

International Journal of Scientific Research and Reviews

Bio-Activity of Secondary Metabolites Extracted From Soil Fungi

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ABSTRACT

Secondary metabolites of microbial origin, either as pure compounds or crude extracts, provide opportunities for new drug development because of their chemical diversity. The aim of the present study is to isolate pharmaceutically important bioactive compounds from soil fungal secondary metabolites. The crude extracts of soil inhabitant fungal isolates were tested against pathogenic bacteria by disc diffusion method. The efficient strain was identified using 18S rRNA sequence analysis. To enhance the production of fungal metabolites, the selected strain was grown under various parameters such as suitable medium, pH, temperature, incubation period and minimal inhibition concentration. The *Penicillium brasilianum* which was selected as efficient strain from 63 pure fungal isolates obtained from soil samples of the Nilgiris district, Tamil Nadu, showed potent antibacterial activity against test pathogens. The metabolite production was enhanced under various physical and chemical parameters. The present study reveals that the selected fungus, *Penicillium brasilianum* produced potential antimicrobial metabolites which could be further characterized for the development of bioactive compounds for pharmaceutical use.

KEYWORDS: Anti-bacterial, Bioactivity, Metabolites, Optimization, *Penicillium brasilianum*

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INTRODUCTION

Natural products have been selected over millions of years by evolution as effectors of biological processes. Plants, algae, fungi, bacteria etc., coexist in an ecosystem and interact with each other in the form of chemical substances called secondary metabolites and most of these are produced to improve the survival fitness of an organism.¹ Several natural products have been discovered and introduced into the pharmaceutical market every year, but only less than 10% of them are exploited. So, the discovery of novel molecules from nature still remains as an untapped resource. In the current scenario, there is a steady decline in the development of new drugs by the pharmaceutical industries, which results in the need for drugs for certain new diseases,^{2,3} and development of Multi Drug Resistance (MDR) among pathogens etc.⁴ The spread of multidrug resistant bacteria in hospital and community settings remains a widely unresolved problem and a heavy burden to health services.⁵ However, the application of antibiotics over a period of 60 years has led to the development of antibiotic resistance in many bacterial pathogens.⁶ This imbalance has pushed the pharmaceutical sector for the discovery of novel drugs. Natural products can be considered as an essential component in the search for and development for new and safe drugs. This is lead, to an up gradation in pharmacological research. In the recent years many bioactive compounds responsible for the effects of crude extracts were separated and structurally elucidated. Arnold⁷, estimated that about 1 million natural products have been isolated, from microbes of which approximately 40% are biologically active.

Bacteria, actinomycetes and filamentous fungi are the major source of microbial natural products. These compounds possess unique structures and profound bioactivity that make them distinct from molecules of other organisms. Discovery of natural drugs has intensified the research towards micro organisms resulting in an exploration of 23,000 bioactive compounds of which 42% are produced by fungi.⁸ Fungi are known to produce an array of bioactive molecules that possess antimicrobial (Cephalosporin), immunosuppressive (Mycophenolic acid), antiviral (Beauvericin), Cholesterol-lowering (Lovastatin) and anticancer activities (Asperlin).⁹⁻¹⁴ Fungi are as diverse as plants and animals with an estimated 1.5 million species; of which only 10% have been described.¹⁵ A more recent publication estimated the number as between 2.2 and 3.8 million¹⁶.

Penicillium is one of the promising and diverse fungal genera with 354 accepted species.¹⁷ This genus is of considerable relevance in different scientific fields such as food spoilage, biotechnology, plant pathology and medicine¹⁸, and shows various kinds of habitats, ranging from necrotrophic pathogenicity to entophytic mutualism. A variety of secondary metabolites including antimicrobial substances is reported from *Penicillium* species.¹⁹⁻²¹ The production of secondary metabolites has been reported to be affected by various factors such as medium, composition, pH,

water activity, temperature, light and oxygen availability.^{23, 23} Keeping in view the above results, the objectives of the present research was carried forward on to the isolate the efficient soil fungal species for antibacterial activity and to observe the effect of medium, pH, incubation days and temperature, to increase quality and quantity of metabolite production and to determine the minimum inhibitory concentration and minimum bactericidal concentration against the test pathogens.

