics are of major utility as these are the molecules secreted by one microbe to invade the function of another microbe. The antibiotics are also broadly categorized under the category known as "defensins". The antibiotics secreted by the positive antibiotic producers are able to inhibit the growth of pathogenic microbes. The present study is about the investigation of twelve isolates from different soil samples i.e only one isolate was obtained from Garden soil (Sample A), seven isolates were obtained from Near the Banks of Ganga River (Sample B) and four isolates were obtained from soil sample from local gardens (Sample C) of Uttarakhand. The isolates were named as R/D on the basis of site of location (say Rishikesh and Dehradun) to avoid ambiguity on the culture plates and slants. The isolates were maintained on Nutrient agar plates and slants. The isolates were further screened for gram staining and different biochemical tests. The results showed that amongst twelve isolates obtained, 6 were gram positive bacilli, 3 were gram negative bacilli and 3 were gram positive cocci. The antimicrobial activity of the supernatant/ethyl acetate fraction and pellet fractions of bacteria isolates was determined by well diffusion method against the selected bacterial and fungal pathogens. The pellets of the isolates was rinsed in N-saline and later evaluated for antimicrobial effect while the supernatant/was taken as such for evaluation of antimicrobial activity. The results showed that, supernatant/ethyl acetate fraction and pellet fraction of the isolates possessed antimicrobial activity. It was found that *E. coli* NCIM 2065, *Lactobacillus plantarum* NCIM 2083, *Micrococcus luteus* ATCC 9341, *Salmonella abony* NCIM 2257 and MRSA 8 (isolated from pus) were the most resistant pathogens which showed no activity against supernatant/ethyl acetate fraction of any of the isolates of soil samples collected from Rishikesh and Dehradun regions of Uttarakhand. Only B2 supernatant/ethyl acetate fraction showed significant antibacterial activity against MRSA 35 (isolated from blood). Fractions, B1-B7 and C1-C4 showed antifungal activity against *Candida albicans* NCIM 3471 and *Aspergillus niger* NCIM 1196. The results indicate that *E. coli* NCIM 2065, *Salmonella abony* NCIM 2257, MRSA 8 (isolated from pus) and MRSA 35 (isolated from blood) were the most resistant pathogens which showed no activity against pellet fraction of any of the isolates. The pellet fractions B2, C2 and B6 showed antibacterial activity against *Micrococcus luteus* ATCC 9341. The pellet fractions, A1, B5, B6, C1 and C6 showed antibacterial activity against *Lactobacillus plantarum* NCIM 2083. The pellet fractions, A1, B2 and B3 showed antifungal activity against *Aspergillus niger* NCIM 1196 while *Candida albicans* NCIM 3471 was found to be sensitive against B1, B2, B5 and B6 pellet fractions. The supernatant/ethyl acetate fractions of the potent isolates, B2, B5 and C2 were analyzed by thin TLC plate using chloroform and methanol (60:40) as solvent system and erythromycin as control/standard antibiotic. Each extract produced spots when the chromatogram was visualized under iodine vapor. The spot was near the solvent front with Rf value of 0.54 for B2, 0.56 for B5 and 0.57 for C2. Standard antibiotic, Erythromycin however formed a tailed spot with Rf of about 0.5.

Keywords: Soil isolates, antibiotic producers, antibiotic, pathogens and drug resistant strains, Uttarakhand region (Rishikesh and Dehradun).

