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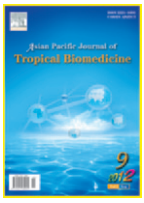

Document heading

Cytotoxic (A549) and antimicrobial effects of *Methylobacterium* sp. isolate (ERI-135) from Nilgiris forest soil, India

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ABSTRACT

Objective: To assess the antimicrobial and cytotoxic effects of *Methylobacterium* sp. isolated from soil sample of Doddabetta forest, Nilgiris, Western Ghats of Tamil Nadu. **Methods:** Isolation of *Methylobacterium* was performed from soils by serial dilution plate technique. The strain was grown in modified nutrient gulucose agar (MNGA) medium to study the morphology and biochemical characteristics. *Methylobacterium* sp. was screened for its antimicrobial activity against pathogenic bacteria and fungi. The strain was subjected to 16S rRNA analysis and was identified as *Methylobacterium* sp. The nucleotide sequence of the 16S rRNA gene of the isolate exhibited close similarity with other *Methylobacterium* sp. and has been submitted to Genbank. The antibacterial substances were extracted using chloroform and ethyl acetate from MNGA medium in which ERI-135 had grown for 5 d at 30 ℃. Cytotoxic effect was also studied. GC-MS analysis was carried out. The antimicrobial activity was assessed using broth micro dilution technique. **Results:** Ethyl acetate extract showed activity against bacteria such as *Bacillus subtilis, Klebsiella pneumoniae (K. pneumoniae), Pseudomonas aeruginosa, Salmonella typhimurium, Shigella flexneri, Enterobacter aerogenes, Staphylococcus aureu* and *Staphylococcus epidermidis (S. epidermidis)* and fungi such as, *Candida albicans* and *Trichophyton rubrum.* The lowest minimum inhibitory concentrations were: 250 μg/mL against *S. epidermidis* and 250 μg/mL against *K. pneumonia*. The isolate had the ability to produce enzymes such as protease. The exyract showed cytotoxic effect in human adenocarcinoma cancer cell line (A549). GC-MS analysis showed the presence of isovaleric acid (3.64%), 2-Methylbutanoic acid (5.03%), isobutyramide (5.05%), N,N-oimethylformamide-di-t-butylacetal (9.79%), benzeneacetamide (15.56%), octyl butyl phthalate (3.59%) and diisooctyl phthalate (5.79) in the extract. **Conclusions:** *Methylobacterium* sp. (ERI-135) showed promising antibacterial and cytotoxic activity. This is the first report in the antimicrobial and cytotoxic effect of *Methylobacterium* sp.

1. Introduction

Over the past 20 years there has been a lot of interest in the investigation of natural materials as sources of new antibacterial agents[1]. The genus *Methylobacterium* was first proposed in 1976 to accommodate Gram-negative bacteria that have the ability to utilize methane and other more complex organic compounds such as carbon and other energy sources. *Methylobacterium* sps are natural sources for the production of industrially important compounds and an alternative to current bacterial expression systems for the production of recombinant proteins. Furthermore, *Methylobacterium* is a promising microorganism for the commercial production of natural products, including polyhydroxybutyrate (PHB) and the very valuable co-polymer P (HB/HV). Particularly *Methylobacterium* sps possess one or more characteristics of plant-growth promoting bacteria (PGPB)[2]. *Methylobacterium* sp. have been recognized as common environmental isolates from such habitats as leaf surfaces, leaf nodules of plants, soil, water, grass, sewage, air, and rice grains[3]. *Methylobacteium* stimulated seed germination, plant development, contributed to plant flavour and inhibited the bacterial infection[4]. The present study was aimed at assessing the antimicrobial activity of a new *Methylobacterium* sp. isolate (ERI-135), obtained from Doddapetta forest soil, Nilgiris (Southern

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Western Ghats of Tamil Nadu), India.

2. Materials and methods

2.1. Sample collection

The soil samples were collected from the depth of 5-15 cm at Doddapetta forest, (southern Western Ghats), Tamil Nadu, India.

2.2. Isolation of methylobacterium

Isolation of *Methylobacterium* was performed by serial dilution using dilution plate technique. One gram of soil was suspended in 9 mL of sterile distilled water. The dilution was carried out up to 10^{-6} dilutions. Aliquots (0.1 mL) of 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were spread on the isolation plates containing Modified Nutrient agar. The plates were incubated at 28 ℃ for 5 d.