MATERIALS AND METHODS

Collection and isolation of soil fungi:

Soil samples were collected from the Nilgiris hills region (Lat 11°8' N to 11°37' N, Long 76°27' E to 77°4'E), of Tamil Nadu, India. The fungal communities were isolated by Soil dilution plate method. The soil samples were sieved through a 2mm sieve in order to avoid large soil particles and decomposed plant materials. The samples were then dispensed into sterile polythene bags and were brought to the laboratory. Fungal communities were isolated by mixing 1g of soil samples containing decomposed plant material in 10ml of sterile distilled water were spun at 100rpm for 15min at room temperature. After 6-fold serial dilution 0.1ml of the suspension was spread onto sterile Potato Dextrose Agar (PDA) medium containing chloramphenicol (150mg/l) for isolation of fungal strains.²⁴

Fermentation and extraction of secondary metabolites:

Extraction of secondary metabolites was carried out by using the method of.²⁵ All the fungal isolates were inoculated into 250 ml Erlenmeyer flasks containing 100ml potato dextrose broth and incubated at room temperature for 21 days under stationary conditions. The broth culture was filtered to separate the mycelia and the filtrate. To the filtrate equal volume of ethyl acetate was added, mixed well for 10 minutes and kept for 5 minutes until two clear immiscible layers were formed. The upper layer of ethyl acetate containing the extracted compounds was separated using a separating funnel. The culture filtrate extracts were pooled and evaporated to dryness in hot air oven. The extract residue was dissolved in Dimethyl sulfoxide (DMSO) and stored at 4°C to be used as stock solution for antimicrobial assay.

Test microorganisms:

For antibacterial assay three gram-negative bacteria (*Alcaligenes faecalis* (Acc No: MTCC3104), *Acinetobacter baumannii* (Acc No: MTCC1425) and *Escherichia coli* (MTCC1261) obtained from the King Institute of Preventive Medicine and Research Chennai, were used as test

pathogens. The bacterial cultures were maintained on nutrient agar (NA) slants (Peptone 5g, Yeast extract 2g, NaCl 5g and Agar 18g, Distilled water 1000ml, pH 7.0) and incubated at 37°C.

Antibacterial assay:

Antibacterial activity assay was performed by disc diffusion method.²⁶ Muller Hinton Agar (Hi-Media) served as the basal medium to carry out the assay. To adjust the turbidity of bacteria, 0.5 McFarland Standard was used prior to carrying out the microbial assay. The discs were placed on to the bacteria- seeded plate along with 5% DMSO which served as negative control to detect the solvent effects. Commercial antibiotic discs (Streptomycin 10mcg) served as the positive control. The plates were then incubated at 37°C for 24 hours. Each test was carried out in triplets.

Molecular Characterization of the Selected Fungal Isolate:

DNA Extraction:

Total genomic DNA of the fungus was extracted using InstaGene™ Matrix (Catalog # 732-6030) genomic DNA isolation kit, following the supplier instructions. The freshly cultured cells were pelleted by centrifuging for 2 minutes at 10,000 rpm. The pellet was resuspended in 1ml of sterile double distilled water and centrifuged at 10,000 rpm for 1 minute. The supernatant was discarded and 200µl of Insta Gene matrix was added to the pellet. This mixture was evenly mixed by using magnetic stirrer and incubated at 56°C for 15–30 minutes. Later the mixture was vortexed at high speed for 10 seconds and placed in 100°C heat block for 8 minutes. Again the mixture was vortexed at high speed for 10 seconds and spun at 10,000 – 12,000 rpm for 2-3 minutes. The supernatant was used for DNA amplification using polymerase chain reaction (PCR).

PCR Amplification:

PCR was performed using the diluted genomic DNA of the selected isolate. 18S rRNA amplification and sequencing were done using the forward primer, ITS-1: TCCGTAGGTGAACCTGCGG and reverse primer ITS2 (5'-TCCTCCGCTTATTGATATGC-3').²⁷ The PCR product was purified using Montage PCR Clean up kit (Millipore) and then sequenced using Big Dye Terminator and ABI 3730xl sequencer (Applied Biosystems). The phylogenetic tree was constructed through alignment of the sequences of the NCBI GenBank data using molecular and evolutionary genetics analysis (MEGA) software (version 4.0).

Optimization:

Selection of culture medium for bioactive compound:

In order to select the best suitable medium for production of secondary metabolites, four different culture media namely Potato Dextrose Broth (PDB), Sabouraud Dextrose Broth (SDB), Czapek Doxbroth (CDB) and Glucose Yeast Broth (GYB) were used. The potential of bioactive compounds extracted from selected fungal isolate was assessed by measuring the activity of crude extract against bacterial pathogens.