INTRODUCTION

Soil organisms contribute a wide range of essential services to the sustainable functioning of all ecosystems by acting as the primary driving agents of nutrient cycling; regulating the dynamics of soil organic matter, soil carbon sequestration and greenhouse gas emission; modifying soil physical structure and water regime; enhancing the amount and efficiency of nutrient acquisition by vegetation; and enhancing plant health. [1] These services are not only essential to the functioning of natural ecosystems but also constitute an important resource for the sustainable management of agricultural systems. The bacterial genera *Bacillus* and *Streptomyces* along with the fungal genera *Penicillium* and *Cephalosporium* are commonly found in soil. [2-5] The genus *Streptomyces* are the most prolific antibiotic producers and, although bacteria are a unique subgroup of bacteria called the Actinomycetes. [6] Although soil has historically been used to find new antibiotic producers, at present many of the 'old' antibiotics are now being manipulated in the lab and chemically modified to form new versions of older antibiotics. The actinomycetes produce about 70% of these, and the remaining 30% are products of filamentous fungi and non-actinomycete bacteria. Of the plethora of known bioactive compounds, approximately 160 are currently used in clinical practice. *Streptomyces* species produce about 55% of these. Most of the widely used antibacterial drugs have specific target sites in the physiological processes of microbial cells, which include (1) inhibition of cell wall synthesis, (2) inhibition of protein and nucleic acid synthesis, (3) inhibition of enzyme activity. [7] Since the introduction of antibiotics into clinical use in the mid 1940s, microorganisms have shown a remarkable ability to protect themselves by developing antibiotic resistance through different mechanisms. The major genetic mechanism for antibiotic resistance is mainly through mutation or acquisition of new gene(s) through genetic exchange mechanisms, like conjugation, transduction, and transformation. Both chromosomal and plasmid-encoded genes are important in the development and dissemination of antibiotic resistance genes. Various antibiotic-resistance mechanisms are known in bacteria but the major mechanisms include destruction or modification of antibiotics (e.g. production of α-lactamases and amino-glycosides modifying enzymes), prevention of access to the target (e.g. alteration of permeability), and alteration of the target site. [8-10]

METHODOLOGY

The chemicals and reagents used were of Analytical Grade and were procured from Ranchem and CDH, India. The media used during the study was procured from Hi-Media Ltd. Mumbai, India.

2.1 Collection of soil samples

The soil samples were collected from different sites viz. Garden soil (Sample A), Near the Banks of Ganga River (Sample B) from Rishikesh region and soil sample from local gardens (Sample C) of Uttarakhand. Soil samples were collected using sterile screw-capped glass bottles and stored at 4ºC until analysis in the laboratory. [11]

2.2 Isolation of bacterial isolates

The soil samples collected were serially diluted with N-saline. The maximally diluted samples were streaked on Nutrient Agar medium plates. The plates were further stored at 37^0C for 18-24 h in BOD incubator for the growth of the isolate. [12] The total viable bacterial counts (TVBCs) at 37ºC were determined using the standard plate count. Each of the single colonies obtained were further collected and inoculated separately within Nutrient slants and Nutrient agar petriplates and Nutrient broth. The pure cultures were labeled as "R"

(for Rishikesh isolates) and "D" (for Dehradun isolates). These pure cultures were further used for characterization and further studies.

2.3 Identification of bacterial isolates

The isolates were further screened for gram staining [13] and biochemical tests viz. starch hydrolysis, casein hydrolysis, MRVP test, Urease test, Indole test, H2S production and Citrate test were performed for confirmation of the nature of bacterial isolates. Pure isolates were maintained at -70^0 C in LB broth supplemented with 15% (w/v) glycerol.

2.4 Determination of *in vitro* antimicrobial activity of bacterial isolates against pathogenic strains and Methicillin resistant *Staphylococcus aureus* (MRSA)

The pure bacterial isolates obtained were inoculated in 250 ml Erlenmeyer flasks containing Nutrient broth. The flasks were incubated on a rotary shaker (200 rpm) at 30°C for two to three days. After incubation the culture was extracted by using equal volume of ethyl acetate. Ethyl acetate was evaporated and the residue was dissolved in 1:1 methanol: DMSO solvent. Further each of the culture was centrifuged to obtain the pellets. [14]

2.4.1 Culture Media

The media used for determination of antibacterial activity and growth of pathogenic bacterial cultures was Nutrient agar/broth while Sabouraud's dextrose agar/broth was used for determination of antifungal activity and growth of pathogenic fungal cultures.

