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Exploitation of bacteria from forest ecosystem for antimicrobial compounds

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ABSTRACT

This study deals with bacterial prospecting from forest soil with special reference to antimicrobial substances. Total of 25 morphologically different bacterial colonies were isolated from soil samples collected from Anaimalai forest and Parambikulam tiger reserve forest, Western Ghats, India. About 12 (48%) out of 25 isolates showed antibacterial activity in which strain AF1 showed inhibitory activity against more number of test pathogens. Bioactive substance from strain AF1 was produced by adopting submerged fermentation and extracted using ethyl acetate and chloroform. In disc diffusion method, ethyl acetate extract showed good antibacterial activity (9-17 mm zone of inhibition). Active fraction present in the ethyl acetate extract was determined by thin layer chromatography based bioautography. Findings of this work supported that the forest ecosystems investigated in this study will be potential place for bacterial bioprospecting.

Keywords: Western Ghats, bacteria, antimicrobial activity, bioautography.

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INTRODUCTION

The discovery of antibiotic penicillin by Fleming in 1929 from a fungus has drawn Scientists' interest on microorganisms' importance in biology, chemistry, and life science. Since then, different chemical groups of microbial secondary metabolites have been discovered as remarkable biological principles as well as chemical tools. Although, penicillin was originally known merely as an antimicrobial agent, the biological scope of these metabolites generally called antibiotics (Lami and Osada 1993). Over five thousand antibiotics have been identified from the culture of gram positive, gram negative and filamentous fungi but only hundred antibiotics have been commercially used to treat human, animal and plant disease. A major feature of industrial antibiotic production is directed to screening programmes for new potent antibiotic producing organism either from natural sources or from established cultures (Srividhya *et al.*, 2009). Screening of microbial natural products continues to represent as important route for the discovery of novel chemicals for the development of new therapeutic agents (Courtis *et al.*, 2003; Gillespie *et al.*, 2002). Rare microorganisms are of strategically importance in the discovery of new bioactive microbial secondary metabolites. It may also be true that a reduction in interest in natural products for use in drug development has happened as a result of people dealing with the traditional source of bioactive compounds, including plants of the temperate zones and microbes from a plethora of soil samples collected from different parts of the world (Strobel and Daisy, 2003).

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Therefore, it is worthwhile isolating microorganisms of a different ecosystem to find new compounds. Forest is characterized by a wealthy biological diversity, millions of plants, animals and microorganisms (Lami and Osada, 1993). Searching of less explored ecosystems like forest ecosystems leads to the isolation of novel microbes and lead compounds to fight against drug resistant pathogens (Onifade, 2007). There are numerous reports on diversity of fungi and bacteria in forest ecosystems at global level. But there is no encouraging report on anti-infective agents from microorganisms from forest ecosystems. The present study reports bacterial bioprospecting from an unexplored forest ecosystem with special reference to antimicrobial substances.

MATERIALS AND METHODS

Sample collection and isolation of bacteria

Soil samples were collected from Anaimalai and Parambikulam tiger reserve forest, Western Ghats, Tamilnadu using sterile polythene bags and transported to laboratory. About one gram of soil sample was serially diluted using sterile distilled water blanks. About 0.1 ml of aliquot from each dilution was inoculated into nutrient agar plates supplemented with filter sterilized nystatin (20µg/ml). Plating was done in triplicate and all the plates were incubated at 28°C for 3-5 days (Srividhya *et al.*, 2009). Morphologically different isolates were selected, sub cultured and maintained on nutrient agar slants and preserved at 4°C.

Preliminary screening for antibacterial activity

About 10% of bacterial inoculum prepared in nutrient broth was transferred into each 10 ml of fermentation medium and incubated in a rotary shaker at 28°C for 120 hours. After incubation the culture broth was centrifuged at 10,000 rpm for 10 minutes and the cell free supernatant was stored in sterile vials. Antimicrobial activity of cell free supernatant was tested by agar well diffusion method (Valgas *et al.*, 2007). Test bacterial strains used in this study include *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Salmonella typhi*. Bacterial growth with 0.5 McFarland standard was inoculated into nutrient agar plates using sterile cotton swab. About 5 mm size well was made and 100 µl of cell free supernatant was added into it. All the plates were observed for zone of inhibition after incubation at 37°C for 24 hours. Bacterial isolate which showed good inhibition against test bacterial pathogens was selected for further investigations.

Production of antimicrobial compounds from selected isolates

For the preparation of inoculum, the potential strain was inoculated on the nutrient broth and incubated at 28°C for 18 hours. About 10% of bacterial inoculum adjusted to 0.5 McFarland standard was transferred into each 100 ml of fermentation medium (Grams/ litre: L-Glutamic acid 5.0; KH₂PO₄ 0.5; K₂HPO₄ 0.5; MgSO₄.7H₂O 0.2; MnSO₄. H₂O 0.01; NaCl 0.01; FeSO₄.7H₂O 0.01; CuSO₄.7H₂O 0.01; CaCl₂.H₂O 0.015; Glucose 10 with pH 7 was incubated in rotary shaker for 120 hours at 28°C (Hasan *et al.*,

2009). After incubation, the culture broth was separated by centrifugation at 5000 rpm for 10 minutes. The bioactive compounds present in the cell free supernatant was extracted by adapting liquid-liquid extraction method using ethyl acetate and chloroform For 24 hour (Chellaram *et al.*, 2004). The solvent portion was collected and concentrated by evaporation.

Table. 1: Antimicrobial activity of crude compound by well diffusion method and disc diffusion method.