Effect of pH:

Initial pH range of medium was adjusted starting from 4, 5, 6, 7 and 8, and incubated for 21 days at room temperature under stationary condition. The bioactivity of metabolite production was estimated by measuring the zone of inhibition against target bacterial pathogen.

Effect of temperature:

The selected culture was grown under different temperatures to observe the effect on bioactive metabolite production. The culture was inoculated in 250ml flask containing 100ml of PDB and incubated for 21 days at three different temperatures 25°C, 30°C and 35°C.

Effect of incubation period:

The selected fungal strain was incubated and sampled at seven day intervals to observe the activity of the crude extract. Erlenmeyer flask of 250 ml volume containing 100 ml broth (PDB) was inoculated with equal diameter (5mm) of fungal 'mat cake'. All flasks were incubated at 30°C for 7, 14, 21 and 28 days respectively. Activity of crude extract against target bacterial pathogens was assessed by performing disc diffusion assay.

Determination of minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) of fungal crude extract:

MIC and MBC were determined by macro broth two-fold serial dilution method.²⁸ All tests were performed with Muller-Hinton broth. Bacteria were cultured overnight at 37°C. The fungal crude extract was dissolved in 10 per cent DMSO to obtain 2mg/ml. For determination of MIC and MBC, 8 sterile screw capped test tubes were used. A volume of 1 ml of nutrient broth was dispensed into each of the test tubes, 1-6 and 2mL into test tube 7 served as control and incubated at 37°C for 24 hrs. After incubation, the lowest concentrations, which did not show any growth of tested organism was considered Minimum Inhibitory Concentration (MIC). From the test tubes with no

growth (no turbidity), 0.1 ml was taken and spread over the surface of Mueller Hinton agar plates. After incubation at 37 °C for 24 hrs, the colonies were observed and the MBC was determined.

Statistical analysis:

All the results are expressed as mean± SD from triplicate. Values were statistically significant. The mean values and standard deviation were calculated using the Excel program from Microsoft Office 2010 package.

RESULTS AND DISCUSSION

Isolation of soil fungi:

The present study involves the screening of novel antibiotic producing fungi isolated from soil samples collected from the Nilgiris district, Western Ghats, India. These fungal isolates were grouped into 19 genera, based on their physical appearances and growth morphologies. The 19 fungal strains belong to 8 sp. of *Penicillium*, 6 sp. of *Aspergillus*, 3 sp. of *Trichoderma*, and 3 sp. of *Chaetomium*. The present investigation recorded more number of *Penicillium* sp. and *Aspergillus* sp. followed by *Chaetomium* sp. Asan²⁹, studied the flora of Edrin and reported that 16 species belong to *Penicillium* and 2 are *Aspergillus*. Saravanakumar and Kaviyaran³⁰, have reported the *penicillium* sp. and *Aspergillus* sp. have been dominantly isolated from forest soil of Nilgiris district. The density and diversity of microflora depends on the environmental factors such as pH, moisture, temperature, organic carbon sources³¹ etc.

Antibacterial activity of soil fungi:

Results showed that all the fungal isolates used in this study showed antibacterial activity against one or more of the tested organisms. Among them *Penicillium* sp. (Isolate 48) showed prominent activity against all the three pathogenic bacteria (**Tabale 1**). The crude ethyl acetate extract of *Penicillium* sp. showed the highest activity against *A. faecalis* (27.66±0.22), followed by *E.coli* (23.33±0.66) and *A.baumannii* (19.83±0.55). Positive control (streptomycin) shows inhibition zone ranging between 13 and 14mm and no zone of inhibition was observed in the negative control (DMSO). Several metabolites have been purified from many *Penicillium* species.³² One of the important mechanisms that contributed to their biocontrol activities has been proposed to produce antibiotic compounds.^{33,34}

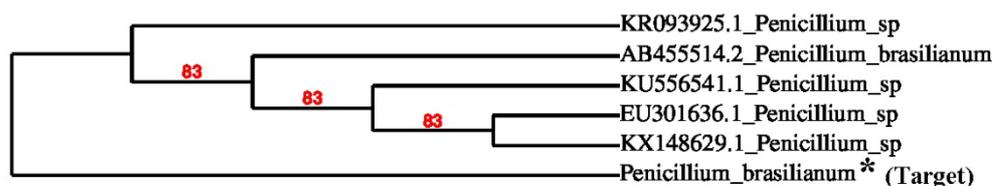
Table 1: Antibacterial activity: Zone of inhibition for soil fungal crude extracts against the test pathogens.