2.4.2 Inoculum

The pathogenic bacteria cultures were inoculated separately into nutrient broth and incubated at 37 ^{0}C for 4 h. The suspension was checked to provide approximately 10^5 CFU/ml. Similar procedure was done for fungal strains by inoculating in Sabouraud's dextrose broth for 6 h.

2.4.3 Microorganisms used

Pure cultures of various pathogenic bacterial and fungal cultures viz. *E. coli* NCIM 2065, *Lactobacillus plantarum* NCIM 2083, *Micrococcus luteus* ATCC 9341, *Salmonella abony* NCIM 2257, MRSA 8 (isolated from pus) and MRSA 35 (isolated from blood), pure fungal cultures viz. *Candida albicans* NCIM 3471 and *Aspergillus niger* NCIM 1196 were used for the study. The bacterial and fungal pathogens were procured from Roorkee Research & Analytical Labs Pvt. Ltd., Roorkee (Uttarakhand) and MRSA cultures were procured from Shooloni University, Solan (H.P), India.

2.4.4 Agar well diffusion method

The agar well diffusion method [15] was modified. Nutrient Agar medium (NAM) was used for bacterial cultures. The culture medium was inoculated with the bacterial strains separately suspended in nutrient broth. Sabouraud's dextrose agar (SDA) was used for fungal cultures. The culture medium was inoculated with the fungal strains separately suspended in Sabouraud's dextrose broth. A total of 8 mm diameter wells were punched into the agar and filled with bacterial supernatant, bacterial pellet (dissolved in N-saline), solvent blank (N-saline) and positive control (Erythromycin, 1 mg/ml) separately in the bacterial cultured plates separately. The bacterial plates were then incubated at 37 ^{o}C for 18 h. The same procedure was used for determining antifungal activity but in this case standard antibiotic (Fluconazole, concentration 1 mg/ml) was used as positive control. Further fungal culture plates were incubated at 37°C for 72 h. The antibacterial and antifungal activity was evaluated by measuring the diameter of zone of inhibition observed.

2.5 Separation of antimicrobial substance by TLC

The concentrated ethyl acetate fractions from bacterial isolates were separated by commercially available silica gel coated chromatography sheets (50×20 cm size MERC). [16] The ability of different mobile phases on the separation of ethyl acetate fraction on TLC sheets was determined and the Chloroform: methanol 60:40 was found to be best for separation.

The crude extracts of antimicrobial agent from the isolate were spotted on the preparative TLC plate as a single line or streak and the chromatogram was performed with the solvent system Chloroform: methanol (60:40). After drying the sheet was kept in closed iodine chamber to visualize the separated compound as clear spots. The spots were scrapped, mixed with ethyl acetate and centrifuged at 3000 rpm for 15 minutes. The supernatant was collected in a sterile vial and kept for evaporation. The partially purified bio-active compound obtained was pooled out from TLC plate for further analysis of the antimicrobial compound.

RESULTS & DISCUSSION

In the present study, the soil samples were collected from Rishikesh and Dehradun region of Uttarakhand. The study was localized to isolate and screen the antibiotic producers from soil.

3.1 Isolation and screening of antimicrobial isolates

Total of twelve isolates were obtained from different soil samples i.e only one isolate was obtained from Garden soil (Sample A), seven isolates were obtained from Near the Banks of Ganga River (Sample B) and four isolates were obtained from soil sample from local gardens (Sample C) of Uttarakhand. The isolates were named as R/D on the basis of site of location (say Rishikesh and Dehradun) to avoid ambiguity on the culture plates and slants. The isolates were maintained on Nutrient agar plates and slants. The isolates were further screened for gram staining and different biochemical tests. The results showed that amongst twelve isolates obtained, 6 were gram positive bacilli, 3 were gram negative bacilli and 3 were gram positive cocci. The results of gram staining and biochemical tests are shown in Table 1. Positive biochemical tests shown by isolates are recorded in Figure 1.