2.3. Cultural characterization

Active isolate (ERI-135) was characterized morphologically and physiologically following the directions given by the Bergey's Manual of Systematic Bacteriology[5]. Cultural characteristics of pure isolates were recorded after incubation for 5 d at 28 ℃ in different media. Active isolate (ERI-135) was identified using different pH levels, NaCl concentration, temperature and utilization of carbon sources following standard methods[6]. The ability to produce enzymes was also studied[7].

2.4. Extraction of bioactive metabolites

Culture inoculate of the isolate (ERI-135) was taken in 500 ml Erlenmeyer flasks containing 150 mL of modified nutrient gulucose agar (MNGA) medium and incubated at 30 ℃ in a shaker (200 rpm) for 5 d. After 5th day the culture broth was centrifuged at 10 000 rpm for 15 min to remove the biomass. Equal volumes of chloroform and ethyl acetate (1:1 v/v) were added. The organic solvent layer was transferred to a clean conical flask. The organic layer was concentrated using vacuum rotary evaporator at 40 ℃. The extract was transferred to a 5 mL sterile vial.

2.5. Microbial organisms

The following bacteria and fungi were used for the experiment. Bacteria: *Bacillus subtilis (B. subtilis)* MTCC 441, *Klebsiella pneumoniae (K. pneumoniae)* MTCC 109, *Pseudomonas aeruginosa (P. aeruginosa)* MTCC 741, *Salmonella typhimurium (S. typhimurium)* MTCC 1251, *Shigella flexneri (S. flexneri)* MTCC 1457, *Enterobacter aerogenes (E. aerogenes)* MTCC 111, *Staphylococcus aureus (S. aureus)* MTCC 96 and *Staphylococcus epidermidis (S. epidermidis)* MTCC 3615; fungi: *Candida albicans (C. albicans)* MTCC 227 and *Trichophyton rubrum (T. rubrum)* 57/01. The reference cultures were obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India-160 036. All the other cultures were obtained from the Department of Microbiology, Christian Medical College, Vellore, Tamil Nadu, India.

2.6. Antimicrobial assay

Antibacterial and antifungal activities were carried out using disc-diffusion method[8]. Petri plates were prepared with 20 mL of sterile Mueller Hinton agar (MHA) (Hi-media, Mumbai). The test cultures were swabbed on the top of the solidified media and allowed to dry for 10 min and a specific amount of crude extract 2 mg/disc was added to each disc separately. The loaded discs were placed on the surface of the medium and left for 30 min at room temperature for compound diffusion. Negative control was prepared using respective solvents. Streptomycin (10 μg/disc) was used as positive control against bacteria. Ketoconazole was used as positive control for fungi. The plates were incubated for 24 h at 37 ℃ for bacteria and for 48 h at 28 ℃ against fungi. Zones of inhibition were recorded in millimetres and the experiment was repeated twice.

2.7. Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration studies of the crude extract were performed according to the standard reference methods for bacteria[9], for filamentous fungi[10] and yeasts[11]. The required concentrations (2 000 μg/mL, 1 000 μg/mL, 500 μg/mL, 250 μg/mL, 125 μg/mL, 62.5 μg/mL and 31.25 μg/mL) of the extract were dissolved in DMSO (2%), and diluted to give serial two-fold dilutions that were added to each medium in 96 well plates. An inoculum of 100 from each well was inoculated. The antifungal agents ketoconazole and fluconazole for fungi and the antibacterial agent streptomycin and ciprofloxacin for bacteria were included in the assays as positive controls.

2.8. Cytotoxicity effect on A549 cancer cell lines

A549 human adenocarcinoma cell lines were maintained in complete tissue culture medium DMEM with 10% fetal Bovine serum and 2 mM L-Glutamine, along with antibiotics (about 100 IU/mL of penicillin, 100 μg/mL of streptomycin) with the pH adjusted to 7.2. The cytotoxicity was determined according to the method of Hsu *et al*[12]. The cytotoxicity against cancer cells was determined by measuring the absorbance of the converted dye at 570 nm in an ELISA reader. Cytotoxicity of each sample was expressed as IC_{50} value.