Fungal Isolate No	<i>Alcaligenes faecalis</i>	<i>Acinetobacter baumannii</i>	<i>Escherichia coli</i>
1	13±0.66	17±0.66	-
2	-	-	-
3	-	-	-
4	14.83±0.55	14.66±0.22	22±0.66
5	19±0.66	10±0	-
7	14.33±0.44	14±0	-
8	12.16±0.22	16.83±0.55	-
13	12.33±0.22	11±0	-
14	-	-	-
15	11.16±0.55	12.5±0.33	-
17	14±0	-	-
17t	20.16±0.22	10±0.66	-
18	15±0	9±0	-
20	12±0	11±0	-
21	14±0.66	12.33±0.44	-
33	-	-	-
34	13.33±0.44	-	-
38	14±0	14.16±0.22	-
48	27.66±0.22	19.83±0.55	23.33±0.66
Streptomycin	13±0	13±0	14.83±0.55
DMSO	-	-	-

Molecular identification of efficient fungus by 18S rRNA sequence:

The selected fungus *Penicillium* sp. was characterized on the basis of 18S rRNA gene sequencing, illustrates the phylogenetic tree showing the evolutionary relationship of the isolate *Penicillium* sp. with other closely related taxa (**Figure.1**). The isolate showed 96% similarity with *Penicillium brasilianum* (AB455514.2) of the NCBI Gene bank data. The sequence has been deposited in Gene bank with Acc. No. MH198044.

CTGCGTTTTTCATCGATGCCGAAAGAGATCCGTTGTTGAAAGTTTTAACTGATTT
AGCTAATCTACTCAGACTGCAATCTTCAGACAGAGTTCAATGGTGTCTTCGGCGGGGCGC
GGGCCCCGGGGGCGAGTGCCCCCGGCGGCCGTGAGGCGGGCTCGCCGAAGCAACAAGG
TACAATAAACACGGGTGGGAGGTTGGACCCAGAGGGCCCTCACTCAGAAGCCTTCCGC
AGGTT CACCTACGGAA

**Figure 1- Phylogenetic tree of *Penicillium brasilianum***

Optimization of culture conditions for the production of bioactive metabolites:

Effect of culture media

In the present study, *Penicillium brasilianum* was grown on different culture media such as PDB, CDB, SDB and GYP. The significant quantity of bioactive metabolite production was observed in Potato dextrose broth (PDB) with maximum zones of inhibition 24.5 ± 0.33 , 17 ± 0.66 and 23 ± 0.66 against *A. faecalis*, *A. baumannii*, and *E. coli* respectively (**Figure 2**). Potato dextrose broth has been used for the production of tropolone, an antimalarial antibiotic from the insect pathogenic fungus *Cordyceps* sp.³⁵

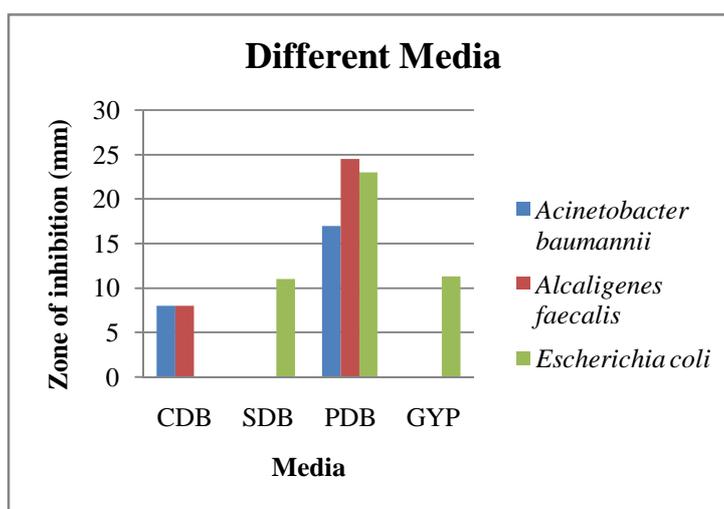


Figure 2 - Effect on different growth media on production of fungal metabolites

Effect of pH:

The pH of culture medium is one of the determining factors for the metabolism.³⁶ In order to determine the optimum pH for maximum antimicrobial metabolite production, the selected isolate, *Penicillium brasilianum* showed variations in antimicrobial activity when subjected to different pH values. Maximum activity was found at a pH value of 5, with a zone of inhibition of 21 ± 0.33 against *A. faecalis* (**Figure 3**). Bioactive metabolite production of *Aspergillus fumigatus* reached maximum at pH 5³⁷. However, Digrak et al.,³⁸ reported that highest production of biomass by *F. equiseti* was at pH 8.