3.2 Antimicrobial activity of bacteria isolates

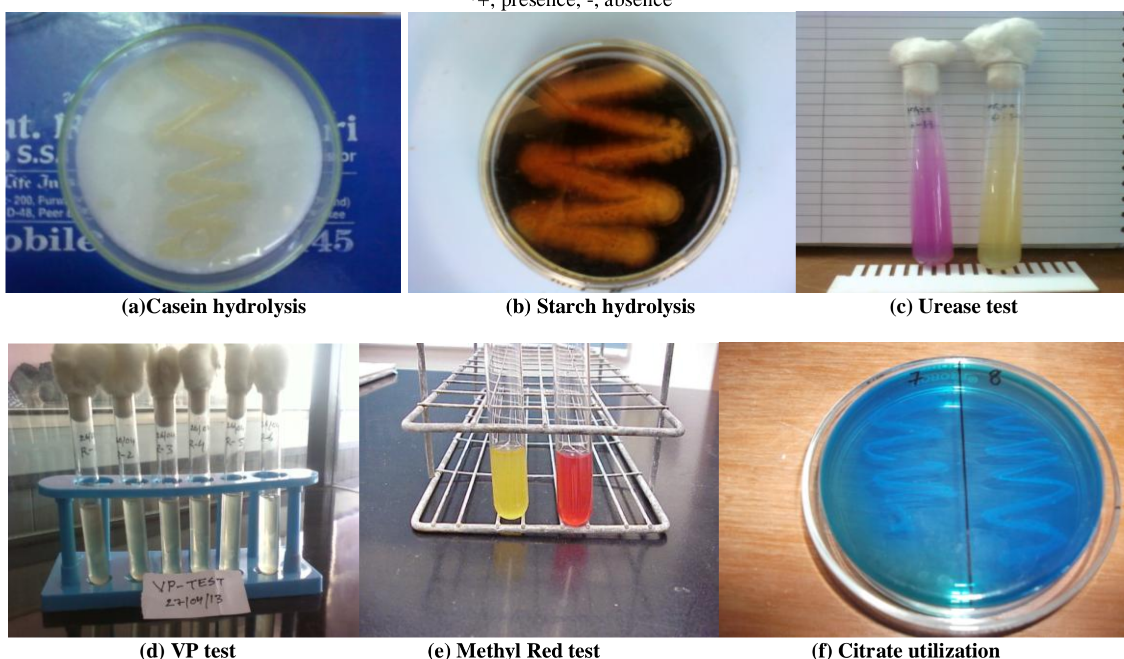
The antimicrobial activity of the supernatant/ethyl acetate fraction and pellet fractions of bacteria isolates was determined by well diffusion method against the selected bacterial and fungal pathogens. The two fractions were assessed for antimicrobial activities in order to evaluate the presence of antimicrobial substance in supernatant or in pellet in order to confirm whether it is membrane bound or non-membrane bound. The results thus confirms about the nature of antimicrobial substance. The pellets of the isolates was rinsed in N-saline and later evaluated for antimicrobial effect while the supernatant/was taken as such for evaluation of antimicrobial activity. It was found that supernatant/ethyl acetate fraction and pellet fraction of the isolates possessed antimicrobial activity. The study thus states that some isolates produce membrane bound antimicrobial substance while some isolates produce non membrane bound antimicrobial substance. It was found that *E. coli* NCIM 2065, *Lactobacillus plantarum* NCIM 2083, *Micrococcus luteus* ATCC 9341, *Salmonella abony* NCIM 2257 and MRSA 8 (isolated from pus) were the most resistant pathogens which showed no activity against supernatant/ethyl acetate fraction of any of the isolates of soil samples collected from Rishikesh and Dehradun regions of Uttarakhand. Only B2 supernatant/ethyl acetate fraction showed significant antibacterial activity against MRSA 35 (isolated from blood). Fractions, B1-B7 and C1-C4 showed antifungal activity against *Candida albicans* NCIM 3471 and *Aspergillus niger* NCIM 1196. The results are shown in Table 2 and Figure 2 (a & b). The results indicate that *E. coli* NCIM 2065, *Salmonella abony* NCIM 2257, Methicillin resistant S. aureus, MRSA 8 (isolated from pus) and MRSA 35 (isolated from blood) were the most resistant pathogens which showed no activity against pellet fraction of any of the isolates. The pellet fractions B2, C2 and B6 showed antibacterial activity against *Micrococcus luteus* ATCC 9341. The pellet fractions, A1, B5, B6, C1 and C6 showed