Test organism	Inhibition zone (mm in diameter)	
	Well diffusion method	Disc diffusion method
<i>Staphylococcus aureus</i>	10	16
<i>Bacillus spp.</i>	12	17
<i>Escherichia coli</i>	-	-
<i>Klebsiella spp.</i>	6	9
<i>Pseudomonas spp.</i>	9	14
<i>Salmonella typhi</i>	11	16

Antibacterial activity of crude extracts

Crude ethyl acetate and chloroform extract was tested for antibacterial activity by disc diffusion method. 0.25 gm of crude extract was impregnated into the sterile filter paper disc, dried and placed over nutrient agar plates inoculated with test bacterial pathogens. The diameter of the inhibition zone was measured after 24 hours of incubation at 37°C (Balagurunathan and Subramanian, 1994).

Purification of crude extract

The crude extract which showed good activity was subjected to purification by thin layer chromatography (TLC) (Selvameenal *et al.*, 2009) using commercially available silica gel coated chromatography sheets. To find out the best solvent system to separate the crude compound, the solvents such as methanol, chloroform, acetic acid, n-butanol, n-hexane and water were used in different proportions. The crude compound was dissolved in 200µl of ethyl acetate and the sample was spotted at the bottom of silica gel coated sheet with the help of capillary tube. Then the chromatogram was developed using different solvent systems. After running, the sheet was kept in room temperature for the complete drying of the plates. Then the sheet was kept in closed iodine chamber to visualize the separated compound as clear spots.

Table. 2: Antimicrobial activity of active spot obtained from TLC.

Test organism	Inhibition zone (mm in diameter)
<i>Staphylococcus aureus</i>	19
<i>Bacillus spp.</i>	20
<i>Escherichia coli</i>	-
<i>Klebsiella spp.</i>	12
<i>Pseudomonas spp.</i>	17
<i>Salmonella typhi</i>	20

Bio autography

The bioautography procedure described by Selvameenal *et al.*, (2009) was followed for the detection of active compound separated in TLC. Chromatogram developed as described above was placed in a sterile bioassay petridish and overlaid with 10 ml of molten nutrient agar seeded with 0.2ml of *S. aureus* and incubated at 37°C for 24 hours. Reference chromatogram was also prepared. The R_f value of the inhibition zones on test

chromatogram was compared with the Rf of reference chromatogram. The corresponding spots that showed antibacterial activity were collected and used for further studies.

MIC of partially purified compound

Preparative TLC was performed to separate the active compound from the crude extract. The active fraction separated in TLC was scrapped and its minimum inhibitory concentration was determined by adopting broth dilution method (Igbinosa *et al.*, 2009).

Characterization of potential strain:

Microscopic, cultural and biochemical characteristics of potential strain were studied by adopting standard procedure and the potential strain was identified with the help of Bergey's manual of Systematic Bacteriology.

RESULTS & DISCUSSION

The present investigation was attempted for bioprospecting of bacteria special reference to antibacterial compounds from Western Ghats, Tamilnadu. In the present study, totally 25 bacterial colonies were yielded from forest soil. Several studies have reported from forest ecosystem even from Western Ghats (Srividhya *et al.*, 2009). Maruthu Ramachandran *et al.*, (2007) isolated soil fungi from Kodaikanal hills and produced antimicrobial compounds.

Among 25 strains, 12 strains were selected for antimicrobial studies in that particularly strain AF1 showed prominent activity against all the tested bacterial pathogens which include both Gram positive and Gram negative bacteria. But AF1 does not exhibit any activity against *E. coli*. It was clearly stated that, the potential strain possessed broad spectrum of antibacterial activity against prokaryotic organisms.

Most of the Antibiotics produced from bacteria are mainly through the submerged fermentation process and the crude bioactive compounds produced from bacteria by submerged fermentation were extracted using solvents for the antimicrobial activity (Srividhya *et al.*, 2009). In this study also the AF1 strain produced good level of growth and bioactive compounds in submerged fermentation process. The crude bioactive compound produced by AF1 strain in fermentation broth was extracted using different solvents for antimicrobial activity. Of the various solvents with different polarity used for the extraction of pigments from bacteria, encouraging amount of pigment activity was showed by ethyl acetate, on medium polar solvent extract. In this study ethyl acetate was proved effective for the extraction of extra cellular pigments from AF1 strain.

The antimicrobial activity of potent strain was tested by disc diffusion method (Marudhu Ramachandran *et al.*, 2007). In this study, ethyl acetate extract of the potent strain AF1 was tested for antibacterial activity by disc diffusion method. Ethyl acetate extract of the strains showed activity against *S.aureus*, *S. typhi*, and *Bacillus spp.*, tested with inhibition zone range from 9-17mm. Chellaram *et al.*, 2004 extracted the crude compound from

fermentation medium by ethyl acetate which yielded a brownish yellow pigment. Selavameenal *et al.*, (2009) studied the separation of yellowish crude pigment compound by TLC by various solvents system. In this present study the crude compound greenish (AF1), was purified by n-butanol: acetic acid: water (70:30:10). This has selected as a best solvent where showed single spot with Rf value 1.66. Bioautography allows localizing antimicrobial activity of an extract on the chromatogram. This method avoids the need of previous purification of the substance, reducing the cost of the initial screening (Selvameenal *et al.*, 2009). In this present study activity of purified compound was detected in TLC sheet itself by agar overlay bioautography. In bioautography, the separated spot showed activity against the drug resistant pathogens tested. Based on the results of bioautography, the crude compound was purified and Minimum inhibitory concentration (MIC) of compound was tested. The MIC of the crude compound was found to be 8.5µg/ml *Bacillus species*. Megha *et al.*, 2007 isolated and identified 52 Pseudomonads from the soils of different forests in the Western Ghats possessed antimicrobial activity and multiple beneficial traits. In this present study, based on the micro morphological, physiological and biochemical characteristics the potential strain showing antimicrobial activity AF1 was identified as *Pseudomonas sp.*

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