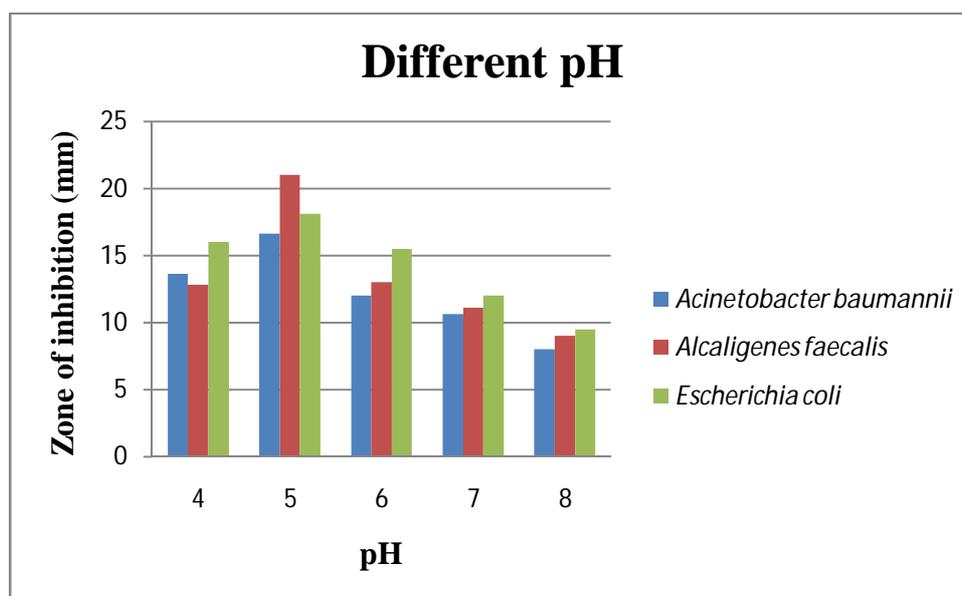


Figure 3 - Effect of different pH of growth medium on production of fungal metabolites.

Effect of temperature:

Physical factors such as incubation temperature can exert different effects on the growth and production phases of secondary metabolism.³⁹ The effect of temperature is significant for the production of antimicrobial metabolites of the isolate *penicillium brasilianum* that remained active at all of the rendered temperature values. The most significant production was observed at 30°C with the inhibition zone of *A. faecalis* 23.3 ± 0.88 (**Figure 4**). Ritchie *et al.*,⁴⁰ reported the incubation temperature ranging from 20°C to 25°C to be an optimum for the mycelial growth of the fungi *Rhizoctonia solani*. Huang *et al.*,⁴¹ also reported the isolation of antifungal and anti-tumour agent from endophytic fungi at 25°C. Bhattacharyya and Jha⁴² reported that, the increase of the incubation temperature from 25°C to 30°C enhanced the growth of mycelia and production of bioactive metabolite in *Aspergillus* strain.

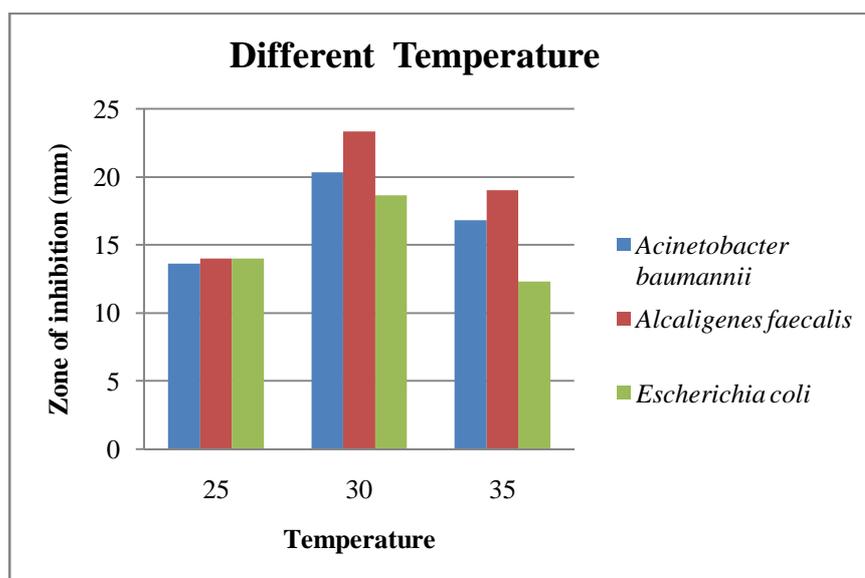


Figure 4: Effect of temperature on production of fungal metabolites.