antibacterial activity against *Lactobacillus plantarum* NCIM 2083. The pellet fractions, A1, B2 and B3 showed antifungal activity against *Aspergillus niger* NCIM 1196 while *Candida albicans* NCIM 3471 was found to be sensitive against B1, B2, B5 and B6 pellet fractions. The results are shown in Table 3 and Figure 3 (a & b).

Table 1 (a): Biochemical Tests for confirmation of bacterial isolates recorded from soil samples of different sites of Rishikesh and Dehradun

Isolate	Gram staining	Starch hydrolysis	Citrate utilization	Indole test	H2S production	Methyl red test	VP test	Casein hydrolysis	Urease test
A1	+Cocci	+	+	_	_	+	_	+	+
B1	+Rod	+	+	_	_	+	_	_	+
B2	+Rod	_	+	_	_	+	_	_	+
B3	+Rod	_	+	_	_	+	_	_	+
B4	_Rod	_	+	_	_	+	_	_	+
B5	_Rod	_	+	_	_	+	_	_	+
B6	_Rod	_	+	_	_	+	_	_	+
B7	+Cocci	+	+	_	_	+	_	+	+
C1	+Cocci	_	+	_	_	+	_	_	+
C2	+Rod	_	+	_	_	+	_	_	+
C3	+Rod	_	+	_	_	+	_	_	+
C4	+Rod	_	+	_	_	+	_	_	+

*+, presence; -, absence



(a)Casein hydrolysis (b) Starch hydrolysis (c) Urease test

(d) VP test (e) Methyl Red test (f) Citrate utilization

Figure 1: Positive Biochemical Tests as recorded by bacterial isolates from soil samples

Table 2: Antimicrobial activities of supernatant/ethyl acetate fractions of isolates obtained from soil

Isolate supernatant	Diameter of zone of inhibition (mm)							
	Pathogens studied							
	E.coli	S. abony	M. luteus	L. plantarum	MRSA 35	MRSA 8	niger	C.albicans
A1	NA	NA	NA	NA	NA	NA	05	03
B1	NA	NA	NA	NA	NA	NA	04	02
B2	NA	NA	NA	NA	NA	NA	03	03
B3	NA	NA	NA	NA	NA	NA	02	04
B4	NA	NA	NA	NA	NA	NA	03	03
B5	NA	NA	NA	NA	NA	NA	04	07
B6	NA	NA	NA	NA	NA	NA	03	NA
B7	NA	NA	NA	NA	23	NA	02	NA
C1	NA	NA	NA	NA	NA	NA	05	NA
C2	NA	NA	NA	NA	NA	NA	07	NA
C3	NA	NA	NA	NA	26	NA	04	NA
C4	NA	NA	NA	NA	NA	NA	06	NA
Erythromycin (1 mg/ml)	18	12	15	15	10	12	NT	NT
Fucanazole (1 mg/ml)	NT	NT	NT	NT	NT	NT	12	15