Effect of incubation days:

The effect of incubation period on the growth and production of antimicrobial metabolites was studied by incubating *Penicillium brasilianum* isolate for 7, 14, 21 and 28 days. Maximum antimicrobial activity was observed from the fungal cultures incubated for 14 days with inhibition range between 22 ± 0 and 26.6 ± 0.44 (Figure 5). Endophytic fungi *A. fumigatus* strain KARVS04 reached maximum growth at 12 days of incubation.³⁸ Lovastatin production by *Aspergillus fumigatus* increased in 9 days.⁴³

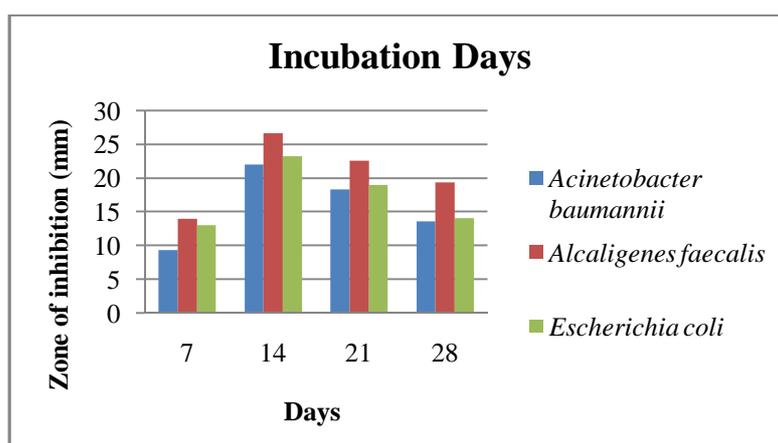


Figure 5 – Effect of incubation period on production of fungal metabolites.

MIC and MBC:

Minimum inhibitory concentration (MIC), defined as the lowest concentration of antibiotics, did not show any growth of tested pathogens at a minimum concentration.⁴⁴ To determine the degree

of antibacterial activity, the crude extract was subjected to MIC) assay by serial two fold dilution method.⁴⁵ The results revealed that the crude extract of the fungus possessed good antibacterial activity against all the three test pathogens (**Table 2**). The MIC values were found at 100µg/ml for test bacteria. The Minimum Bactericidal Concentration of crude extract was recorded as 100µg/ml against *Alcaligenes faecalis* and 200µg/ml for *Acinetobacter baumannii* and *Escherichia coli*. Santos et al.,⁴⁶ reported that the minimum inhibitory concentration of *Nigrospora. sphaerica* against *Pseudomona aeruginosa* showed as 1.56mg/ml and MBC as 12.5mg/ml.

Table 2: MIC and MBC values of fungal crude extract against test pathogens.

Pathogens	Crude extract µg /ml		Standard µg/ml	
	MIC	MBC	MIC	MBC
<i>Acinetobacter baumannii</i>	100	200	25	100
<i>Alcaligenes faecalis</i>	100	100	25	50
<i>Escherichia coli</i>	100	200	50	100

CONCLUSION

This study indicates that fungi isolated from soil environment produce many pharmaceutically important bioactive compounds with antibacterial potential under optimum conditions. *Penicillium brasilianum* showed better antimicrobial activity against test pathogens. Soil fungi revealed their potential to yield bioactive metabolite which may play an important role in providing biomolecules for drug designing in pharmaceuticals industry. Hence, further studies have to be carried out for purification, characterization and identification of bioactive metabolites of *Penicillium brasilianum* for drug discovery and exploitation.

ACKNOWLEDGEMENT

Authors gratefully acknowledge the support and facilities extended by the Secretary, Ramakrishna Mission Vivekananda College, Mylapore, Chennai for conducting the present study.

CONFLICTS OF INTEREST

All authors declare that they have no conflicts of interest.

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