*NA, No Activity; NT, Not Tested

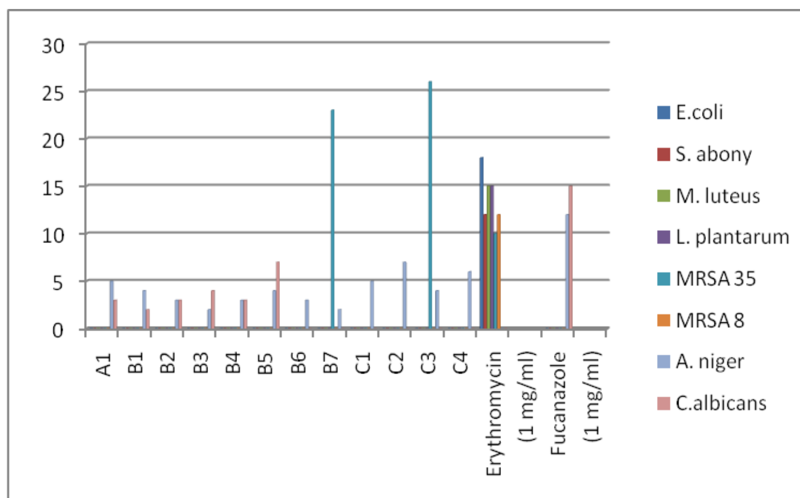


Figure 2 (a): Graphical representation of antimicrobial activity of supernatant/ethyl acetate fraction obtained from soil isolates against pathogenic strains

Figure 2 (b): Antimicrobial activity of supernatant/ethyl acetate fraction obtained from soil isolates against pathogenic strains

3.3 TLC profile of potent antibiotic fraction

The supernatant/ethyl acetate fractions of the potent isolates, B2, B5 and C2 were analyzed by thin TLC plate using chloroform and methanol (60:40) as solvent system and erythromycin as control/standard antibiotic. Each extract produced spots when the chromatogram was visualized under iodine vapor. The spot was near the solvent front with Rf value of 0.54 for B2, 0.56 for B5 and 0.57 for C2. Standard antibiotic, Erythromycin however formed a tailed spot with Rf of about 0.5. The results are shown in Table 4 and Figure 4.

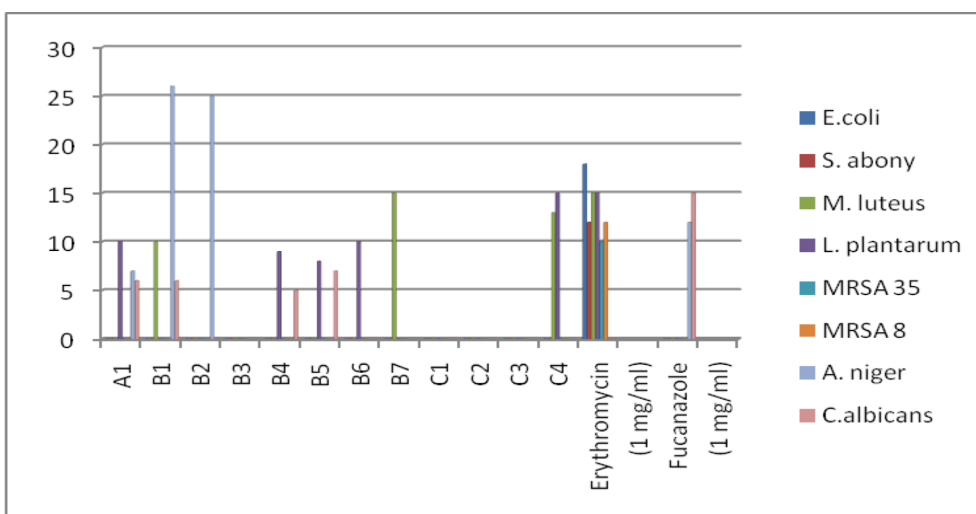


Figure 3 (a): Graphical representation of antimicrobial activity of pellet fraction of isolates obtained from soil

Figure 3 (b): Antimicrobial activity of pellet fraction of isolates obtained from soil

Table 3: Antimicrobial activities of pellet fractions of isolates obtained from soil

Isolate supernatant	Diameter of zone of inhibition (mm)							
	Pathogens studied							
	E. coli	S. abony	M. luteus	L. plantarum	MRSA 35	MRSA 8	niger	C. albicans
A1	NA	NA	NA	10	NA	NA	07	06
B1	NA	NA	10	NA	NA	NA	26	06
B2	NA	NA	NA	NA	NA	NA	25	NA
B3	NA	NA	NA	NA	NA	NA	NA	NA
B4	NA	NA	NA	09	NA	NA	NA	05
B5	NA	NA	NA	08	NA	NA	NA	07
B6	NA	NA	NA	10	NA	NA	NA	NA
B7	NA	NA	15	NA	NA	NA	NA	NA
C1	NA	NA	NA	NA	NA	NA	NA	NA
C2	NA	NA	NA	NA	NA	NA	NA	NA
C3	NA	NA	NA	NA	NA	NA	NA	NA
C4	NA	NA	13	15	NA	NA	NA	NA
Erythromycin (1 mg/ml)	18	12	15	15	10	12	NT	NT
Flucanazole (1 mg/ml)	NT	NT	NT	NT	NT	NT	12	15

*NA, No Activity; NT, Not Tested

Table 4: TLC profile of potent antibiotic fraction

S.No.	Ethyl acetate/supernatant fraction	Rf values
1.	B2	0.54
2.	B5	0.56
3.	C2	0.57
4.	Erythromycin	0.5

Figure 4: TLC profile of potent antibiotic fraction

The results showed that antimicrobial substance produced by some isolates was membrane bound while some of the isolates produce non membranous bound antimicrobial substance. It was found that *E. coli* NCIM 2065, *Lactobacillus plantarum* NCIM 2083, *Micrococcus luteus* ATCC 9341, *Salmonella abony* NCIM 2257 and MRSA 8 (isolated from pus) were the most resistant pathogens which showed no activity against supernatant/ethyl acetate fraction of any of the isolates of soil samples collected from Rishikesh and Dehradun regions of Uttarakhand. Only B2 supernatant/ethyl acetate fraction showed significant antibacterial activity against MRSA 35 (isolated from blood). Fractions, B1-B7 and C1-C4 showed antifungal activity against *Candida albicans* NCIM 3471 and *Aspergillus niger* NCIM 1196. The results indicate that *E. coli* NCIM 2065, *Salmonella abony* NCIM 2257, MRSA 8 (isolated from pus) and MRSA 35 (isolated from blood) were the most resistant pathogens which showed no activity against pellet fraction of any of the isolates. The pellet fractions B2, C2 and B6 showed antibacterial activity against *Micrococcus luteus* ATCC 9341. The pellet fractions, A1, B5, B6, C1 and C6 showed antibacterial activity against *Lactobacillus plantarum* NCIM 2083. The pellet fractions, A1, B2 and B3 showed antifungal activity against *Aspergillus niger* NCIM 1196 while *Candida albicans* NCIM 3471 was found to be sensitive against B1, B2, B5 and B6 pellet fractions. The results correlate the previous findings reported by large number of researchers showing the screening of antimicrobial isolates from soil samples. [17-21]

CONCLUSION

The results of this study strongly support that the metabolite may be used in the management of microbial infection and the present finding highlights the importance for further investigation towards the goal of obtaining novel antimicrobial agent. The recovery of strains with

antimicrobial activity suggests that water represents an ecological niche which harbours a largely uncharacterized microbial diversity and yet unexploited potential in the search for new secondary metabolites. The present study thus can be utilized further to explore the microbial biodiversity in sulphur water springs which are able to produce antibiotics that can be utilized in cure of skin infections etc. Further analysis of compounds by spectrometric, chromatographic techniques and strain improvement studies provides detailed information about the compounds produced by the isolates and the ability of the strains in producing newer compounds.

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Conflict Of Interest

The author declares no conflict of interest.

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